Evaluating the Efficiency of Immunohistochemical Methods in Diagnosis of Endometrial Status in Women with Uterine Infertility

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The aim of the investigation was to evaluate the efficiency of immunohistochemical methods of diagnosis in complex examination of women with uterine factor infertility.

Materials and Methods. A total of 100 patients aged 27 to 42 years were examined, in 59 of the women immunohistochemical markers (ER, PR, Ki-67, CD34, CD56, CD68, CD138, VEGF) were revealed.

Results. The study showed significant reduction of CD56-positive cells in all the women, decreased or absent CD138 expression, increased CD34 expression and abnormal VEGF expression. Thus, immunohistochemical markers may serve as additional criteria for evaluation of receptor activity, the adequacy of proliferative and reparative functions of the endometrium prior to pregnancy planning.

Key words: infertility; implantation; immunohistochemical examination; endometrial hyperplasia; chronic endometritis; intrauterine synechiae.

Female infertility remains one of the most important problems of modern medicine. According to epidemiological research data, the incidence of infertility fluctuates from 8 to 18% worldwide, and in Russia it amounts to 17.5% with no tendency to decline [1–3]. The uterine factor in the structure of infertility occupies one of the leading positions (50%) along with tubal and peritoneal factors (70%) and endometriosis (46.5%) [4, 5]. Abnormalities in the embryo implantation process at the stage of blastocyst occurring in the middle of the luteal phase (days 19–24 of the menstrual cycle) underlie infertility pathogenesis in uterine pathology [6, 7]. At present histologic and functional assessment methods are applied to study the structure and functions of the endometrium [8–10], however, they provide no opportunity to identify the deep mechanisms of interaction between the ovum and the uterus that ensure pregnancy occurrence and progression throughout its duration. To improve the quality of differential diagnosis of endometrial status, we decided to explore the possibilities of using immunohistochemical methods.

The aim of the investigation was to study the efficiency of determining immunohistochemical parameters for evaluation of endometrial status in women with the uterine form of infertility caused by intrauterine pathology.

Materials and Methods. Complex examination of 100 women with impaired fertility, aged 27 to 42 years, was carried out in V.F. Snegirev Clinic of Obstetrics and Gynecology of I.M. Sechenov First Moscow State Medical University during the period from 2012 to 2014. Forty-six of the patients had endometrial hyperplasia, 35 women had intrauterine synechiae, 19 were diagnosed with chronic endometritis.

The criterion for the patients to be included in the study was the absence of pregnancy in conditions of regular sexual activity without contraception during 1 year or more, against the background of intrauterine pathology.

The study complies with the Declaration of Helsinki (adopted in June 1964, Helsinki, Finland and revised in October 2000, Edinburgh, Scotland) and was performed following approval by the Ethic Committee of I.M. Sechenov First Moscow State Medical University. Written informed consent was obtained from every patient under study.

General clinical methods of examination

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included: clinical and biochemical blood analysis, hemostasiogram, general urine analysis, microscopical and bacteriological examination of flora from the cervical canal and the vagina.

Instrumental methods included colposcopy, hysterosalpingography, transvaginal ultrasound examination of the pelvic organs, hysteroscopy, endometrial biopsy with subsequent morphological and immunohistochemical study of scrapings.

Transvaginal ultrasound examination was performed using the Toshiba SSH-140A ultrasound machine (Toshiba, Japan) with 6.5 MHz curved linear transvaginal transducer. There was assessed the size of the uterus, the structure of the endo- and myometrium, the endometrium thickness, the size and echostructure of the ovaries, presence or absence of functional lesions in the ovaries. The size, quantity, localization and echostructure of pathological mass lesions were also evaluated.

For final confirmation of intrauterine pathology, all the patients underwent office hysteroscopy on days 5–9 of the menstrual cycle. Office fiber optic hysteroscopes (Karl Storz and Rudolf, Germany) 2.7 mm in diameter were used without bullet forceps and cervical dilatation. The cervical canal and uterine cavity shape, the status of cervical mucous membrane, the endometrium, the tubal ostia were evaluated.

Hysterosalpingography was performed to estimate the state of fallopian tubes more accurately.

Morphological study of bioptates was performed in the Inter-Clinic Laboratory of Molecular Diagnostics of I.M. Sechenov First Moscow State Medical University. The bioptates and scrapings of the endometrium and tissue were fixed in 10% neutral buffered (phosphate) formalin and embedded in paraffin. Total fixation and embedding time did not exceed 24 h. No less than ten step sections, each 4 μm thick, were made from each block and stained subsequently with hematoxylin and eosin. The condition of the uterine glands and the stroma, the presence of fibrosis, inflammatory infiltration, the quantity and caliber of spiral arteries, their wall thickness were evaluated in the stained preparations.

