PharmacologyOnLine
Archives • 2021 • vol.3 • 1780-1790

THE ROLE OF THE MORPHOFUNCTIONAL STATE OF ENDOMETRIUM IN THE REALIZATION OF REPRODUCTIVE FUNCTION IN *IN VITRO* FERTILIZATION COMPLICATED BY OVARIAN HYPERSTIMULATION SYNDROME

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Abstract

Ovarian hyperstimulation syndrome (OHSS) remains the most important, potentially life-threatening iatrogenic complication of controlled ovarian stimulation (COS). COS alters the expression profiles of more than 150 genes in the endometrium in both animals and humans. The morphological and functional state of the endometrium in women with OHSS is poorly understood. There is limited and controversial information about pregnancy outcomes in patients with OHSS.

The purpose of the study was to improve reproductive results in women with infertility during in vitro fertilization complicated by OHSS, based on the development of a methodology for preparing patients for the transfer of vitrified / warmed embryos and minimally invasive assessment of endometrial receptivity before embryo transfer.

Material and methods. In the course of experimental modelling of OHSS, the morphofunctional state of the endometrium, myometrium and ovaries was studied in 110 female hybrid mice. Based on the data obtained, a minimally invasive technique for assessing the receptivity of the endometrium before embryo transfer was proposed and a pathogenetic substantiation of the feasibility of individualized preparation of women for the transfer of vitrified / warmed embryos was carried out, an appropriate technique was developed, and its effectiveness was assessed.

Results. Negative changes in the morphofunctional state of the endometrium of mice and women in the presence of OHSS have been proven. In women, changes in the expression of implantation molecules (HOXA-10, VEGF, LIF, IL-6, IL-6R, LIFR, gp130), a decrease in inducible NO-synthase activity, impaired endometrial immune reactivity (expression of lymphocyte subpopulations CD45, CD56, CD138) were revealed. The use of individualized preparation of the endometrium for the transfer of vitrified / warmed embryos, which included drugs that restore the state of the ovarian-endometrial system (anti-inflammatory, decongestants, improve microcirculation, normalize endometrial function, endometrial molecular profile and endometrial immunoreactivity) before embryo transfer increased the number of live births in women with transferred OHSS 2.11 times (OR 3.17±0.50; 95% CI 1.20-8.39; p<0.02).

Conclusions. It is advisable to transfer embryos during in vitro fertilization in case of OHSS no earlier than four months after vitrification, after preliminary treatment aimed at improving the morphofunctional state of the ovaries and endometrium, using a minimally invasive technique for assessing endometrial receptivity.

All human studies were conducted in compliance with the rules of the Helsinki Declaration of the World Medical Association "Ethical principles of medical research with human participation as an object of study". Informed consent was obtained from all participants.

Keywords: : *infertility, in vitro fertilization, ovarian hyperstimulation syndrome, vitrification, morphofunctional state of the endometrium, experimental modelling of ovarian hyperstimulation syndrome, pinopodes, implantation molecules, inducible nitric oxide synthase, endometrial immunoreactivity, transfer of vitrified / warmed embryos, live embryos.*

Introduction

Ovarian hyperstimulation syndrome (OHSS) remains the most important potentially life-threatening iatrogenic complication of controlled ovarian stimulation (COS) [1]. Cases of spontaneous OHSS have been reported to be associated with mutations in the follicle-stimulating hormone (FSH) receptor gene [2].

For complete prevention of OHSS in risk groups, it is recommended to perform CBS with gonadotropin-releasing hormone antagonists (antGnRH), use as a trigger of ovulation agonist GnRH (aGnRH), vitrification of all embryos, cycle segmentation [3, 4]. Prevention of late OHSS is ensured by refusing to transfer embryos in the fresh cycle. Pregnancy after the transfer of thawed embryos is associated with a lower risk of premature birth, low birth weight and perinatal mortality than pregnancy from a transferred fresh embryo [5]. However, the strategy of using antGnRH in COS cycles and cycle segmentation cannot completely eliminate the development of OHSS, as well as its consequences in all patients [6]. Most researchers agree that in the transfer of morphologically high-quality embryos, one of the important reasons for the failure of in vitro fertilization cycles is a violation of the receptivity of the endometrium [7].

