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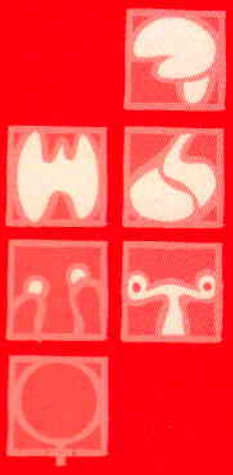

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ORIGINAL ARTICLE

Risk factors vary early preterm birth and perinatal complications after assisted reproductive technology

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Abstract

We conducted a study of markers of endothelial dysfunction and angiogenesis regulation, as well as the identification of the main lymphocyte populations, activated $CD3^+CD95^+$-cells and cytokine-producing $CD4^+IFN\text{-}\gamma^+$-, $CD4^+IL\text{-}4^+$-lymphocytes in the 1st trimester of gestation in women with ART-induced pregnancy and spontaneous pregnancy. We used the same indicators to assess the immune status of ELBW infants at birth and at the post-conceptual age of 38–40 weeks. It was determined that the risk factors of very early preterm delivery are: threatened miscarriage, chronic placental insufficiency, endothelial dysfunction, increased spontaneous production of intracellular cytokines. Adverse perinatal outcomes in ELBW infants from ART-induced pregnancy are associated with lower anthropometric measures, low Apgar scores high level of inflammatory infections (pneumonia), grade II intraventricular hemorrhage, movement disorders in the form of lower paraparesis. Immune status of those infants is characterized by the increase in the number of $CD8^+$- and $CD3^-CD16^+CD56^+$-lymphocytes, the expression level of Fas-receptor by T-cells, and the increased production of intracellular and serum IFNγ against the decrease in the number of $CD4^+$-cells, which indicates enhancing of cytotoxic effector potential and proinflammatory orientation of cell responses.

Keywords

Assisted reproductive technology, extremely low birth weight infants, induced pregnancy

Introduction

One of the key problems of modern reproductive medicine is establishing a direct link between the outcome of pregnancy, induced by assisted reproductive technology (ART), and the causes of infertility of the parents, together with analyzing the impact of a multi-step ART procedure on children's health and development rates [1].

According to published data, ART-induced pregnancy associates with multiple pregnancy, placentation disorders, higher risk of threatened miscarriage and preterm labor, fetal growth restriction, low birth weight, neonatal encephalopathy, and higher perinatal morality rate [2,3].

The incidence of obstetric complications and adverse perinatal outcomes, including preterm labor, is up to three times higher in induced pregnancy than after natural conception give reference! The results mostly associate with multiple pregnancy, and in case of singleton pregnancy – with older age of patients, together with aggravated somatic and obstetric and gynecological history [4–7].

According to some literature data, preterm infants born after assisted conception suffer from immune system insufficiency and slower maturation of humoral response [8]. The formation of the fetal and neonatal immune system is affected by hormonal disruptions in the endocrine system of the mother; the intensity of these disruptions determines the onset of complications during pregnancy that cause fatal and neonatal growth disorders [9].

Problems of perinatal pathology risk assessment and health status of extremely low birth weight (ELBW) infants, born after ART, were covered just in isolated studies [10].

Objectives: The objective of this study is evaluate the risk factors of very early preterm births, perinatal outcomes and immunological indicators in extremely low birth weight infants, born following ART.

Materials and methods

We examined 49 women who delivered at 23–28 weeks of gestation and their newborns. The 1st main group and the 1st subgroup consisted of 24 women with pregnancies, induced by ART and their ELBW infants. The 2nd main group and the 2nd subgroup consisted of 25 women with spontaneous pregnancies and their ELBW infants.

The comparison group (3rd group) consisted of 15 relatively healthy women and their full-term infants with uncomplicated neonatal adaptation period (3rd subgroup).

Exclusion criteria: women with multiple pregnancy, infants with malformations, genetic, or chromosome diseases.

We studied maternal past medical and obstetric and gynecological history, current pregnancy history, clinical examination and laboratory tests results of women (in the first trimester of pregnancy), and their newborn children (umbilical cord blood test at the first day of life and peripheral blood test at the

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postconceptional age (PCA) of 38–40 weeks). All women provided written informed consent for biological sampling.