Immunohistochemical study was performed using two-stage streptavidin-biotin-peroxidase method and antigen retrieval with standard sets of monoclonal antibodies (DAKO, USA). The following immunohistochemical markers were identified: receptors ER and PR, Ki-67, CD34, CD56, CD68, CD138, vascular endothelial growth factor (VEGF). Reaction manifestation was carried out using visualization system Dako Cytomation (USA). Biotin-free detection system Super Sensitive Polymer-HPR Detection System (Biogenex, USA) was applied to visualize primary antibodies.

The results of receptor reaction to estrogen and progesterone were identified based on nuclear or membrane cell staining for corresponding markers, assessing the proportion of stained cells and cell staining intensity. Expression of receptors to estrogen and progesterone was evaluated using a three-point scale (weak, moderate and expressed).

To identify the level of Ki-67 antigen expression in the glands, the proliferation index was calculated: the ratio of stained cell nuclei quantity to the total nuclei number (percentage) in as many as 300 nuclei. Ki-67 expression in the stroma was assessed by calculating the quantity of stained nuclei in the field of vision at 400-fold magnification studying no fewer than 10 fields of vision.

CD34 and VEGF expression activity in the epithelium, endometrial stroma and vascular endothelium manifested itself as epithelial and endothelial cell membrane and cytoplasm staining. The results were evaluated semi-quantitatively, according to standard methodology: (+) up to 20% of positive cells, 2 points; (++) 20–40% of positive cells, 4 points; (+++) over 40% of positive cells, 6 points.

CD56, CD68, CD138 expression was assessed calculating positive cells at 400-fold magnification in 10 fields of vision and more.

Statistical processing of the material was carried out using licensed software Microsoft Excel 2010. Spearman rank correlation coefficient (p) was used as a calculation method in the present study.

Results and Discussion. Sixty-two of 100 examined women with impaired fertility were diagnosed with primary infertility and 38 with secondary infertility. Infertility duration amounted to 5.5±0.5 and 3.2±0.7 years, respectively.

Analysis of obstetric-gynecologic history in women with secondary infertility revealed that only 19 out of 38 patients (50%) had previous deliveries, with 17 women (44.7%) having one delivery, 9 women (23.6%) — two deliveries. Induced termination of pregnancy took place in 23 out of 38 women (60.5%), and in 15 of them (39.4%) it occurred more than twice. Spontaneous abortions were observed in the history of 11 women (28.9%), 6 of them (15.7%) had abortions more than three times. All the patients with primary and secondary infertility had gynecologic surgical interventions in their history (Figure 1).

Speculum examination of the cervix and extended colposcopy enabled revealing cicatricial deformity of the cervix in 8 cases and cervical ectopia in 10 patients.

Hysterosalpingography was performed in 60 women out of 100 (60%), 40 patients underwent this earlier in departments of assisted reproductive technologies prior to IVF (in vitro fertilization) and ET (embryo transfer). In 21 out of 60 women (35%) hysterosalpingography revealed filling defects of the uterine cavity, both partial and complete obliteration, which allowed diagnosing the presence of intrauterine synechiae.

Transvaginal ultrasound examination, which was performed during the first phase of the menstrual cycle, showed increased echogenicity, heterogeneous structure of the endometrium with multiple small anechoic inclusions.