Mild forms of OHSS are common, of less clinical significance, and occur in approximately 20–33% of assisted reproductive technology (ART) cycles. Because the symptoms of mild OHSS can be erased and go unnoticed, their incidence is likely to be underestimated. More clinically significant moderate and severe forms of OHSS occur in approximately 3-8% of ART cycles (3 to 6% moderate and 0.5 to 5% severe) [2, 8].

OHSS is characterized by bilateral cystic enlargement of highly luteinized ovaries. Secondary complications include increased vascular permeability as well as hemorrhagic ovarian cysts. Moderate clinical manifestations of OHSS manifest themselves in the form of bloating, nausea and vomiting, as well as poor appetite. In total, 1.9% of patients with OHSS are hospitalized for effects such as hepatorenal insufficiency, acute respiratory distress syndrome, ovarian rupture bleeding, and thromboembolism. Severe cases of OHSS can even lead to death [2].

It is known that COS changes the expression profiles of genes in the endometrium in both animals and humans. Differences in the expression of more than 150 genes were found, including genes that regulate angiogenesis and early implantation [9]. The morphofunctional state of the endometrium in women with OHSS has been little studied, which requires further research. It is important to find non-invasive methods that would assess the receptivity of the endometrium directly in the cycles of embryo transfer. There are conflicting data on the effectiveness of different methods of preparing the endometrium for the transfer of vitrified / warmed embryos - from the absence of any difference to the benefits of one method or another [10].

The purpose of the study was to increase reproductive outcomes in women with infertility *in vitro* fertilization complicated by OHSS, based on the development of methods for preparing patients for the transfer of vitrified / warmed embryos and minimally invasive assessment of endometrial receptivity before embryo transfer.

Materials and methods

The study was conducted at the clinical bases of Donetsk National Medical University (Lyman) and Odessa National Medical University (Odessa) in accordance with the planned research, was approved by bioethics commissions and consisted of experimental and prospective stages.

Animal studies have been conducted in accordance with existing legal documents and guidelines, their nutrition and manipulation with them were carried out in accordance with the provisions of the European Convention on the Protection of vertebrate animals used for research and other scientific purposes (Strasbourg, 1986).

During the experimental simulation of OHSS, the morphofunctional state of the endometrium and myometrium in 110 female hybrid mice (CBA × C57BL) was studied. Control group A included 24 female mice at the stage of estrus, which corresponded to spontaneous ovulation. Experimental group B consisted of 86 animals at the stage of estrus, which were intraperitoneally administered different doses of serum

gonadotropin of serum of mares (GSHK), and after 48 hours - HCG.

In *a prospective study* of the results of in vitro fertilization analyzed features of laboratory, clinical and instrumental parameters, morphofunctional state of the endometrium during the expected window of implantation in 108 women, including 78 women with early OHSS (OHSS group) and 30 women without OHSS. The control group of KII (n = 30) included conditionally somatically and gynecologically healthy fertile patients. Based on the data obtained at this stage, a minimally invasive method for assessing the receptivity of the endometrium before embryo transfer is proposed and pathogenetic substantiation of the feasibility of individualized endometrial preparation for the transfer of vitrified / warmed embryos and developed a method. To evaluate the developed method, the OHSS group was randomized into two groups: group I (n = 39) - using the developed method of endometrial preparation and group II (n = 39) - with standard correction of morphofunctional state of the endometrium before transfer of vitrified / warmed embryos.

General clinical, laboratory and instrumental examinations of all women were performed in accordance with existing protocols for inclusion in the *in vitro* fertilization program.

