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Immunophenotypic profiles of peripheral blood lymphocytes on the day of embryo transfer in women undergoing in vitro fertilization

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Abstract: Evaluation of different types of lymphocyte subpopulations in the peripheral blood has unknown and controversial significance in diagnosis of infertility. The aim of the study was to evaluate selected blood lymphocytes in patients treated with intracytoplasmic sperm injection (ICSI). Materials and methods: women were divided into three groups: (1) control fertile group (n=18), (2) infertile women that achieved (n=32), and (3) did not achieve a pregnancy after ICSI (n=26). The following types of leukocytes were analyzed by three-colour flow cytometry by detection of specific CD antigens: lymphocytes T (CD3+), B (CD19+ and CD5+CD19+), T and B (CD5+), NK cells (CD56+CD16-, CD56-CD16+, CD56+CD16+, $CD56^{bright}CD16-$, $CD56^{dim}CD16+$). Additionally, the antigen of early activation (CD69) was evaluated on T, B and NK cells. The results were presented as a percentage and total counts of all lymphocytes. Results: The percentage of total NK cells (CD56+CD16+, CD56+CD16- and CD56-CD16+) did not differ between pregnant and non pregnant women and was lower comparing to control group. Fractions of CD56-CD16+ cells were higher in pregnant vs. non-pregnant women. The percentages of $CD56^{bright}CD16-$ NK cells were higher in control group comparing to both ICSI treated groups. Other fractions of lymphocyte subpopulations, including activated cells (with CD69 expression) did not differ between the analyzed groups. Total counts of CD56-CD16+ cells were higher in pregnant vs. non-pregnant group, and the $CD56^{bright}CD16-$ cells was more abundant in control group vs. women with unsuccessful ICSI. Conclusions: Testing of peripheral blood NK cells and the others lymphocytes has limited value as a prognostic factor in ICSI treated patients. The antigen of early lymphocytic activation (CD69) has not any predictive value in prognosis of ICSI outcome.

Key words: Lymphocytes - NK cells - IVF - Infertility - Reproductive failure

Introduction

Idiopathic infertility and IVF failure may have immunological background. However, the immunological assessment has a lot of limitations. Only a few examinations have significance in reproductive medicine. Evaluation of different types of lymphocyte subpopulations in the peripheral blood and in endometrial tissue has unknown and controversial significance in diagnosis of infertility or recurrent miscarriages [1,2]. Since there is no clear cause for at least of 20% of infertility cases, immunologists try to link reproductive failure with immunological background. Some laboratories offer infertile patients the evaluation of reproductive immunophenotypes. The count of total peripheral blood NK cells (CD56+CD16-, CD56+CD16+ and CD56-CD16+) should be lower than 12% of lymphocytes. However, some authors report higher normal levels, even up to 29% of lymphocytes [3,4]. On the other hand, several authors do not confirm that the elevated proportions of NK cells have significant influence on reproductive failure [5,6]. The fact that the endometrial NK cells subpopulation differs from peripheral blood NK cells subpopulation can additionally explain the limitation of such a examination. At the time of implantation, NK cells make up 70-90 % of lymphocytes in the endometrium [7]. These NK lymphocytes have significant expression of CD56 and lack of CD16 antigen ($CD56^{bright}CD16-$). They are less cytotoxic than the subpopulation with lower expression of CD56 ($CD56^{dim}CD16+$ or $CD56^{dim}CD16-$) that represents up to 90% of peripheral blood NK cells. The others peripheral blood NK cells have following

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Table 1. The fractions of different lymphocyte subpopulations in peripheral blood, presented as a percentage [%] of total lymphocytes.