Office hysteroscopy revealed inhomogeneously

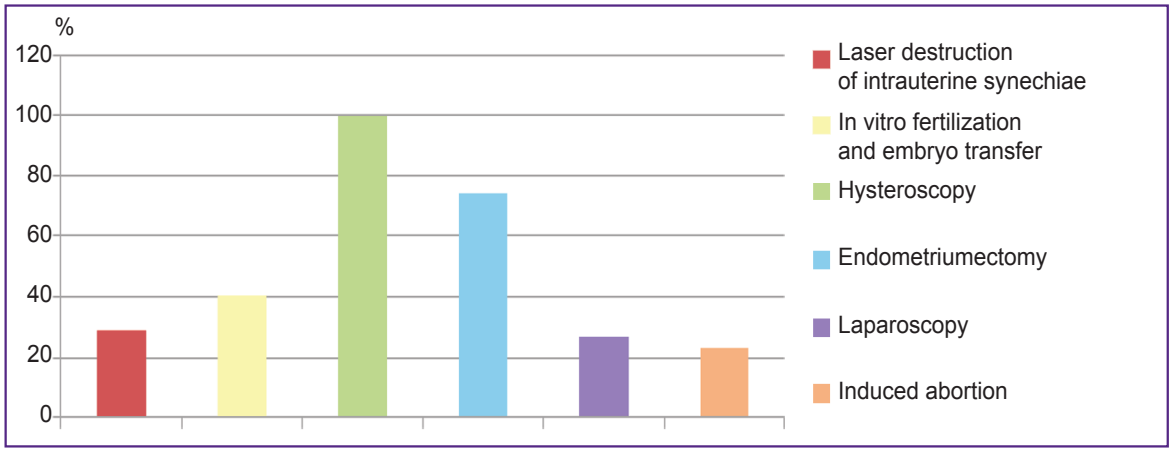

Figure 1. Frequency of gynecologic surgical interventions in women with primary and secondary infertility

thickened mucous membrane with accentuated vascular pattern in 46 women, tubal ostia were visualized. In 7 patients the uterine cavity was obliterated completely, intrauterine synechiae deforming 2/3 of the cavity were found in 4 women, intrauterine synechiae were visualized in the area of tubal angles in 5 women under study, and in the form of single fibrotic folds in 19 patients. In 19 patients there was observed hyperaemia, vulnerability of walls which bled when touched, there were visualized whitish or yellowish islands and hypertrophied, edematous mucous membrane of the uterus.

During hysteroscopy all the patients underwent an endometrial biopsy with subsequent histological analysis and in 59 cases immunohistochemical study of the uterine mucous membrane was performed.

According to the results of histological and immunohistochemical study of the endometrium, 59 patients were divided into four groups: group 1 (n=25) — patients with endometrial hyperplasia; group 2 (n=17) — with intrauterine synechiae; group 3 (n=10) — with chronic endometritis; group 4 (n=7) — with the endometrium in the proliferative phase. The morphological picture in group 1 corresponded to simple endometrial hyperplasia without atypia. There was observed increased number of both glandular and stromal elements, yet, there was no crowding of glands that had rounded shape and various size (Figure 2).

The morphological picture in group 2 was consistent with the diagnosis of intrauterine synechiae, the endometrium corresponded to the proliferative phase, its stroma being largely replaced by fibrous tissue, the glands presenting inactive prismatic epithelium of endometrial type, there were detected avascular tissues, stromal and glandular calcification.

The morphological picture in group 3 corresponded to chronic endometritis. There were observed multiple inflammatory infiltrates mainly consisting of plasmatic cells and lymphoid elements. Focal infiltrates appeared

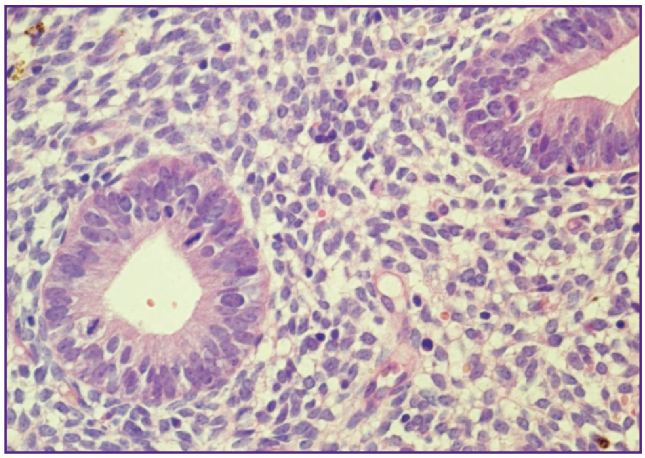

Figure 2. Endometrial hyperplasia; mitoses are seen in the epithelial cells; hematoxylin and eosin staining; ×400

as lymphoid follicles and were located not only in the basal, but in all other areas of the functional layer of the endometrium, consisting of leukocytes and histiocytes (Figure 3). Sclerotic changes in the walls of endometrial spiral arteries and focal stromal fibrosis suggested long-term chronic inflammation.

The morphological picture in group 4 demonstrated that the endometrium corresponded to the proliferative phase, the glands being small, uniformly distributed, the stroma loose, the vessels multiple with thin walls (Figure 4), which spoke of morphologically unchanged endometrium and allowed regarding this group as the control one. The findings of subsequent immunohistochemical studies of the endometrium confirmed their complete correlation with the morphological data.

The findings of immunohistochemical study of the endometrium in patients of all groups are shown in the Table.