Uterine lavage was performed on the 21st day of the menstrual cycle using catheters for embryo transfer and the introduction into the uterine cavity of 5 ml of saline. Samples of uterine washes were placed in tubes with a volume of 5 ml and centrifuged for 15 minutes The supernatant was placed in plastic tubes with a volume of 1.5 ml and for further storage was placed in a freezer at t = -70° C. On the day of analysis, the sample was thawed. Determination of leukemia inhibitory factor (LIF) and glycoprotein 130 (gp130) was performed by enzyme-linked immunosorbent assay using the standard commercial kit Bender MedSystems (Austria) and Quantikine ELISA kit, R&D Systems Inc. (USA), according to the instructions of the manufacturers. The color intensity of the enzymatic reaction product was quantified. Mathematical processing of photometry results according to the calibration curve was performed. The results of the analysis were expressed in ng / ml.

Endometrial immunoreactivity studies were performed using mouse MAB to CD45 (clone PD7 / 26 + 2B11, Diagnostic BioSystems, USA), CD16 (clone 2Y7, Novocastra), CD56 (clone 123C3.D5, Diagnostic BioSystems, USA), to the epitope of human syndecan-1 (CD138) (clone MI15, Dako, Denmark).

Drug microscopy and all morphometric studies were performed on an Olympus AX70 Provis research microscope (Olympus, Japan) using the Analysis 3.2 Pro image analysis program (Soft Imaging, Germany). Examination of foams was performed by scanning electron microscopy (SEM) on a microscope "JEOL SuperProbe 733" (Japan).

Processing and analysis of statistical data was performed by methods of variation, correlation and graphical analysis using the mean (M), standard deviation error (± SE), Student's test, 2-criterion, correlation and regression analysis, odds ratio (OR) with calculation 95% CI. The results were presented in the form of: OR [95% CI].

Results

Hormonal stimulation of the ovaries of laboratory mice using different doses of gonadotropic hormones led to the development of pathological changes in the endometrium in animals. The morphofunctional state of the endometrium of female mice in the experimental modeling of OHSS was characterized by a disorder of local microcirculation in the form of dilation, plethora of thin-walled uterine vessels, especially subepithelial, diapedesis of erythrocytes, prolonged persistent stasis of erythrocytes. The severity of changes in the endometrium depended on the dose of hormones administered (Fig. 1).

The conducted experimental study proved the negative effect of COS and OHSS on the morphofunctional state not only of the ovaries but also on the endometrium of female mice. However, the mouse endometrium differs from the human in response to estrogen and P_4, making it difficult to extrapolate all results directly to in vitro clinical fertilization in women. Therefore, the effect of OHSS on the morphofunctional state of the endometrium in women was evaluated.

It should be noted that in women with in vitro fertilization with OHSS during the manifestation of OHSS there was a significant increase in ovarian volume with the presence of multiple luteal bodies: right - 19.96 and left - 18.97 times, while in

fertilization cycles in vitro without the development of this syndrome, the average ovarian volume increased only 5.89 and 6.08 times. The number of aspirated follicles in the groups of women with OHSS exceeded that in persons without OHSS 2.02 times (Table 1).

Scanning electron microscopy of the superficial epithelium of the uterine mucosa in patients of in vitro fertilization cycles confirmed its pronounced dyssynchrony in patients with OHSS, which was primarily manifested by redistribution of stages of development of foams on the day of the expected implantation window.

Integrins, molecules of the transmembrane glycoprotein family consisting of non-covalently linked α- and β-subunits, have a direct influence on the implantation process. Evaluation of the expression of αVβ3-integrins in the endometrium during the expected implantation window did not reveal a difference between their H-SCORE in the study groups: in the group with OHSS H-SCORE αVβ3-integrins was 278.78 ± 3.28%, in group K - 285, 07 ± 5.02 and in the KII group - 287.83 ± 3.53%.