Phenotypic subpopulations of lymphocytes	Control [%] n=18	Pregnant [%] n=32	Non-pregnant [%] n=26	p value
Total mononuclear lymphocytes	33.4	34.6	26.3	NS
CD3 (T lymphocytes)	73.4	65.8	62.6	NS
CD5 (T and B lymphocytes)	73.3 [a]	65.22	59.03 [a]	<0.05[a]
CD19 (B lymphocytes)	7.87	7.98	8.31	NS
CD5+CD19+ (subpopulation of B lymphocytes)	1.16	0.82	1.08	NS
CD56+ (NK and T cytotoxic lymphocytes)	17.55	15.76	11.63	NS
CD16+ (antigen on NK cells)	12.09	10.29	7.09	NS
CD56+CD16+ (subpopulation of NK)	10.48	8.43	6.00	NS
CD56+CD16- (subpopulation of NK)	7.41	7.33	5.62	NS
CD56-CD16+ (subpopulation of NK)	1.61	1.86 [a]	1.08 [a]	<0.05[a]
Total NK (CD56+CD16+.CD56+CD16- and CD56-CD16+)	19.6[a]	17.62	12.71[a]	<0.05[a]
$CD56^{bright}CD16-$ (NK cells with higher expression of CD56)	0.67[a]	0.14[a]	0.11[a]	<0.01[a]
$CD56^{dim}CD16+$ (NK cells with lower expression of CD56)	9.41	8.99	6.52	NS
CD3+CD4+CD69+ (activated T CD4 lymphocytes)	12.25	13.51	14.94	NS
CD3+CD8+CD69+ (activated T CD8 lymphocytes)	4.51	7.26	6.26	NS
CD19+CD69+ (activated B lymphocytes)	2.51	2.40	3.31	NS
CD56+CD69+ (activated NK cells)	2.50	2.15	2.32	NS
The fraction [%] of CD56+CD69+ to total CD56+	13.47	13.65	19.94	NS
The fraction [%] of CD19+CD69+ to total CD19+	32.50	30.07	39.83	NS

control - fertile patients; pregnant - patients that achieved pregnancy after ICSI; non-pregnant - patients that did not achieve pregnancy after ICSI; NS = not significant; [a]groups with significant differences ($p<0.05$)

phenotypes: CD56-CD16+, $CD56^{bright}CD16+$ or $CD56^{bright}CD16-$.

Lack of evidence that the NK cells has quite a limited value in diagnosis of infertility, causes that also other subpopulations of the lymphocytes or others markers are evaluated. Different panels (CD3, CD4, CD5, CD8, CD16, CD19, CD27, CD56, and CD94) and combinations of CD antigens are used in the studies to define the range of standard values and to determine their impact on reproduction [2,8,9]. That allowed to recognize the T (CD3+, D3+CD4+, CD3+CD8+), B (CD19+, CD5+CD19+), T and B (CD5+) lymphocytes and NK cells (with expressions of CD56 or CD16). Additionally, in the latest studies, an early activation antigen (CD69) was evaluated in this setting. It was reported that increased levels of CD69 positive cells can be responsible for reproductive failure [8]. Objective of the current study was to evaluate the expression of different types of immunophenotypes in lymphocytes of patients treated with intracytoplasmic sperm injection (ICSI).

Materials and methods

Fifty eight women qualified to ICSI with good outcome prognosis treated from November 2006 to May 2007 and 18 fertile women as a control group were enrolled in the study. Patients treated with ICSI were divided into two groups: pregnant women (n = 32) and women that did not achieve a pregnancy after ICSI (n = 26).

The inclusion criteria were: age of <35 years; normal gynecological, hormonal and anatomical state; concentration of the sperm >5 mln/ml; at least three good embryos on the third day of culture, evaluated 18-20h after ICSI procedure as an optimal zygotes according to Scott; easy embryo transfer. The history of abortions, PID, curettage, hydrosalpinx, endometriosis, endocrinological and metabolical diseases; ≥1 ICSI/ET procedure; gynecological interventions (leyomyomas, endometrial polyps, pelvic adhesions removal) were the exclusion criteria. The inclusion criteria for control group were: age of <35 years; normal gynecological, hormonal and anatomical state, confirmed fertility - minimum two deliveries, no history of miscarriages nor complications of the pregnancy.

IVF procedure. Long GnRH agonist protocol with triptorelin (Diphereline SR 3,75; Boufor Ibsen Pharma, France) or short GnRH antagonist protocol with cetrorelix 3 mg (Cetrotide; Merck Serono, Germany) along with recombined follitropin alfa (Gonal F; Serono Pharma, Switzerland) or menotropin (Menopur, Ferring Pharmaceuticalis, Switzerland) and human choriogonadotropin (Ovitrelle; Merck Serono, France) were used. Transvaginal oocyte retrieval was performed 36 hours after Ovitrelle injections under systemic analgesia. All patients underwent ICSI procedure. After oocyte retrieval luteal phase was supported by intravaginal progesterone (Luteina, Adamed, Poland) and oral administration of dydrogesterone (Duphaston, Solvay Pharmaceuticalis, Germany). The embryos were evaluated at the first day 18-20h after ICSI procedure according to the Scott criteria and on the 3rd day of culture. Transfer of two embryos on the 3rd day of culture was performed with Soft Frydman catheter.

