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Research Article

Dynamic of Intracellular Cytokine's Production in Patients with Recurrent Implantation Failure and Thin Endometrium in *In Vitro* Fertilization Program

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ABSTRACT

The article presents results of research of 25 patients with recurrent implantation failure after *In Vitro* Fertilisation (IVF) and the "thin endometrium" in comparison with 30 patients without reproductive loss and normal endometrial thickness. Dynamics of intracellular production of γ-INF, IL-1 and IL-10 by cytotoxic endometrial lymphocytes was studied by flow cytometry.

It was found that before treatment in patients with "thin endometrial" syndrome and recurrent implantation failure after IVF there were a significant (18-fold) decrease in the level of CD8+ cytotoxic/suppressor lymphocytes ($p<0.01$), a 3-fold decrease in the level of CD56+ cells, as well as a sharp inhibition of the level of intracellular production of cytokines- γ-INF (10 times), IL-1 (10 times) and IL-10 (3 times); there was a sharp decrease in intracellular production of γ-interferon by CD8+ lymphocytes-almost 35 times, by CD16+ lymphocytes-6,8 times and by CD56 + lymphocytes-16 times.

During complex therapy including the elimination of pathogens, personalized hormonal therapy with progesterone (Gynoprogest), there were found that the thickness of the M-echo endometrium increased from 6,82 ± 1.3 mm to 13.0 ± 1.5 mm; and there was also a positive dynamics of intracellular cytokine production with a significant ($p<0.01$) increase in the level of IL-1 and IL-10, as well as the level of γ-IFN by endometrial lymphocytes.

Key words: *In Vitro* fertilization, Recurrent implantation failure, IL-1; IL-10, Interferon, Thin endometrium

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ABBREVIATIONS

CD: Cluster of Differentiation; ELISA: Enzyme-Linked Immunosorbent Assay; GnRH: Gonadotropin-Releasing Hormone; γ-IFN: Interferon; IVF: *In Vitro* Fertilisation; IL: Interleukin; JZC: Junctional Zone of Contractions; PCR: Polymerase Chain Reaction; RIF: Recurrent Implantation Failure

INTRODUCTION

Recurrent Implantation Failures (RIF) in IVF programs are the most important problem in a practice of a reproductologist. Today among many reasons for RIF in IVF programs the "thin endometrium" which leads to endometriopathies and implantation disorders plays the important role (Diejomaoh MF, 2015). The thickness, morphological structure and receptivity of the endometrium are the main signs of endometrial maturity and, at the same time, the main criteria for predicting a successful pregnancy (Mamedaliyeva NM, *et al.*, 2016). The preimplantation endometrium characterised by the presence of a developed capillary network, microcirculation, oxygenation of tissues, proliferative activity of epithelial and stromal cells, active metabolism and readiness of the endometrial neuroreception. The optimal receptivity of the endometrium begins on the 6th day after ovulation and lasts 4-5 days, which matches to 20-24 days of a menstrual cycle, and this period is called the "implantation window".

Chronic endometritis is the most frequent pathology of the endometrium, when occur multiple secondary morphofunctional changes, disrupting the cyclic transformation and receptivity of the uterine mucosa. Moreover, the syndrome of "thin endometrium" accompanies chronic endometritis.

The "thin endometrium" is the thickness of the endometrium less than 7 mm and absence of a three-layer structure during the "implantation window". The pathophysiological features of the "thin endometrium" consist in insufficient growth of the glandular epithelium, depletion of blood vessels and impaired expression of a number of regulatory cytokines, growth factors, natural killer cells, and lymphocytes, which reduce the implantation ability of embryos (Mamedaliyeva NM, *et al.*, 2016).

In addition, insufficient production of progesterone can lead to suppression of progesterone receptors in the epithelial cells of the endometrium, and decrease its receptivity during implantation, or "implantation window" is absent, implantation does not occur at all, which leads to infertility, and even if implantation occurs, it is ineffective and miscarriage develops (Seshadri S, Sunkara SK, 2016). Today scientists' interest in intracellular cytokines and growth factors that responsible for the receptivity of the endometrium (Michou VI, *et al.*, 2003; De Maria A, *et al.*, 2011). Intracellular production of pro-inflammatory cytokines by endometrial lymphocytes, in particular γ-interferon and IL-1, is considered as biologically active factors that improve the decidualization process. At the same time, the intracellular production of the IL-10 cytokine improves the receptivity of the endometrium. An integral assessment of pro- and anti-inflammatory cytokines serves as a marker of how the endometrium function works and as a prediction of

the effectiveness of hormonal therapy (Krylov US, *et al.*, 2013).