One of the key angiogenic factors that play an important role in the development of physiological and pathological angiogenesis in the endometrium is VEGF-A. It increases the permeability of blood vessels in the endometrium and the proliferative activity of endothelial cells, inhibits apoptosis, stimulates the release of endothelial cells of spiral arteries of nitric oxide and prostacyclin, which promotes vasodilation. VEGF plays an important role in the process of normal implantation. It is known to be secreted by the corpus luteum, preimplantation embryo and endometrium. VEGF stimulates angiogenesis and is considered critical for trophoblast invasion and spiral artery reconstruction. It is closely regulated by the uterine endometrium, and impaired VEGF expression can be associated with both impaired and excessive trophoblast intervention, which can lead to placental dysfunction syndromes such as preeclampsia. Patients of the OHSS group with a decrease in stroma edema and vasodilation showed a decrease in VEGF production in the stromal compartment (45.90 ± 0.61 per 1 mm^2 of slice area) compared with the KI and KII group (48.95 ± 0.74 and 53,62 ± 0.93 per 1 mm^2 of slice area) (Fig. 2).

Nitric oxide promotes better vascularization of the endometrium, stimulates gene transcription and cell division, as well as regulates the synthesis of sex hormones. Endothelial dysfunction in the endometrium in women with OHSS during the expected implantation window was manifested by a decrease in iNOS production in 56.41% of individuals ($p<0.01$) and an increase in 3.85%, normal iNOS expression occurred in only 39.74% ($p<0.01$) (Fig. 3).

The preparation, which contains dry extract from the seeds of horse chestnut Aesculus hippocastanum, the active substance of which is escin, has a pronounced venotonic, anti-inflammatory and anti-edematous effect in venous diseases. Clinical reports on the vascular efficacy of escin have paid particular attention to improving microcirculation, reducing vascular permeability, increasing venous tone and venous return, all of which reduce edema. It has been suggested that the effects observed result from the protection of endothelial cells from hypoxia and inflammatory stimuli. In fact, as shown in preclinical studies, β-escin retains ATP during oxygen deficiency, reduces histamine response and cytokine release, suppresses serotonin-induced capillary permeability, inhibits extravasation and migration of leukocyte storage. It is also worth noting the data indicating the antioxidant potential of beta-escin and its inhibitory effect on hyaluronidase, the enzyme is involved in the pathogenesis of chronic venous insufficiency. In later works of inflammation, the emollient properties of beta-escin are associated with its modeling effect on tumor necrosis factor-alpha-mediated inflammatory pathways.

Escin is able to break the formed positive feedback, restoring normal hemocirculation in venous vessels and prevents the development of decompensation of diseases. Anticoagulant and antiplatelet effects of the drug make its use effective in the prevention of thrombosis. The presence of anti-inflammatory, anti-edematous, capillary-resistant effect, the ability to reduce the permeability of the plasma lymphatic barrier provide rapid relief of symptoms, which is important for the restoration of ovarian and endometrial function in OHSS. Vitamin E and L-arginine improve corpus luteum function and endometrial growth in patients by increasing blood flow in the uterine artery.

Serratiopeptidase is a natural proteolytic enzyme isolated from the non-pathogenic intestinal bacterium Serratia E15. Has fibrinolytic, anti-inflammatory and anti-edematous effects. Serrathiopeptidase slowly turns into exudate in the site of inflammation and gradually its level in the blood decreases. By hydrolyzing bradykinin, histamine and serotonin, serrathiopeptidase directly reduces capillary dilatation and controls their permeability. Serrathiopeptidase blocks plasmin inhibitors, thereby contributing to its fibrinolytic activity.

Myo-inositol is widespread in human tissues and cells and is a precursor to phosphoinositides involved in signal transduction by stimulating membrane receptors and other secondary messengers, including diacylglycerol and inositol triphosphate. The main function of myo-inositol and its derivatives is to participate in intracellular signaling and ensure the functioning of such important receptors as insulin receptors, reproductive hormones, growth factors, catecholamines and others. Derivatives of myo-inositol interact with specific proteins involved in the functioning of the reproductive system and embryo development. Myo-inositol is an important synergist of folate and other vitamins (B5, PP).