The status of infants at birth was evaluated by the Apgar score, gases homeostasis, and anthropometric measures (body mass and length). We analyzed the structure of morbidity in the developmental period using pneumonography via X-ray unit TMX R+(GE Healthcare, Pittsburgh, PA), brain neurosonography according to standard methods via ultrasound scanner PHILIPS HD 15 (Burlington, MA). We evaluated the degree of central neural system affliction by ultrasound picture according to the Classification of Perinatal CNS lesions by Association of perinatal medicine specialists (Russian Association of perinatal medicine specialists, 2000)

Immunophenotyping of lymphocytes (CD3+, CD4+, CD8+, CD19+, CD16+/56+, CD3+CD95+, CD4+IFN-γ+, and CD4+IL-4+) was performed by flow laser cytometry analyzer FACS Calibur by «Becton Dickinson» (Franklin Lakes, NJ). The level of markers of endothelial function, angiogenic factors, and cytokines content in blood serum was evaluated by a ''sandwich'' principle of enzyme-linked immunosorbent assay (ELISA) using double antibodies in accordance with the recommendations of the sets manufacturers. The detection was performed via ELISA analyzer Wallac 1420 (Victor^2) by PerkinElmer (Turku, Finland) and Multiskan MCC/340 by Labsystems (Vantaa, Finland). The contents of Vascular endothelial growth factor (VEGF-A) and of VEGF soluble receptor (VEGF-1R) were detected using commercial test kits «Bender Medsystems» (Campus Vienna Biocenter 2, Wien, Austria); the concentrations of endoglin and of stable nitric oxide (NO) metabolites (endogenous nitrite total nitrite and nitrate) were determined by using reagents by R&D Systems (Minneapolis, MN); the endothelin level was measured using sets by Biomedica (Wien, Austria). The umbilical cord blood levels of IFN-γ and IL-4 were detected using test systems by ZAO Vector-Best (Novosibirsk, Russia).

Statistical processing of the study results was performed with the help of the application program packages «Microsoft Excel» (2007) and «Statistica for Windows 6.0» (StatSoft, Tulsa, OK). Quantitative data were presented by median (Me), the lower, and upper quartiles (25th and 75th percentiles, LQ-UQ). Testing of statistical hypotheses about the absence of intergroup differences of quantitative traits was performed using the nonparametric Kruskal–Wallis test. For indicators that characterize qualitative traits, we specified absolute value and relative value in percentage; verification of statistical hypotheses about the coincidence of the observed and expected frequencies was performed using Chi-square (χ^2) and Fisher's exact test. We applied Bonferonni adjustment in order to overcome the multi-sample problem. We settled the critical level of significant discrepancy (p), in which the null hypothesis of no difference was rejected and the alternative one was accepted, as equal to 0.017; while $0.05 < p < 0.017$ was considered as the tendency towards the change in the parameter.

Results

Median age of all examined women was comparable (29 (29.0–34), 30 (24–35), and 30 (30–33) years old in the 1st, the 2nd, and the 3rd group relatively, $p > 0.05$ in all cases). The percentage of multigravida women in the groups was 75%, 56%, and 80% relatively.

In the structure of extragenital pathology of women who delivered prematurely, cardiovascular diseases were observed in 37.5% and 20% of cases, endocrine disorders – in 25% and 24%, and urinary system problems – in 12.5% and 12% of cases ($p > 0.05$).

Obstetric history of all examined women was burdened with artificial abortions (62.5%, 40%, and 30%), spontaneous abortions (14.3%, 8%, and 0%), and fetal death during the previous pregnancy (0%, 8%, and 0%, $p > 0.05$ in all cases).

The current pregnancies in women who gave birth to children with ELBW were complicated with the threat of miscarriage (70.8% versus 64%, $p_{1-2} > 0.05$). Moderate preeclampsia was not presented in the first group, but it was presented in 8% of cases in the second group ($p_{1-2} > 0.05$). Severe preeclampsia was diagnosed in 37.5% women in the first group, versus 16% of women in the second group ($p_{1-2} > 0.05$). Violation of utero-placental blood flow of different severity was detected in 50% and 40% of women who delivered preterm infants ($p_{1-2} > 0.05$). The frequency of placental insufficiency (PI) in women who delivered children with ELBW was equal (75% against 52% in the second group, $p_{1-2} > 0.05$). Hypoamnion is the 1st and the 2nd group was diagnosed in 37.5% versus 20% of cases relatively ($p_{1-2} > 0.05$).