During syndrome of "thin endometrium" pregravidal preparation with selective influence on endometrium which failed receptivity is very important. Successful treatment of chronic endometritis is hormone therapy. Considering that the leading role that leads to implantation failures in IVF programs and miscarriage are assigned to an infectious factor, and according to Sidelnikova VM (Sidelnikova VM, 2015) chronic endometritis is histologically verified in 73% of cases, and in 87% there is persistence of opportunistic microorganisms in the endometrium, and by the decision of the World Congress of Obstetricians and Gynecologists (FIGO, Kuala Lumpur) that all cases of non-developing pregnancy and implantation failure should be associated with the presence of chronic endometritis - an important stage in the treatment strategy is to eliminate the microbial-infectious agent and includes rational antibacterial and immunomodulation therapy. So, the first stage includes antibacterial, antiviral and anti-candidiasis therapy with the use of immunomodulators, eybiotics and other things, that include physiotherapy, potentially capable of restoring the receptivity of the endometrium (Samoilich YA, 2018).

When Marinkin IO (Marinkin IO, *et al.*, 2013) investigated the pathomorphogenesis of recurrent miscarriage they demonstrated a failure in the processes of cellular and intracellular regeneration of endometrial epithelial cells, which leads to insufficient expression of both progesterone and estrogens receptors and decrease endometrial receptivity with a "closed implantation window", and this also applies to women with Recurrent Implantation Failures in IVF programs. Therefore, the second stage of the treatment strategy includes the induction of intracellular regeneration of endometrial epithelial cells: two phases hormonal therapy (estrogen valerate and progesterone). Intramuscular progesterone is effective to support the physiological level of progesterone, but intramuscular forms are painful, can lead to sterile abscess and reverse allergic reactions, even eosinophil pneumonia (Ahuja A, Ikladios O, 2017). Intravaginal forms of progesterone with proven higher bioavailability and efficiency are better way to use. Scientific studies of progesterone's pharmacokinetics have shown that vaginal forms, the concentration of progesterone in the endometrium is significantly higher than with intramuscular injection (Oprishco VI, Nocivec DS, 2016). This is achieved by the effective interaction of progesterone with functionally complete receptors of the endometrial tissue. Furthermore, progesterone is suppressive of Junctional Zone Contractions (JZC), and early progesterone support in the form of vaginal progesterone has been shown to decrease JZC at the time of embryo transfer (Killick S, 2007). An atraumatic embryo transfer with soft end of the catheter, without touching the uterus fundus is important too (Reynolds K, *et al.*, 2010).

In order to evaluate the effectiveness of complex treatment of patients with RIF in IVF programs and "thin endometrium" using two phases hormone therapy (estrogen and progesterone), and as an alternative antiestrogen Tamoxifen has its stimulatory effect on endometrium in 20 mg 3 times per day from 3d to 18th days of menstrual cycles (Ke H, *et al.*, 2018; Ji J, *et al.*, 2020), we studied the dynamics of endometrial thickness and intracellular production of pro-inflammatory cytokines γ-interferon, interleukin-1 (IL-1) and anti-inflammatory cytokine interleukin-10 (IL-10) by cytotoxic CD8+, CD16+, CD56+ endometrial lymphocytes.