The complex of placental regulatory peptides has an immunotropic effect: stimulates the functional capacity of phagocytes of mucous membranes and blood, increases the synthesis of anti-inflammatory cytokines, affects the activity of regulatory subpopulations of lymphocytes. In autoimmune diseases or syndromes reduces the activity of immune-dependent inflammatory process, increases the number of CD4 + / 25 + - and CD8 + / 25 + - cells, especially IL-10 levels in blood plasma. The complex of placental regulatory peptides has a pronounced anti-inflammatory and resorbing effect, reduces the intensity of destructive, infiltrative and proliferative processes in the site of inflammation, reduces the severity of edema, accelerates epithelialization, regeneration, prevents the development of adhesive process.

L-arginine aspartate has a positive effect on the expression of markers of angiogenesis, thereby participating in the formation of new blood vessels in the endometrium. At the same time the strengthened formation of nitric oxide leads to dilatation of peripheral vessels and decrease in the general peripheral vascular resistance that promotes decrease in arterial pressure and decrease in a hypoxia of fabrics. Increasing the enzyme activity of NOS on the background of oral administration of a solution of L-arginine aspartate provides a constant baseline level of nitric oxide, which in turn increases the density of functional vessels of the endometrium, increasing its blood supply.

A study of the effectiveness of the developed measures of individualized correction of the morphofunctional state of the endometrium compared to the standard approach. Indicators such as echostructure and ovarian volume, endometrial echometry data on the 2-3rd day of the first, second and third menstruation after the in vitro fertilization treatment cycle, LIF and gp130 concentration in uterine washes on the day of the expected implantation window were evaluated. second and third menstruation after the in vitro fertilization treatment cycle, and before transfer previously studied immunohistochemical markers in pipette biopsies of the endometrium.

The largest sizes of ovaries were in the first menstrual cycle, reduction of the sizes practically to initial and normalization of an echostructure of ovaries was noted only after the third menstruation. The use of the developed method of endometrial preparation in patients with OHSS before the transfer of thawed embryos led to a faster and greater reduction in ovarian volume: in the group I – OD 270,86±8,81 sm^3 → 121,86±8,81 sm^3 → 28,89±2,02 sm^3 → 14,34±0,64 sm^3 i OS 279,91±8,46 sm^3 → 132,71±8,46 sm^3 → 32,86±2,00 sm^3 → 15,49±0,82 sm^3; in the group II – OD – 288,68±11,95 sm^3 → 173,68±11,95 sm^3 → 41,47±1,06 sm^3 → 17,58±0,94 sm^3 and OS – 286,81±11,48 sm^3 → 180,81±11,48 sm^3 → 43,84±1,04 sm^3 → 18,34±0,98 sm^3.

The developed individualized preparation of the endometrium is effective and can be widely used in clinical practice.

Conclusions

1. Treatment of infertility by in vitro fertilization can lead to the development of potentially life-threatening iatrogenic complication - OHSS.

Disorders of the morphofunctional state of hyperstimulated ovaries lead to changes in target organs, including the endometrium, which has been confirmed experimentally and in clinical trials and adversely affects implantation and delivery of pregnancy.

2. Experimental modeling in female laboratory mice of OHSS by administration of different doses of gonadotropic hormones leads to the development of local microcirculation disorders in the endometrium, dystrophic changes of the integumentary epithelium, numerous zones of spongy layer necrosis, accumulation of macrophages. There is a direct correlation between the severity of pathological changes in the endometrium and the dose of gonadotropins administered.

3. Embryo transfer should be performed no earlier than four months after vitrification, after prior treatment aimed at improving the morphofunctional status of the ovaries and endometrium, which have undergone changes due to OHSS.

4. Transfer of vitrified / warmed embryos should be performed in a cryoprotocol on the background of aGnRH administration followed by estrogen use for 14 days and 6-9 days of progesterone intake, as mostly in women with impaired reproductive function who have undergone OHSS, respectively, there is no regular menstrual cycle. spontaneous ovulation, which is a reference point for embryo transfer in the natural cycle.

5. For women who have undergone OHSS, preparation for the transfer of vitrified / warmed embryos should include drugs that restore the state of the ovarian-endometrial system: anti-inflammatory, anti-edematous, those that improve microcirculation, normalize endothelial function, endothelial function and molecular profile factor.

Conflict of interest

The authors declsre that there are no conflicts of interest.