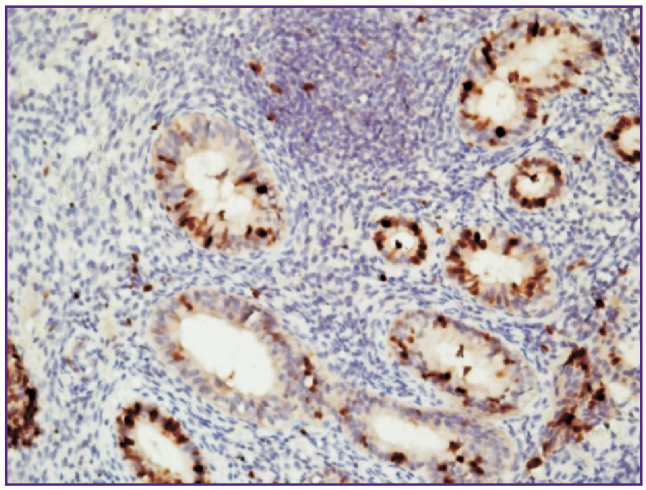

Figure 3. In the upper part of the preparation there is a lymphoid follicle with single dividing cells; immunohistochemical reaction; ×200

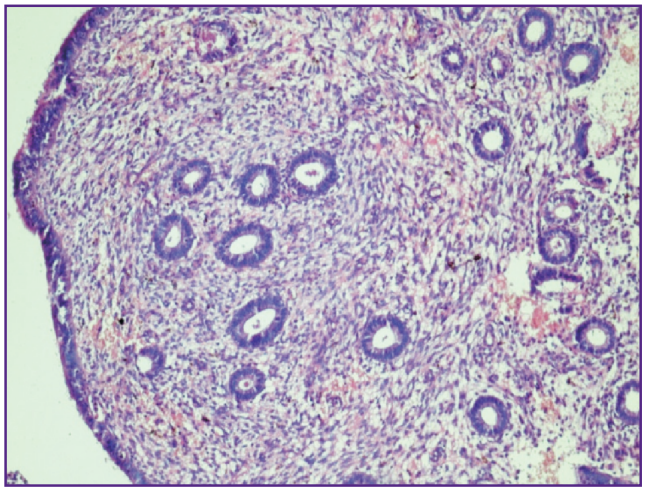

Figure 4. The endometrium in the early proliferative phase; hematoxylin and eosin staining; ×100

The findings of immunohistochemical study of the endometrium in patients of all groups

Indices	Group 1 (n=25)	Group 2 (n=17)	Group 3 (n=10)	Group 4 (n=7)
CD34*	2.1±0.5	2±0	2±0	2.5±0.8
CD56^{+}	45.2±2.1	37.8±2.1	43.2±3.8	77.3±2.1
CD68^{+}	59.5±1.4	35.3±1.6	40.4±1.5	41.8±1.1
CD138$^{\wedge}$	6.8±2.1	—	9.7±3.9	—
Ki-67 (gland)$^{\#}$	39.3±1.2	39.8±2.5	25.8±2.5	26.1±0.4
Ki-67 (stroma)$^{\wedge}$	23.3±2.7	16.3±2.3	22.6±0.7	13.3±1.1
ER$^{\square}$	96.0±2.0	92.9±3.2	77.8±2.4	93.5±2.1
PR$^{\square}$	90.0±3.5	87.4±3.0	74.2±1.3	95.6±1.4
VEGF*	2.0±0	—	2.0±0	2.2±0.7

N o t e s: * quantity of positive cells; $^{+}$ quantity of positive cells in the field of vision at ×400; $^{\wedge}$ quantity of stained stromal cell nuclei in the field of vision at ×400; $^{\#}$ proliferation index (the ratio of stained nuclei number to the total nuclei number in %); $^{\square}$ quantity of stained nuclei in the field of vision at ×400.

When comparing ER (estrogen receptor) and PR (progesterone receptor) expression, no statistically significant differences were found in groups 1, 2 and 4. In the course of investigation, the indices ER:PR were found to equal 1 in the proliferative phase ($p<0.05$). Heterogeneous expression of ER and PR receptors in the stroma and glands was noted, predominantly in the glands. Expression in the group with chronic endometritis (group 3) was decreased and amounted to 77.8 and 74.2%, respectively ($p>0.05$), speaking of impaired estrogen and progesterone receptor synthesis in glandular and stromal cells. Thus, in patients of this group, the endometrium became weakly responsive to estradiol and progesterone.