MATERIALS AND METHODS

The study included 25 patients with recurrent implantation disorders in IVF programs and thin endometrial syndrome (the main group). The comparison group consisted of 30 patients without reproductive loss and presence of normal endometrial thickness on days 20-24th of the cycle. The local Ethics Review Committee approved the study protocol and a written informed consent was obtained from all patients. For each woman an individual medical history was filled and included the results of survey with the study of complaints, somatic and obstetric-gynecological history, data of general and gynecological status, laboratory, as well as special research methods: pelvic ultrasound, hysteroscopy, urogenital smear microscopy, ELISA, polymerase chain reaction for genital transmitted infections, determination of lupus coagulant, genetic consultation and karyotype, test for thrombophilia. Analysis of clinical and anamnestic data showed that in the main group the average age of the patients was 33 ± 4.3 years, in the comparison group-34.8 ± 3.3 years, no significant differences in age were found in the study groups. The average age of the onset of menarche in the main group was 13.2 ± 1.6 years and in the comparative group-13.6 ± 1.4 years. The average level of anti-Müllerian hormone was 1.93 ± 1.3 ng / ml. Violation of the menstrual cycle (irregular menstrual cycle) was observed in the main group-in 9 patients (36%) and includes short phase of proliferation (<11 days) in 12% and long-20% and the presence of cryptomenorrhea was diagnosed in 1 case (4%), menstruations were painless and scanty. 2 patients had moderate cervical dysplasia. The experience of infertility in patients in the main group ranged from 1 to 16 years.

All patients of the main group had a history of one or more episodes of unsuccessful IVF attempts before. When analysing the number of IVF program attempts, we noted that the majority (64%) of patients in the main group had a history of 2-3 IVF stimulations with embryo transfer in fresh and cryocycles, in 36%-4-5 stimulation attempts, and 20%-5 pregnancies induced by the IVF program occurred, but the pregnancy outcomes were spontaneous miscarriages (16%-4 cases) and biochemical pregnancies-2 cases in the same patient. When assessing the parity of pregnancies from the anamnesis in patients of the main group before entering the IVF program, it was revealed that secondary infertility was observed in 6 (24%) cases, including 1 case (4%) with bleeding and repeated curettage of the uterine cavity in the postpartum period and in one (4%) patient had an intrauterine device for more than 5 years. A history of miscarriage was observed in 9 patients (36%) of the main group, medical abortion-in 4 (16%), and two (8%) had a history of ectopic tubal pregnancies with unilateral and bilateral tubectomy. The combination of the female factor of infertility with the male factor was observed in 2 cases. Increased homocysteine and thrombophilia were diagnosed in 4 (16%) patients. The interstitial form of uterine fibroids and endometriosis of the uterus and ovaries were diagnosed in 3 cases (12%).

In the comparison group, 1 pregnancy was noted in 10 patients (33%), 2-3 pregnancies-in 13 patients (43%), 4 pregnancies-in 4 patients (13%). Only childbirth as a pregnancy outcome was observed in 15 women in the comparison group (50%), a combination of childbirth and medical abortion-in 15 women (50%).

All patients of the main and comparative groups underwent transvaginal echography on a Samsung Medison SonoAce R7 (South Korea) using a multi-frequency transvaginal transducer with a frequency of 4-10 MHz, with software for the implementation of a triplex scanning mode (grayscale B-mode in combination of color and pulse Doppler in real time). Dynamic ultrasound examination was performed before and after treatment in phase II (during the "implantation window"). The study began with a transabdominal ultrasound scan (with a filled bladder) to exclude pelvic masses. The study was continued after emptying the bladder and was determined by the position of the uterus in the pelvic cavity. The main attention was the study of the M-echo: thickness, echo structure and other disorders. The normal value of the M-echo was a homogeneous structure, the absence of hypo- or hyperechoic inclusions, the correspondence of its structure to the day of the menstrual cycle, the thickness of the M-echo in the "implantation window" not less than 8 mm. When examining all who applied in the comparison group, the thickness of the endometrium on

the 19-22th day of the menstrual cycle was more than 8 mm. The "thin endometrium" was the thickness of the endometrium less than 7 mm on the 20-24th day of the menstrual cycle.