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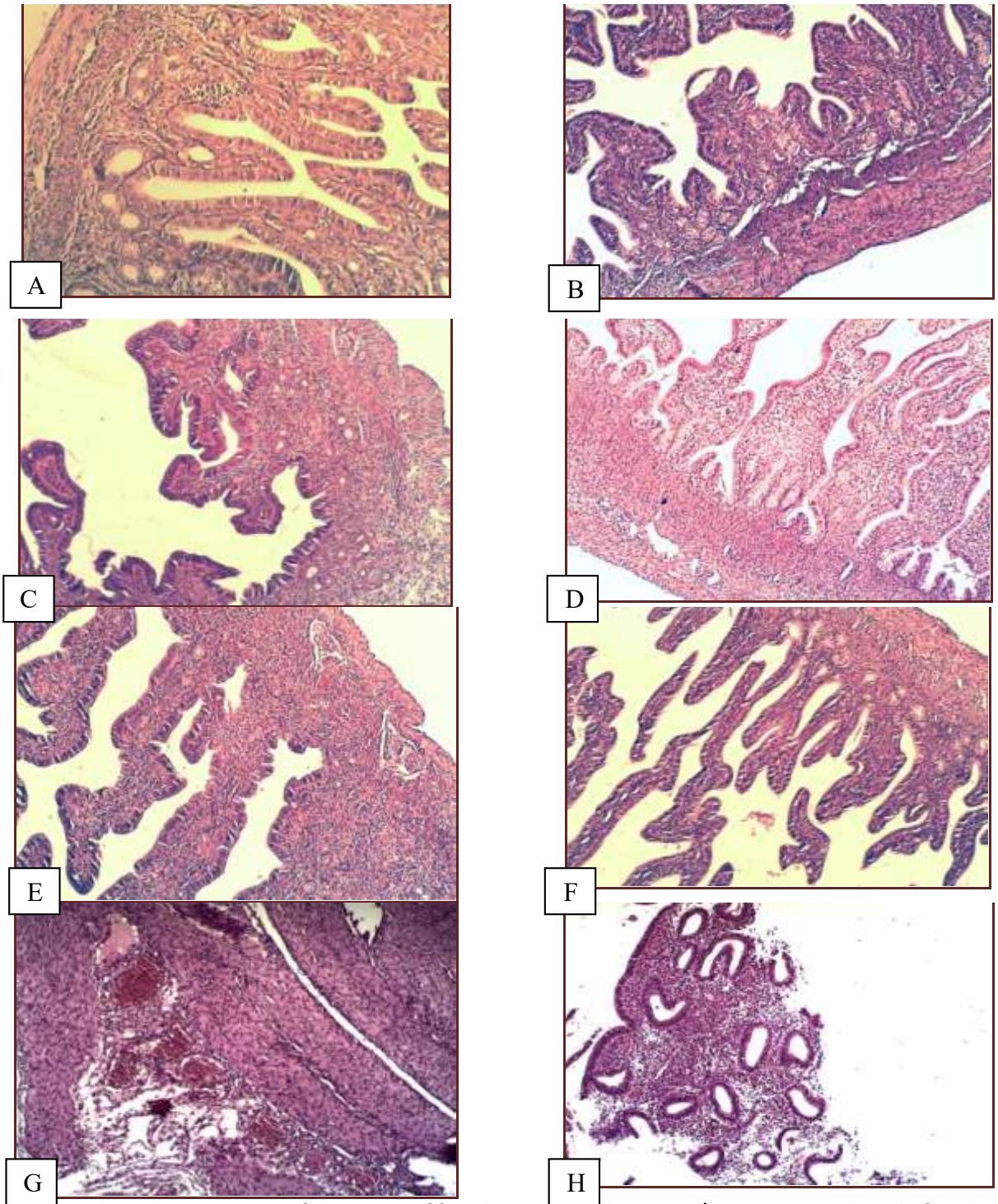