Uneven, low proliferative activity was found in patients of all groups. Ki-67 expression level was noted to be decreased both in the glandular epithelium and stromal cells ($p<0.05$). There was no correlation between the obtained data and receptor expression to estradiol and progesterone, which may suggest the presence of inadequate implantation window.

The study revealed significant decrease in major immunocompetent endometrial cells (CD56-positive cells) in all the groups ($p<0.05$), which showed as low cytotoxic activity (Figure 5) and reduction of macrophages CD68 in the endometrium ($p<0.05$), whereas the quantity of these cells is considered [5, 8] to increase in the first phase of the menstrual cycle to ensure normal implantation.

Expression level of plasmatic cell marker CD138 in the endometria of patients from group 1 was rather low and in patients of groups 2 and 4 CD138 expression was absent ($p<0.05$). This points to insignificant inflammatory changes in the endometria of patients from group 1 and the absence of inflammatory processes in groups 2 and 4. The obtained data speak of insignificant role of inflammatory endometrial changes in impaired implantation in patients with endometrial hyperplastic processes and intrauterine synechiae, while in patients with chronic endometritis increased CD138 expression is a factor preventing implantation and indicating the presence of inflammatory changes in the endometrium.

In all groups, expression of CD34 (a marker responsible for early stages of blood-forming) showed

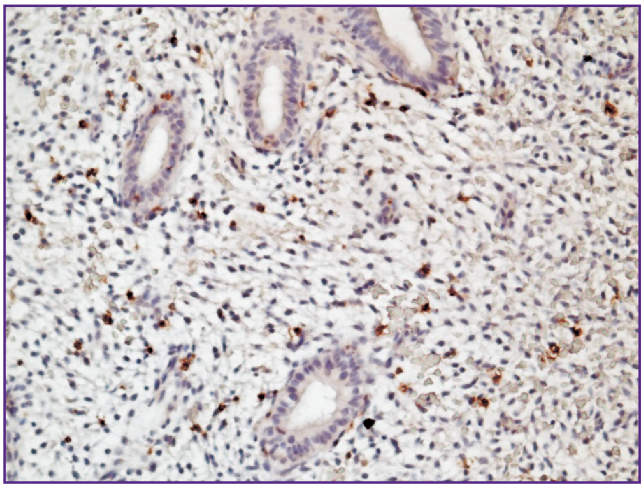

Figure 5. Scarce CD56-positive lymphocytes in the endometrial stroma; immunohistochemical reaction; ×200

as positive intensive reaction in the endothelium of the spiral arteries, multiple capillaries and stromal cells, which implied normal processes of vascular formation and adequate proliferation of endotheliocytes ($p<0.05$). However, in group 4 there were noted increased indices for the given stage of the menstrual cycle (proliferation), suggesting intensification of endotheliocyte proliferation processes in the basal layer and endometrial stroma. Normally, increased CD34 expression in the basal layer and endometrial stroma is noted in the second phase of the cycle. Thus, the patients of group 4 are unlikely to have fibrosis of endotheliocytes, though it may be present in patients of groups 1, 2 and 3.

VEGF expression in groups 1 and 3 ($p<0.05$) manifested itself as positive reaction in separate stromal cells and vascular endothelium, which suggested decrease in the processes of neoangiogenesis, causing insufficient blood supply and tissue hypoxia due to reduced oxygen diffusion. Low VEGF level leads to poor vascular formation which further reduces vascular blood flow in the endometrium. VEGF expression in group 4 manifested itself as positive reaction in all stromal cells and vascular endothelium, however, it was absent in group 2, which spoke of impaired neoangiogenesis in patients with intrauterine synechiae.

Thus, immunohistochemical methods of diagnosis, included in complex examination of women with uterine pathology and infertility, allow us to better evaluate the status of the endometrium and verify its morpho-functional deviations. Changes in expression of ER and PR receptors, reduction of immunocompetent cells CD56 and macrophages CD68 as well as decrease in the processes of neoangiogenesis and subsequent tissue hypoxia clearly illustrate the picture of implantation process impairment, help evaluate the possibilities of its implementation. Low level of Ki-67 expression points to insufficient proliferative activity of the endometrium, which also interferes with implantation.

Conclusion. Immunohistochemical markers may serve as additional criteria for evaluation of receptor activity, the adequacy of proliferative and reparative functions of the endometrium prior to pregnancy planning.

Introducing immunohistochemical methods of examination into clinical practice offers the possibility to substantially improve the quality of diagnosing endometrial impairments in women with uterine infertility and administer adequate treatment.

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