In addition to ultrasound examination all patients of the main group underwent diagnostic hysteroscopy with endometrial biopsy (Karl Storz, Germany) and histology, because visualisation of the intrauterine cavity is highly sensitive and specific. It was found that in all patients with recurrent implantation disorders and 'thin endometrium" in the 1st phase of the menstrual cycle 100% observed changes in the endometrium such as: 80% showed signs of endometritis (hyperemia, stromal edema, micro polyps <1 mm), in one case the remainder of the trophoblast shell was diagnosed as hypotrophy of the entire uterine cavity with a loose micropapillary texture (the edges are well bounded and neurotic plaques that bleed were visible), polyps of the cervical canal-8%, submucous fibroids-4%, endometrioid cyst-8%, intrauterine adhesions-32% (the uterine cavity was asymmetric, intertwined like a cobweb between the walls of the endometrium, tissue fibrosis with lymphoid infiltration, without vascularization). In the remaining infertile patients, the only finding was endometrial hypoplasia and atrophy (the functional layer of the endometrium is thinned or absent, pale, vascularization was reduced) *(Table 1)*. Moreover, more than 70.1% patients with such pronounced hysteroscopic changes were observed in the older age group (over 30 years).

Table 1: Results of diagnostic hysteroscopy and subsequent histology in infertile patients with "thin endometrium" in the proliferative phase of the menstrual cycle

Researched parameters	Identified by hysteroscopy (n=25)	Confirmed by histology (n=25)
Endometritis	20 (80%)	22 (88%)
Endometrioid cyst	2 (8%)	2 (8%)
Cervix polyps	5 (20%)	4 (16%)
Atrophy	1 (4%)	1 (4%)
Submucosal fibroids	1 (4%)	1 (4%)

The material for the immunological study was the uterine endometrium biopsies obtained by Pipelle biopsy using a Goldstein catheter. Isolation of immunocompetent cells from endometrial tissue was performed by using an enzyme-free method. The endometrial fragments were placed in a Medicon container (Becton Dickenson/USA), phosphate buffer was added and ground in a Medimachine homogeniser (Becton Dickenson / USA) for several minutes. In result the cell suspension was centrifuged for 30 min in a ficoll-verographin density gradient (d=1.078). Labeled monoclonal antibodies CD8PE, CD16PE, CD56PE (labeled with PE-Phycoerythrin) were added to tubes with mononuclear cells to stain surface receptors. After permeabilisation of the cell membrane with Cytofix/Cytoperm solution, antibodies for intracellular staining of γ-INFFITC, IL-1FITC, and IL-10FITC (labeled with FITC-Fluorescein Isothiocyanate) were added. The relative content of endometrial cytotoxic lymphocytes and intracellular cytokine production were determined on a BD FACS CALIBUR flow cytometer (USA) using the CELLQuest program.

Patients of the main group took complex therapy. First of all, to all patients in main group despite the methods of stimulation ovulation (IVF in natural cycle and modified cycles; with minimal stimulation, long protocols with Gonadotropin-Releasing Hormone (GnRH) agonist, short protocols with GnRH antagonist) we offered total elective embryo freezing unless endometrium thickness increase and gain trilaminar pattern.

At the first stage, antibacterial therapy reduce risks of inflectional contamination second time were prescribed to both male and female of the couple in cases of positive infections (2 cases with PCR+Chlamydia). Loop excision of the cervix was performed after preliminary treatment of the infection in patients suffered with cervix pathology (2 cases).

All patients in the main group despite PCR and ELISA tests took antibacterial, antiviral, antifungal and physiotherapy (Viferon; Superlymph suppositories 25 mg intravaginal-10 days or Polyoxidonii 12 mg suppositories intravaginally-10 days; Doxicyclin 500 mg × 2 times during 14 days+Metronidosol 500 mg × 3 times during 10 days; Mycosist 150 mg in the 1st and 10th day of antibacterial therapy). In trompohilia cases anticoagulation and antiagregant therapy were prescribed (Kardiomagnil, Trombo-ass, Kurantil, Fraxiparine, Klexan); to strengthen tissue exchange, activation of energy exchange, elimination of tissue hypoxia and to improve metabolic processes in endometrium we use metabolic therapy, vitamins, antioxidants, systematic ensymopathy (Elevit, Vitazhenal, Folacin, Vobenzyme, L-arginine 6 g/day, tocopherol 400 mg/day given over several months), plasmopheresis.