Figure 1. Histological preparations of the uterus of female mice: A (subgroup Aa) - intact control on the first day after fertilization, a large number of glands, thickened endometrium; B (subgroup (B1b) - large epithelial cells of glands with light cytoplasm, a large number of basophilic leukocytes in the stroma; C (subgroup BI) - pathologically altered vacuolated muscle fibers of the myometrium, enlarged lumen of single uterine glands BI cellular stroma of the endometrium, coarsening of the collagen component of the uterine mucosa; D (subgroup BIId) - paresis and blood stasis in the deep layers of the mucosa; E (subgroup BIIIb) - cells of the integumentary epithelium dystrophically altered, increasing the number of muscle fibers; F (subgroup BIIIc) - vessels are paralytically dilated, yawning, malnutrition of myometrial muscle fibers is determined; C (subgroup BIIId) - cystic formations in the uterine mucosa, diapedetic hemorrhages.

Table 1. Embryological data in women in the treatment cycle of fertilization *in vitro*

Indicator	Women with OHSS		Women without OHSS
	Group I (n=39)	Group II (n=39)	Group KI (n=30)
Number of aspirated follicles	30,00±0,73 [kl]	30,08±0,77 [kl]	14,90±0,41
The number of oocytes obtained	24,46±0,65 [kl]	19,46±0,77 [kl]	11,50±0,41
% oocytes from aspirated follicles	81,38±1,33	65,20±2,38 [kl]	77,37±2,05
Number of mature oocytes	13,85±0,39 [kl]	13,31±0,46 [kl]	9,43±0,40
% of mature oocytes from the total number of oocytes	58,40±2,17 [kl]	69,83±1,80 [kl]	81,77±1,41
Number of embryos	11,08±0,35 [kl]	10,85±0,34 [kl]	7,70±0,44
% of embryos from the total number of oocytes	80,49±1,80	82,88±2,05	81,29±2,47
Number of embryos of excellent and good quality	7,67±0,34 [kl]	7,13±0,33 [kl]	5,63±0,40
% of embryos of excellent and good quality of the total number of embryos	69,60±2,41	66,42±2,55	71,69±2,66
Note. [kl] - statistically significant difference with the indicators of the group KI, p <0,05.			