Endothelial function assessment shown that the patients who delivered ELBW infants after ART have the highest levels of endothelin-1: 2.36 (1.85–6.11) versus 0.22 (0.007–0.94) and 0.11 (0.004–0.27) fmol/ml in the 2nd and the 3rd groups ($p_{1-3} = 0.001$, $p_{2-3} = 0.021$).

The content of total nitrite ($NO2^-$) and nitrate ($NO3^-$) in women, delivered ELBW infants, was considerably lower the same indicators in the comparison group – 14.64 (13.78–16.26) and 12.8 (12.11–14.72) μmol/l in the first group, 15.56 (11.62–15.67) and 11.5 (10.04–15.64) μmol/l in the second group, versus 20.3 (17.27–23.55) and 18.56 (15.19–22.42) μmol/l in the third group ($p < 0.017$ in all cases). Women of the 1st group had lower levels of endogenic NO_2^- – 1.14 (0.45–1.66) versus 1.57 (0.41–2.41) and 1.8 (1.37–2.16) in the 2nd and the 3rd group. A significant increase in the production of one of the main vasoconstrictors, accompanied by a decrease in the release of vasodilators, ascertained on early stages of pregnancy in women from main groups indicates the endothelium functional state disorder in supraphysiological hormonal support in assisted pregnancy. Women of the second group demonstrated the tendency to the increase of endothelin-1 level associated with the significant decrease in total NO_2^- and NO_3^-. In assessing the production of key regulators of angiogenesis, we found that the women of the 1st group had significantly higher levels of proangiogenic VEGF, and in the 2nd group, there was an uptrend – 0.37 (0.12–5.72) and 0.38 (0.0–3.9) versus 0.07 (0.0–0.44) pg/ml in the comparison group. The level of anti endoglin factors and soluble receptor for VEGF in women of the 1st group was also significantly reduced – 4.75 (4.02–5.56) pg/ml and 0.06 (0.01–0.68) pg/ml versus 7.66 (6.36–10.08) ng/ml and 1.01 (0.71–1.87) pg/ml in the control group ($p < 0.017$ in all cases). In the 2nd group, we found no significant difference 7.66 (6.66–10.32) ng/ml and 0.47 (0.23–1.46) pg/ml. Abnormality of formation of placenta vasculature is one of the main factors leading to the implementation of pathological conditions. Thus, the increase in the number of preterm births in induced pregnancy can be associated with endotheliopathy which causes dysregulation of angiogenesis and implementation of vascular component mediated pathological conditions. Women from the 1st group have significantly increased levels of proangiogenic VEGF. Decreased release of antiangiogenic factors in induced pregnancy may indicate a strategy to neutralize the body's mechanisms for down-regulation in order to intensify the vascularization processes in the background of prevalence of vasoconstrictor effects.

Determination of the surface phenotype of peripheral blood lymphocytes showed that women from the main groups have disrupted cell-mediated immunological mechanisms of regulation of gestation. Thus, patients who gave birth to children with ELBW after induced pregnancy had higher levels of absolute number of leukocytes and lymphocytes, as compared with a group of women with uncomplicated pregnancy (Table 1). Absolute and relative number of B-cells exceeded the parameters of women who gave birth to full-term babies from spontaneous pregnancies, there was a significant increase in the number of natural killer cells.

Table 1. Immunological parameters of women who delivered children with ELBW from spontaneous and induced pregnancy, Me (LQ-UQ).