After preparation therapy, at the same time began hormonal replacement therapy preparation for frozen embryo transfer according to the artificial preparation of endometrium protocol. Patients received estradiol valerate (Proginova) 2,0 mg, two tablets (up to 3 tablets in some patients) daily and from the 16th day of the menstrual cycle, progesterone (Gynoprogest) 200 mg, one tablet three times daily-10 days (in cycles which ended with embryo transfer till a positive pregnancy test and further). The longest period of complex therapy before cryoembryo transfer in different patients was 4 months. But on the sixth day of the progesterone application the thickness of patient's endometrium was 6,8-7,5 mm after ultrasound monitoring in most of them. Then instead of estradiol valerate Tamoxifen 20 mg 3 times daily increase endometrium thickness in 5 patients up to 13 mm-14,5 mm *(Table 2)*.

Table 2: Dynamics of M-echo thickness data before and after treatment

Indicator, mm	Before treatment (n=25)	After treatment (n=25)
M-echo	6,82 ± 0,45	13,0 ± 1,5*
*the difference is significant between the parameters before and after treatment at p <0.05		

The results were statistically analysed using Student's t-test. Differences between compared groups and numbers were considered significant when the probability of error was $P \leq 0.05$ and 0.01.

RESULTS AND DISCUSSION

The effectiveness of complex treatment of patients with RIF in IVF programs and the "thin endometrium" by using two phase hormone therapy (estrogen and progesterone) was studied by the dynamics of intracellular production of pro-inflammatory cytokines IL-1 and anti-inflammatory cytokine IL-10 with cytotoxic CD8+, CD16+, CD56 + endometrial lymphocytes before and after treatment (3-4 months) and by the dynamics of endometrial thickness (M-echo).

The most significant for the prognosis of the implantation capacity of the endometrium are the absence of disorders inside the uterus (Krasnapolskaya KV, *et al.*, 2016). At the same time, to determine the potential of the endometrium, it is necessary to take into account not one criterion, but assess of the receptivity of the endometrium by using immunological markers of implantation (Nicolaeva MA, *et al.*, 2021). At the next stage we studied the features of the intracellular production of cytokines γ-interferon, IL-1 and IL-10 by cytotoxic CD8 +, CD16 +, CD56 + endometrial lymphocytes. The data characterise the features of the subpopulation composition of endometrial cytotoxic lymphocytes in patients of the main group are presented in *Table 3*.

Table 3: Dynamics of indicators of the relative content of endometrial cytotoxic lymphocytes in patients with recurrent implantation failures in IVF programs and thin endometrial syndrome

Indicator, %	The comparison group, n=30	The main group	1 (4%)
		Before treatment	After treatment
		(n=25)	(n=18)
CD8+	8,5 ± 1,7	0,45 ± 0,28**	2,3 ± 1,04@@
CD16+	2,3 ± 0,6	1,48 ± 0,39	3,54 ± 1,57
CD56+	3,7 ± 1,0	1,22 ± 0,21*	1,98 ± 0,46
total γINF+	4,4 ± 1,0	0,42 ± 0,33**	2,28 ± 1,62
γ INF+CD8+	1,42 ± 0,48	0,04 ± 0,08*	0,3 ± 0,28
γ INF+CD16+	1,51 ± 0,39	0,22 ± 0,16	1,5 ± 1,12
γ INF+CD56+	2,47 ± 0,89	0,15 ± 0,13**	0,48 ± 0,34@
total IL1+	8,0 ± 2,7	0,75 ± 0,92*	5,9 ± 0,3##
total IL10+	4,6 ± 1,3	1,48 ± 0,62*	5,7 ± 0,7##
* the difference is significant between the parameter's comparison and main groups at p<0.05			
** difference is significant between the parameter's comparison and main groups at p<0.01			
## difference is significant between the parameter's before and after treatment at p<0.01			
@ the difference is significant between comparison and after treatment groups at p <0.05			
@@ difference is significant between comparison and after treatment groups at p<0.01			

It was found that in patients with RIF in the IVF program and the "thin endometrial" syndrome endometrial receptivity was decreased, which was expressed in a significant (18-fold) decrease in the level of CD8+ cytotoxic/suppressor lymphocytes (p<0.01), as well as a 3-fold decrease of level of CD56+ cells. The level of natural killer cells with phenotype CD16+ tended to decrease.