Table 2. The total counts [n/μl] of different subpopulations of lymphocytes in peripheral blood.

Phenotypic subpopulations of lymphocytes	Control [%] n=18	Pregnant [%] n=32	Non-pregnant [%] n= 26	p value
Total mononuclear lymphocytes	2223	2450	2290	NS
CD19 (B lymphocytes)	173	195	190	NS
CD5+CD19+ (subpopulation of B lymphocytes)	27	21	25	NS
CD56+ (NK and T cytotoxic lymphocytes)	405	371	244	NS
CD16+ (antigen on NK cells)	242	237	149	NS
CD56+CD16+ (subpopulation of NK)	249	191	126	NS
CD56+CD16- (subpopulation of NK)	165	180	118	NS
CD56-CD16+ (subpopulation of NK)	34	46[a]	23[a]	<0.05[a]
Total NK (CD56+CD16+. CD56+CD16- and CD56-CD16+)	453	416	269	NS
$CD56^{bright}$CD16- (NK cells with higher expression of CD56)	14.7[a]	3.2[a]	2.3[a]	<0.001[a]
$CD56^{dim}$CD16+(NK cells with lower expression of CD56)	224	201	162	NS
CD3+CD4+CD69+ (activated T CD4 lymphocytes)	264	304	384	NS
CD3+CD8+CD69+ (activated T CD8 lymphocytes)	98	165	156	NS
CD19+CD69+ (activated B lymphocytes)	58	57	81	NS
CD56+CD69+ (activated NK cells)	61	47	44	NS

control - fertile patients; pregnant - patients that achieved pregnancy after ICSI; non-pregnant - patients that did not achieve pregnancy after ICSI; NS = not significant; [a]groups with significant differences ($p<0.05$)

Flow cytometry. Three-color flow cytometry (FACS Calibur) was used to evaluate different subpopulation of the mononuclear cells. The examination was done on the fresh blood on the day of the transfer. The differently labeled monoclonal antibodies in following combinations were used: CD3 PerCP, CD45 PerCP, CD5 FITC CD19 PE, CD16 FITC CD56 PE, CD19 FITC CD 69PE, CD56 FITC CD69 PE, CD56 FITC, CD4 FITC CD69 PE, and CD8 FITC CD69 PE. All the reagents were produced by Becton Dickinson (B-D Biosciences USA). The subpopulations of mononuclear lymphocytes with following CD antigens were analyzed: CD3, CD5, CD19, CD5+CD19+, CD19+CD69+, CD3+CD4+CD69+, CD3+CD8+CD69+, CD56+CD16-, CD56-CD16+, CD56+CD16+, CD56+CD69+ and depending on expressions of CD56 - $CD56^{bright}$CD16 and $CD56^{dim}$CD16+.

The results were presented as a percentage of total lymphocytes and after calculation as a total count in n/μL

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Ethical issues. All women gave informed consent according to the Medical Ethical Committee guidelines.

Statistical analysis. Non-parametric ANOVA was used to detect statistical differences in mean percentages and counts of leukocyte subpopulations. P value <0,05 was considered as significant. All calculations were performed using Statistica for Windows 7.1 (StatSoft Inc., Tulsa;USA).

Results

The mean age between women who achieved, did not achieve a pregnancy and control group did not differ significantly (31,6 years vs. 32,6 years vs. 32,4, respectively). The pregnancy ratio among ICSI-treated and included in the study patients was 55,1% and it was significantly higher comparing to 42% among all patients treated with ICSI between November 2006 and May 2007 (126 patients, 53 pregnancies). Levels of NK cells (total CD56+CD16+, CD56+CD16- and CD56-CD16+) do not differ between patients that achieved and did not achieve pregnancy after ICSI. Surprisingly, the level of total NK was significantly higher in control group in comparison to not-pregnant women (19,6% vs.12,71%).

Population of CD56-CD16+ was significantly higher in pregnant women comparing to non-pregnant (1,86% vs. 1,08%, respectively). The evaluation of CD56 expression revealed that the percentage of NK cells with high expression of CD56 ($CD56^{bright}$) is significantly higher in control group comparing to both pregnant and non-pregnant patients. Other subpopulations, such as a total mononuclear lymphocytes, CD3+, CD19+, CD5+CD19+, CD3+CD4+CD69+, CD3+CD8+CD69+, CD19+CD69+, CD56+CD69+ did not differ between analyzed groups of patients. Only percentage of lymphocytes T and B (CD5+) was higher in control group comparing to women that did not achieve pregnancy after ICSI. Results are shown in Table 1.