Parameters		Women who have given birth to newborns with ELBW conceived using ART (group 1, $n=24$)	Women who have given birth to newborns with ELBW of conceived naturally (group 2, $n=25$)	Women who have given birth in full-term newborns conceived naturally (group 3, $n=15$)	p
Leukocyte	10^9/l	7.95 (6.68–9.15)	6.6 (5.4–7.2)	6.25 (5.28–6.98)	$p_{1-3}=0.001$
Lymphocytes	%	34.0 (27.25–43.25)	30.0 (26.0–35.0)	38.0 (29.0–48.0)	$p_{2-3}=0.03$
	10^9/l	2.57 (2.07–3.33)	2.04 (1.63–2.3)	2.22 (2.06–2.73)	$p_{1-3,\ 1-2}=0.02$
$CD3^+$	%	67.0 (62.0–74.0)	74 (62.0–78.0)	71.0 (67.5–75.5)	$p_{1-3,\ 1-2}=0.04$
	10^9/l	1.76 (1.4–2.32)	1.52 (1.22–1.69)	1.53 (1.41–1.95)	$p_{1-2}=0.04$
$CD19^+$	%	11.5 (9.0–15.0)	9.0 (7.0–11.0)	9.0 (8.0–13.0)	$p_{1-3}=0.010$
					$p_{1-2}=0.014$
	10^9/l	0.31 (0.22–0.44)	0.19 (0.14–0.25)	0.21 (0.18–0.29)	$p_{1-3}=0.001$
					$p_{1-2}=0.001$
$CD3^+CD4^+$	%	38.0 (32.25–44.75)	44.0 (40.0–51.0)	39.0 (35.5–46.0)	$p_{2-3}=0.010$
					$p_{1-2}=0.010$
	10^9/l	1.01 (0.76–1.29)	0.91 (0.84–1.06)	0.85 (0.71–1.07)	
$CD3^+CD8^+$	%	25.0 (22.0–31.0)	23.0 (21.0–26.0)	28.0 (24.0–31.0)	$p_{2-3}=0.025$
	10^9/l	0.73 (0.5–0.89)	0.48 (0.39–0.72)	0.69 (0.5–0.8)	$p_{2-3}=0.025$
					$p_{1-2}=0.010$
$CD3^-CD16^+56^+$	%	16.0 (11.5–21.75)	18.0 (11.0–21.0)	13.0 (10.0–19.5)	
	10^9/l	0.41 (0.32–0.59)	0.29 (0.22–0.41)	0.32 (0.24–0.43)	$p_{1-3}=0.017$
CD4/CD8		1.46 (1.16–1.89)	2.16 (1.74–2.32)	1.38 (1.16–1.78)	$p_{2-3}=0.010$
					$p_{1-2}=0.010$
$CD4^+IFN\text{-}\gamma^+$, %	Spont.	4.0 (2.28–6.1)	6.68 (2.86–10.1)	3.41 (1.8–3.91)	$p_{1-3,\ 2-3}=0.010$
	Ind.	7.82 (4.49–11.08)	12.7 (6.22–15.7)	6.77 (4.83–9.11)	$p_{2-3}=0.010$
$CD4^+IL\text{-}4^+$, %	Spont.	3.73 (3.01–5.23)	3.16 (1.94–5.6)	2.46 (1.61–3.86)	$p_{1-3}=0.010$
					$p_{2-3}=0.04$
	Ind.	7.32 (5.05–10.0)	5.06 (3.93–10.8)	5.55 (4.29–6.62)	$p_{1-3}=0.04$
Polarization Index, standard units	Spont.	1.04 (0.87–1.5)	1.38 (0.88–2.54)	1.07 (0.85–1.58)	$p_{1-2,\ 2-3}=0.03$
	Ind.	1.04 (0.71–1.3)	1.44 (1.14–2.76)	1.11 (0.99–1.74)	$p_{1-2,\ 2-3}=0.04$
IFN-γ, pg/ml		3.99 (0.46–4.99)	0.47 (0.08–4.67)	1.42 (0.18–3.11)	
IL-4, pg/ml		0.04 (0.0–0.77)	2.06 (1.04–2.86)	1.42 (0.64–2.38)	$p_{1-3,\ 1-2}=0.010$
IFN-γ/IL-4, standard units		3.27 (0.64–4.81)	0.09 (0.02–0.26)	0.11 (0.0–1.45)	$p_{1-3,\ 1-2}=0.010$

$p_{1-3,\ 2-3,\ 1-2}$ – level of significance of differences between groups (criterion Mann–Whitney). spont., spontaneous level; ind., induced level.

In the group of women who delivered ELBW infants after spontaneous conception, we observed a tendency to a reduction in the relative number of lymphocytes and the decrease of CD8+-cells. The percentage of T-helpers was higher than in groups of women with induced pregnancy and women delivered full-term infants, accordingly the patients from the 2nd group had increased immunoregulatory balance rates.

