EXPERIMENTAL BIOLOGY

Cytotoxic Activity of Endometrial Immunocompetent Cells Determines the Outcome of Embryo Implantation

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The data on the formation of blastocysts in culture during therapeutic cycles of extracorporeal fertilization and on cytotoxic index of the endometrium in women with different clinical pathogenetic variants of sterility are presented. Analysis of correlations of these parameters with the incidence of effective implantation and pregnancy revealed a strict correlation between cytotoxic activity of endometrial immunocompetent cells and human embryo implantation.

Key Words: *sterility; endometrial leukocytes; cytotoxic index; blastocyst; implantation*

Diagnosis and treatment of infertility remains a pressing problem because of high incidence of infertile marriages in Russia and other countries. In the structure of infertile marriages 20-30% cases are due to endocrine sterility, the same number due to endometriosis [3].

In vitro fertilization (extracorporeal fertilization: ECF) used in the treatment of infertility are a unique method for the detection of unknown aspects in the pathogenesis of diseases of the female reproductive system. Infertility in these diseases can be attributed to not only chronic anovulation, characteristic of these conditions, but also abnormalities of oocyte fertilization, changes in the preimplantation development of the embryo, and subsequent implantation disorders [1,8].

Optimal conditions for implantation are created by interactions between numerous signal and effector molecules produced by the fetus and maternal organism [2]. Therefore, the fact of implantation characterizes the quality of embryos (including those in the ECF program) and endometrial function, on the one hand, and the female reproductive function, on the other [5,14].

Endometrial immune system is an important component of local microenvironment of the embryo during the periimplantation period. Populations of lymphocytes infiltrating the endometrium and their cytotoxic activity markedly increase in the "implantation window" (period most favorable for blastocyst implantation) [11].

The defense functions of endometrial immunocompetent cells are determined by their appurtenance to subpopulations of lymphocytes with killer activity. $CD8^+$ and $CD56^+$ lymphocytes (large granular lymphocytes: LGL) predominate among leukocytes during the periimplantation period. These cells realize their cytotoxicity by the Fas-dependent or perforin-mediated mechanisms [10]. In addition, cytotoxic activity of endometrial LGL is determined by production of Th1 cytotoxins: α- and γ-IFN, IL-1, -2, and -4, TNF, and other molecules [11].

The imbalance in the regulation of immunocompetent cell activity and increase of cytokine production by these cells are the causes of implantation failure or

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disorders in the embryo development and early spontaneous abortions [12,13]. The balance between these processes can be evaluated by the ratio of $CD3^+$ and $CD8^+$ T-lymphocytes to endometrial $CD56^+$ LGL [7].

On the other hand, our knowledge on the role of embryonic and endometrial factors in the pathogenesis of implantation failure, including cases associated with endocrine imbalance, remains incomplete, which impedes pathogenetic therapy of sterility and prediction of the efficiency of accessory reproductive technologies.

We studied the implantation potential of the endometrium and embryos in women with different forms of infertility after treatment by ECF methods.

MATERIALS AND METHODS

Oocytes, embryos, and specimens of endometrial tissue were collected from 136 women of reproductive age with disorders of the menstrual and reproductive functions and 106 women with regular cycle suffering from tuboperitoneal sterility (group 1 control). No hormone therapy was carried out 3 months before the study. The patients' ages varied from 20 to 35 years (mean age 30±2 years), duration of current sterility 2-10 years (mean duration 7.1±0.6 years).

Insufficiency of the luteal phase (group 2) was diagnosed in all women of the reference group by functional and ultrasonic methods and measurement of progesterone level during phase II of the cycle. Functional hyperprolactinemia (group 4) without thyroid dysfunction was detected in 57 patients, mixed hyperandrogenia (group 3) in 36 patients, combined hyperandrogenia and hyperprolactinemia (group 5) in 7 women. External genital endometriosis mainly (stages I-III) was diagnosed in 36 patients (group 6). Repeated analysis of ejaculate specimens from sexual partners of these patients confirmed normospermia.

Immunohistochemical studies of the endometrium were carried out on days 6-9 and 20-22 of the cycle using endometrial tissue collected by aspiration biopsy with Pipell system (C.C.D.).

Immunohistochemical studies of CD3 (T lymphocyte surface antigen), CD8 (cytotoxic T lymphocyte surface antigen), and CD56 (LGL surface antigen) were carried out on paraffin sections of endometrial specimens with preliminary demasking of the antigen in the tissue, as described previously [6]. Monoclonal antibodies (Novocastra) were used in immunohistochemical reaction. The results were assessed by quantitative methods: CD3, CD8, and CD56 counts were determined as the mean number of cells expressing this antigen in three visual fields (×400).

Superovulation was stimulated in all therapeutic ECF cycles by gonadotropin-releasing hormone agonists (decapeptil daily) in combination with human menopausal gonadotropin (humegone, menogone). Pregnil (10,000 U) was used as ovulation inductor.