Total counts of analyzed subpopulations of lymphocytes differed significantly only in CD56-CD16+ subpopulation in pregnant and non-pregnant group (46 cells/μL vs. 23 cells/μL, respectively), but while compared to fertile patients the statistical significance was

not seen (34cells/μL vs. 46cells/μL and vs. 23cells/μL). Although the level of NK cells was higher in control and pregnant group than in non-pregnant group (453cells/μL vs. 416cells/μL vs. 269cells/μL, respectively) the differences were not significant. The evaluation of CD56 expression showed that in control group the level of NK cells with high expressions of CD56 was significantly higher when compared to pregnant and non-pregnant patients. The other subpopulations did not differ significantly between the studied groups of patients. Results are shown in Table 2.

Discussion

Many studies try to confirm connection between levels of certain lymphocyte subpopulations and reproductive failure. The best documented studies were performed on patients with recurrent miscarriages [1,4]. The increased levels of NK cells is proposed to be connected with higher risk of pregnancy loss and idiopathic infertility. Some authors consider that cut off value of 12% of NK cells should be recognized as a reference level [1,3,10]. In the current study the fraction of NK cells was increased - 17,62% in pregnant, 12,71% in non-pregnant women and surprisingly - 19,6% in fertile patients. The percentage of NK cells observed in our study was relatively high and reached the levels in recurrent abortions or in patients with repeated IVF failure reported in publications [10,11]. Surprisingly, the fractions of NK cells in our study was the highest in control group (fertile patients). Elevated levels of NK cells has been reported to be associated with lower pregnancy rates in assisted reproduction and higher risk of spontaneous abortion [1,3,12]. Our study did not confirm that thesis. This lack of usefulness of NK cells examination in patients with recurrent spontaneous abortions and in idiopathic infertility has recently been also shown in other studies [2,6] and in terms of reported NK counts cells corresponds to our results.

In our study, the percentage and the total counts of CD56+CD16- NK cells subpopulation was relatively higher than reported by other authors [12,13]. Some studies have shown that the levels of $CD56^{bright}CD16-$ cells may be used in diagnosis of idiopathic infertility and recurrent miscarriages because of its phonotypical similarity to the uterine endometrial subpopulation of NK cells. Increased levels of this subpopulation may be observed in fertile women when compared to infertile patients or those with recurrent spontaneous miscarriages [11,13]. But the study results are controversial and are not confirmed in randomized studies. In our study we showed that the total counts and the percentage of $CD56^{bright}CD16-$ in control group were significantly higher than in women treated either successfully or unsuccessfully with ICSI. The level of NK cells with low expression of CD56 ($CD56^{dim}CD16+$) was comparable in every studied group.

Recent studies try to assess the early activation marker on some types of lymphocytes. It has been reported that the elevated proportions of CD56+CD69+ are connected with infertility and recurrent abortions [5,8,12]. In the current study, the proportion of CD56+CD69+ to total CD56+ cells was high, and it was comparable to the fractions of CD56+69+ cells reported for infertile patients and patients with recurrent miscarriages. However, in our study we did not see the difference between fertile patients (control group) and both ICSI treated groups. Other studies reported that the levels of CD56+69+ in peripheral blood in fertile patients is significantly lower comparing to groups with recurrent spontaneous abortions and with infertility [11,14,16], however this has not been reflected in our experiments.

Other subpopulations of lymphocytes like lymphocytes T (CD3) or B (CD19) were similar in the studied groups, as it was also shown in other reports [9,15]. Also, the presence of early activation marker CD69 on the CD4, CD8 and CD19 positive cells did not differ significantly in both ICSI treated groups and control fertile patients. Also these sets of results were comparable to data reported by other authors, concluding that the fractions and levels of CD69+ positive cells did not correspond with any clinical form of the reproductive failures [2,16,17].

Selection of patients may limit conclusions originating from our study. Idiopathic infertility is a diagnosis from exclusion, and as such may not be clearly identified. Needless to say, such a limitation concerns vast majority of the published studies, therefore discordant results on the role of different subpopulations of blood lymphocytes in pathogenesis of reproductive failure will blur the true significance of immunity in human reproduction.

Nevertheless, also keeping in mind the limitations of the current study, our results adhere largely to data presented in numerous recent publications not recommending immunophenotyping as an important diagnostic tool in reproductive medicine [6].

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