According to the intracellular cytokine production analysis, women from the main groups had increased spontaneous expression of IFN-γ+ and IL-4 + by T-helpers. Under cell stimulation women delivered ELBW infants after induced pregnancy demonstrated increased production of IL-4, and women with ELBW infants after spontaneous conception showed increased production of IFN-γ, in comparison with women, delivered full-term infants. This showed a significant functional reserve of the immune system in the production of regulatory cytokines in the case of induced pregnancy and of proinflammatory cytokines in case of spontaneous pregnancy.

In blood serum tests for cytokines, all groups of examined women showed low levels of IL-4 and IFN-γ, still women who delivered ELBW infants as a result of ART-induced pregnancy displayed statistically significant decrease of IL-4 level, compared with the results for the 2nd and the 3rd groups.

Studies have shown that gestational age of children in main subgroups at birth was comparable 26 (25.25–27.25) and 27 (25–27) weeks, respectively. According to the Fenton charts (2015), weight and length of ELBW infants corresponded with their gestational age in 50% and 44% of cases. Meanwhile, ELBW infants born following ART had lower birth weight 790 (647.5–908.75) versus 840 (790–950) r ($p<0.015$) and length 28.5 (26.5–31.25) versus 32 (30–34.25) cm ($p_{1-2}<0.0001$) that correlates with published data [11]. The share of birth through operative delivery was 75%, 58.3%, and 66.7% in the 1st, 2nd, and 3rd groups ($p>0.05$). Indications to preterm delivery included moderate and severe preeclampsia and subcompensation or decompensation of utero-fetal blood flow. Indications for urgent delivery (by cesarean section) in a group of relatively healthy women included high myopia and abnormal labor.

Infants from the 3rd subgroup were born full-term, in satisfactory condition, with birth weight and length correlating with their gestation age (birth weight 3580 (3122–3965) g, length 52 (50.5–54) cm, with the Apgar score on the 1st and 5th minute 7 (7–7)/8 (8–8) points, $p_{1-3}<0.0001$, $p_{2-3}<0.0001$ in all cases). Infants from the 1st subgroup were more often born in critical condition (37.5% versus 16% in the 2nd subgroup), which was reflected in significantly lower Apgar scores: 3.5 (3–4)/5.5 (5–6) versus 4(3–5)/6(6–6) points for infants from the 2nd subgroup ($p_{1-2}<0.017$). Their critical condition was caused by acute respiratory failure due to respiratory distress syndrome, neurological symptoms, the threat of realization of intrauterine infection, extremely low birth weight, and prematurity. All ELBW infants were on special protective nursing mode, with the use of infant incubators, oxygen therapy, parenteral nutrition, the calculation of fluids gastrogavage, limited manipulations.

Due to severe cardiorespiratory adaptation distress, all preterm infants needed respiratory support in early neonatal period. ELBW infants of 100% and 84% of were in artificial lung ventilation since birth ($p_{1-2}>0.005$). In 16% of cases, infants from the 2nd subgroup were in BNCPAP respiratory support.

Evaluation of neurological status in preterm infants showed the acute period of the perinatal hypoxic brain damage, which was recorded in 100% of cases in both main groups. According to

neurosonography, at the first 24 h of life all ELBW infants had severe cerebral ischemia in comparison with the rates of full-term infants, which correlates with the data by Russian authors [12]. Intraventricular hemorrhage of various severity was found equally frequently in preterm infants (25% versus 28% of cases, $p_{1-2}>0.05$). The incidence of inflammatory infections among infants born following ART was significantly higher (the incidence of radiologically and clinically confirmed pneumonia was 75% versus 36%, $p_{1-2}<0.01$, sepsis was detected in 33.3 and 12% of cases, $p_{1-2}=0.09$).

Anemia is one of the reasons that impair the quality of life of ELBW infants. It occurs so often that repeated blood transfusions are an integral component of the traditional treatment of this cohort of infants. Congenital anemia in ELBW infants was diagnosed in 37.5% and 32% of cases relatively ($p_{1-2}>0.05$). By the full-term post-conception age, the number of blood transfusions was higher in infants born following ART (2 (0–3) versus 0 (0–1) times, $p_{1-2}=0.05$). Infants of the 1st subgroup had longer period of hospitalization in neonatal intensive care and early rehabilitation unit (15 (3–29) versus 11 (7–21), $p_{1-2}>0.05$, and 77 (59–93) versus 64 (54.5–66.5) d relatively, $p_{1-2}=0.014$).