Oocytes and embryos were cultured routinely using Medi-Cult media and reagents. Two to four embryos were transferred into the uterus 72 h after puncture. The embryos left after the transfer were cultured for 48-72 h until the formation of blastocysts. This parameter was estimated in percent as the ratio of embryos forming blastocysts to the total number of embryos in this group.

The incidence of effective implantations in the group was estimated as the ratio of effective implantations to the total number of embryo transfers and expressed in percent, the incidence of pregnancies was estimated as the number of pregnancies confirmed by ultrasonic diagnosis to the total number of embryo transfers (in %).

All studies were carried out in accordance with biomedical ethics regulations, informed consent was obtained from all patients.

The data were processed statistically using Biostat software. The significance of differences was evaluated using Student, Mann—Whitney and *Z* tests and analysis of correlations.

RESULTS

The embryo takes an active part in the process of implantation producing effector compounds interacting with endometrial signal molecules and maternal immunocompetent cells [2]. It is obvious that the best-quality embryos, the formation of which is impaired in the majority of cases with female endocrine sterility, possess the highest implantation potential.

Decreased blastocyst formation in groups 3 and 6 patients in comparison with the control ($p<0.05$, Fig. 1),

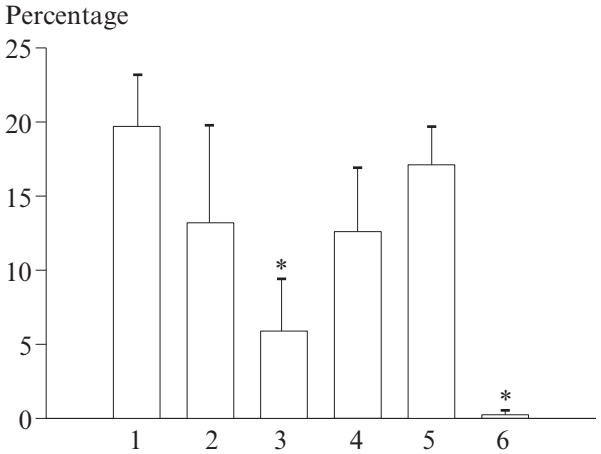

Fig. 1. Percentage of embryos forming blastocysts in the culture left after transfer. Here and in Figs. 2 and 3: *1*) group 1 (control); *2*) group 2; *3*) 3; *4*) 4; *5*) 5; and *6*) 6. *$p<0.05$ compared to the control group.

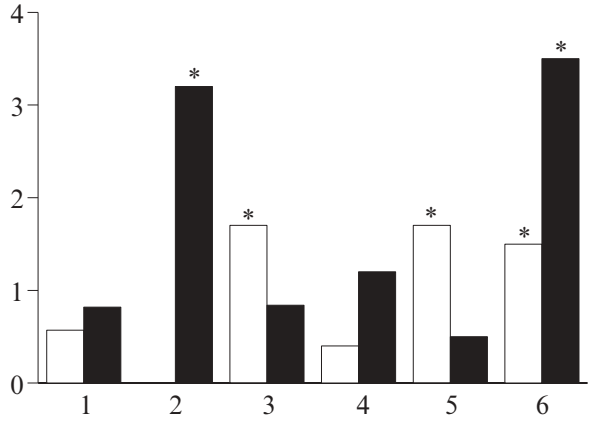

Fig. 2. Cytotoxic index of endometrium in clinical pathogenetic variants of female sterility on days 7 (light bars) and 21 (dark bars) of the cycle.

confirms the hypothesis on the direct effects of pathological factors forming this or that clinical pathogenetic variant of previous sterility on the type of the preimplantation embryogenesis. These effects are realized, among other things, through impairment of hormonal regulation of folliculogenesis processes leading to the formation of deficient oocytes (from the viewpoint of their development potential). These findings are in line with previous data [1] indicating that embryos from women suffering from endometriosis and patients with polycystic ovaries and chronic anovulation most often undergo pronounced fragmentation of blastomers and rarely reach the blastomer stage 8 on day 3 of culturing.

Blastocyst maturation in culture is often used as an indicator predicting the outcome of therapeutic ECF [14], and we therefore carried out analysis of correlations of this criterion with the incidence of pregnancy for all studied forms of sterility and detected a weak positive correlation (r=0.23; p>0.05) between these parameters.

These findings can be explained by the presence of chromosome abnormalities in these embryos leading to early abortion [15]. High incidence of early reproductive losses in this group indirectly confirms implantation of virtually nonviable embryos in cases with endometriosis [4].

The study of the cytotoxic index of endometrium ($CD3^+$+$CD8^+$/$CD56^+$) showed accumulation of cytotoxic lymphocytes in the endometrium in comparison with the control group: during phase II of the cycle in group 2, during phase I in groups 3 and 5, and during both phases of the cycle in group 6 (p<0.05; Fig. 2).