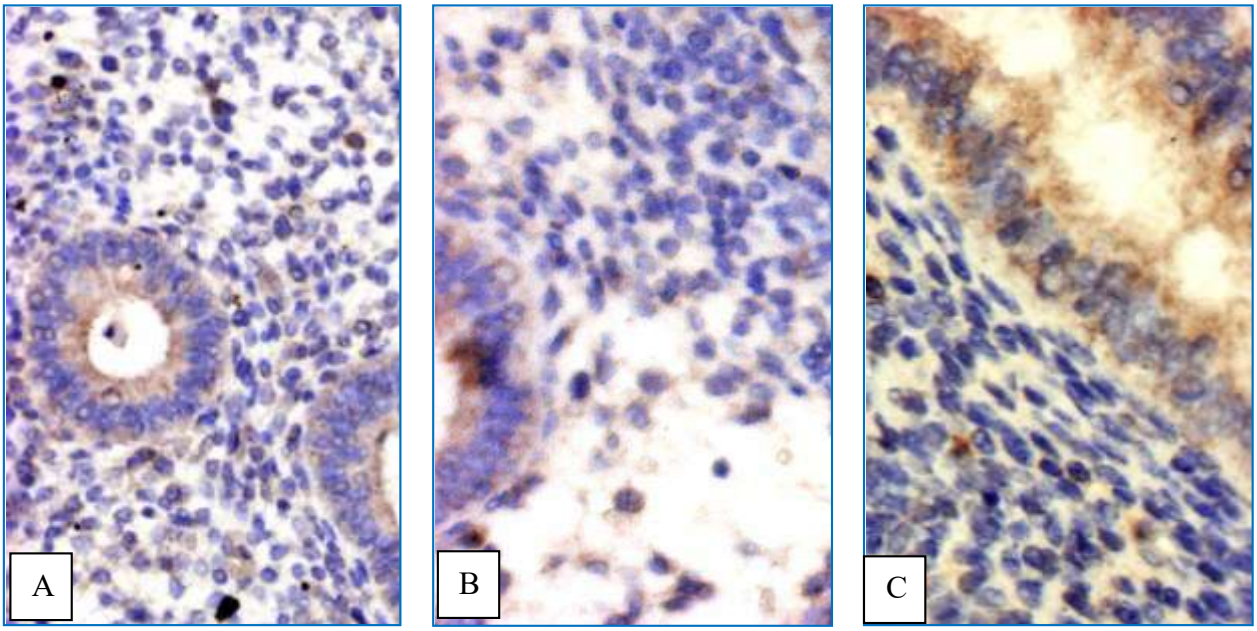

Figure 2. Expression of VEGF in the endometrium during the expected window of implantation in a healthy fertile woman of group KII (A), in a patient in the treatment cycle of *in vitro* fertilization in the group with OHSS (B) and in women of group KI in the treatment cycle of *in vitro* fertilization without OHSS (C). IHC with MAB against VEGF, magnification ×300.

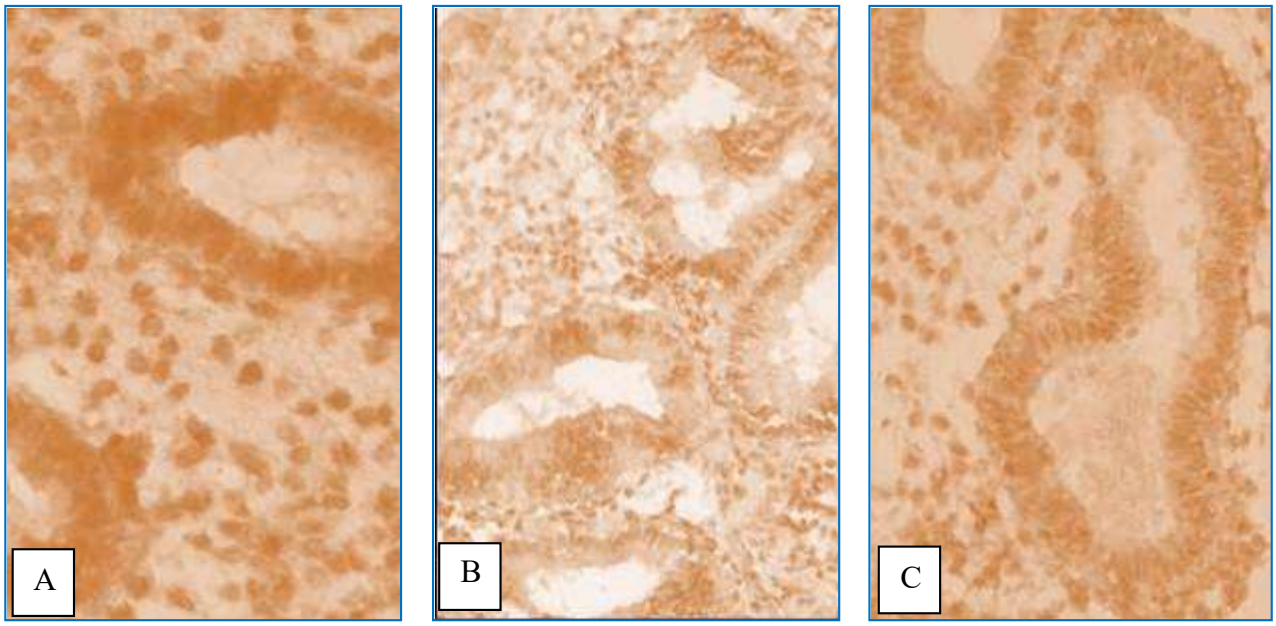

Figure 3. Expression of iNOS in the endometrium during the expected window of implantation in a healthy fertile woman of CII (A), in a patient in the treatment cycle of *in vitro* fertilization with OHSS (B) and in women of the group KI in the *in vitro* fertilization cycle without OHSS (C). IHC with PAB to iNOS, magnification ×400 (A); magnification ×300 (B, C).