In patients with RIF in IVF programs and "thin endometrial" syndrome there was a sharp decrease in intracellular production of γ-interferon by endometrial lymphocytes - by 10 times; by cytotoxic CD8+ lymphocytes-almost 35 times, by CD16+ lymphocytes-in 6,8 times and by CD56+ lymphocytes-16 times.

It should be noted that patients with RIF in IVF programs and the «thin endometrial» syndrome also had a decreased production of intracellular cytokines both pro-inflammatory interleukin-1 by 10 times, and anti-inflammatory interleukin-10-by 3 times compared with similar indicators in the comparison group. The IL1+/IL10+ index tended to decrease, but no significant differences were found, which is associated with decrease in both proinflammatory IL1 and anti-inflammatory IL-10 cytokine. Thus, the revealed changes in the level of immunocompetent cells indicate that in the pathogenesis of RIF in IVF programs with the syndrome of "thin endometrium" there is a pronounced decrease in the level of CD8+ cytotoxic/suppressor endometrial lymphocytes and CD56+ lymphocytes, as well as a sharp decrease in intracellular production of γ -interferon, IL-1 and IL-10 by endometrial lymphocytes.

As you know, the implantation process can go as an inflammatory reaction that promotes attachment and invasion of the embryo into the endometrium, providing the necessary interaction with the maternal vascular system. Deficiency of special molecules and their synthesis of proteins, which occurs in the syndrome of "thin endometrium" is accompanied by disruption of peri-implantation mechanisms, including the regulatory action of sex steroid hormones (Nicolaeva MA, *et al.*, 2021; Vartanyan EV, *et al.*, 2018; Shneiderman MG, 2020).

During treatment the dynamics of the subpopulation composition of endometrial cytotoxic lymphocytes in patients of the main group was as follows *(Table 3).*

In the main group, in patients with RIF in IVF programs and the «thin endometrial» syndrome, the level of CD8+ cytotoxic/suppressor lymphocytes after treatment tended to increase, but was still significantly (p<0.01) low compared to the same indicator in the comparative group. The level of natural killer cells CD56+ and CD16+ cells tended to increase.

The level of intracellular production of γ-interferon by endometrial lymphocytes tended to increase. At the same time, the production of γ-interferon by CD56+ lymphocytes were higher than that before treatment, but it was still significantly (p<0.05) low compared to the same indicator in the comparative group. After treatment, an increase in the level of intracellular cytokines IL-1 and IL-10 was recorded (p<0.01).

We have estimated the pregnancy rate in the patients of the main group. Human chorionic gonadotropin positive analysis was in 3 patients (12%), biochemical pregnancy was recorded in 2 (8.3%), in 1 (2,5%) clinical pregnancy ended in full-term delivery, this patient was diagnosed isthmic-cervical insufficiency during pregnancy (correction was carried out) and was threat of miscarriage.

Ultrasound data are presented in Table 2. During ultrasound examination in most patients of the main group in addition with thin endometrium (the selection criterion for this study is the thickness of the M-echo on the day of the proposed implantation window less than 7 mm) were changes in its echo structure. In 53.3% of patients the endometrium was echo-heterogeneous, in 36.7% the echo structure of the endometrium did not correspond to the day of the menstrual cycle. In the dynamic of treatment, the thickness of the M-echo according to transvaginal echography significantly (p<0.05) increased from 6,82. ± 0.45 mm to 13.0 ± 1.5 mm.

CONCLUSION

Thus during complex therapy that includes the elimination of pathogens, hormonal therapy by using high doses of estrogens valerate (Proginova) and addition of antiestrogen and anticoagulants, vitamins and progesterone (Gynoprogest) intravaginally, there was a significant increase in endometrial thickness (6.82 ± 0.45 mm to 13.0 ± 1.5 mm); and there was also a positive dynamics of intracellular cytokine production with a significant (p<0.01) increase in the level of IL-1 and IL-10; tendency to increase production of γ-interferon by lymphocytes. The results shows that its necessary to prescribe complex therapy to patients with recurrent implantation disorders in IVF programs and with the syndrome of the "thin endometrium" that include two-stage preparation of the endometrium-restoration of receptivity followed by induction of regeneration by using hormonal therapy with estrogen and progesterone and antiestrogen as alternative.

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