Evaluation of the incidence of effective implantations as the positive outcome of therapeutic cycle showed significant decrease (p<0.05) of this parameter in groups 2, 3, 4, and 6 in comparison with the control. The incidence of pregnancies was lower in group 2 (p<0.05; Fig. 3).

The incidence blastocyst formation did not decrease and the cytotoxic index of the endometrium remained unchanged in group 4 in comparison with the control, which corresponded to high incidence of pregnancy onset in this disease. The incidence of pregnancy onset in hyperprolactinemia was comparable to that in the control, which can be explained by intact mechanisms of steroidogenesis regulation in this disease, which plays the key role in the realization of the reproductive function and, specifically, in the preparation of the endometrium to implantation [5].

The lowest values of blastocyst formation and the highest cytotoxic index were observed in group 6, which corresponded to significantly lower incidence of effective implantations in these patients (p<0.05). According to some reports [9], oocytes in endometriosis develop under conditions of changed steroidogenesis, many-fold enhanced production of IL-1 and -6 and platelet growth factor-β, with decreased production of the vascular endothelial growth factor in the ovaries

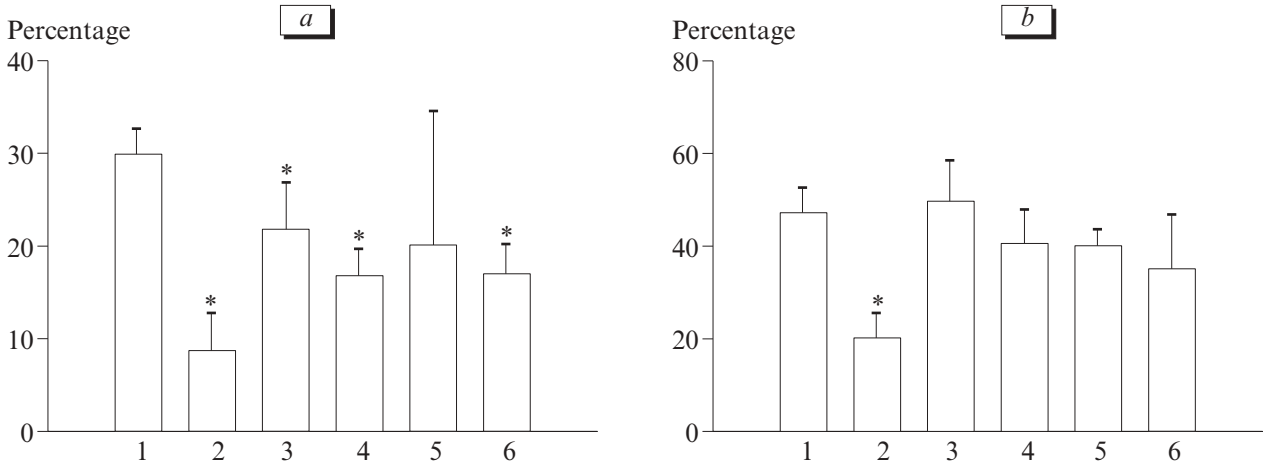

Fig. 3. Incidence of implantation (*a*) and pregnancy onset (*b*) in women with different clinical pathogenetic variants of sterility.

and high production of IL-6 and -12 in the endometrium. These factors deteriorate the quality of embryos in women with endometriosis, which was confirmed in some studies [1]. Moreover, endometriosis is associated with lower percentage of pregnancies eventuating in full-term delivery and higher incidence of stillbirth [4].

A strict negative correlation between the endometrial cytotoxic index and implantation (r=-0.72; p=0.16) and onset of pregnancy (r=-0.98; p=0.002) was observed in all groups of women.

Hence, the capacity of embryos to reach the stage of blastocyst is impaired in some forms of female sterility, the embryos in women with hyperandrogenism and endometriosis possessing the lowest potential for the formation of blastocysts. High-quality embryos in the ECF program is not the key factor for effective implantation, as the formation of blastocysts just slightly correlates with subsequent implantation and onset of pregnancy.

The endometrial factors, including accumulation of immunocompetent cells with cytotoxic potential towards the developing embryo, is the key factor in the pathogenesis of implantation disorders in the studied forms of sterility. This type of changes in the local immune system of the endometrium can lead to the formation of local humoral microenvironment incapable of supporting implantation of the embryo. In addition, the mechanisms of programmed cell death of the developing embryo with subsequent arrest of its development initiated by the endometrial cytotoxic lymphocytes can also have a negative impact on the outcome of implantation.

Hence, the predominant role of endometrial factors in disorders of human embryo implantation detected in our study prompts the development of methods of pathogenetic therapy of reproductive disorders aimed at correction of local endometrial immune system. In addition, creation of available methods for evaluation of endometrial immune status will appreciably improve the protocols and outcomes of therapeutic ECF cycles.

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