By the PCA of 38–40 weeks, moderate and severe bronchopulmonary dysplasia was diagnosed in 75% and 64% of preterm infants ($p_{1-2}>0.05$). Severe cerebral ischemia was detected in 100% of cases in both main groups. Grade II intraventricular hemorrhage (IVH) of was recorded more frequently in preterm born following ART – 25% versus 8% ($p_{1-2}=0.022$). IVH III was observed only in infants from the 2nd group (12%, $p_{1-2}>0.05$). Periventricular leukomalacia as a result of severe hypoxic CNS affection was 1.4 times more frequently diagnosed among infants born following ART (62.5% versus 44%, $p_{1-2}>0.05$); here movements disorders in the form of lower paraparesis (40% versus 12.5%), which correlates with the data by foreign authors [13]. The incidence of retinopathy III had no significant difference (66.7% versus 40%).

Umbilical cord blood count showed leukopenia in all ELBW infants, which may be caused both by peculiarity of early ontogenesis and by leukopoiesis defect in bone marrow under intrauterine distress. The lowest number of white blood cells and the highest number of lymphocytes was observed in infants from induced pregnancies, compared with the infants from the 2nd and 3rd subgroups (Table 2). The relative content of lymphocytes in 1st subgroup infants was higher than in infants born after spontaneous pregnancy. The absolute number of $CD4^+$-cells was significantly lower. We should note the decreased $CD4^+/CD8^+$-cells ratio and the increased number of $CD8^+$- and NK-lymphocytes in 1st subgroup infants, compared with 2nd and 3rd subgroup infants. The increase in the number of cells expressing the marker of readiness to programmed cell death, in 1st subgroup infants indicates the elevated circulation of clones of activated lymphocytes in blood.

Assessing the production of intracellular cytokines in infants born following ART-induced pregnancy, we observed the decrease in relative number of $CD3^+IL\text{-}4^+$-cells compared with the rates of 2nd and 3rd subgroups, and a shift of the immune response towards Th-1 (IFN-γ/IL-4). The content of serum IFN-γ in 1st subgroup infants exceeded the rates of ELBW infants born from spontaneous pregnancy and infants in the comparison group 2.5 and 4 times. The level of IL-4 in infants born from induced pregnancy was 5.5 and 7.7 times lower than the similar parameters of the 2nd and 3rd groups, resulting in the increase of IFN-γ/IL-4 rate.

At the full-term PCA, peripheral blood of infants from main groups showed significant leucopenia and relative lymphocytosis (Table 3), indicating a high risk of the implementation of infection in preterm infants. The number of T-cells was significantly decreased in all ELBW infants. Infants born following induced pregnancy showed the decrease in the absolute number of T-helper cells. We observed the increase in the number of B-cells in the group of ELBW infants born from spontaneous pregnancy,

Table 2. Populations and subpopulations of lymphocytes and parameters of spontaneous and induced production of intracellular cytokines cord blood of newborns with ELBW, Me (LQ-UQ).

Parameters		Newborns with ELBW conceived using ART (subgroup 1, $n=24$)	Newborns with ELBW conceived naturally (subgroup 2, $n=25$)	Full-term newborns conceived naturally (subgroup 3, $n=15$)	p
Leukocyte	10^9/l	4.9 (3.3–5.6)	5.85 (4.05–7.13)	12.47 (10.11–14.01)	$p_{1-2}=0.025$, $p_{1-3,\ 2-3}=0.001$
Lymphocytes	%	81 (75.0–92.0)	71.5 (65.5–79.0)	39 (33.5–47.5)	$p_{1-2,\ 2-3,\ 1-3}=0.010$
	10^9/l	4.51 (2.47–5.0)	3.68 (2.67–4.87)	4.9 (4.25–5.85)	
$CD3^+$	%	63.0 (55.0–68.0)	39.0 (31.0–65.25)	60.0 (37.5–64.25)	
	10^9/l	2.8 (0.96–3.11)	1.76 (0.85–2.78)	2.79 (1.94–3.21)	
$CD19^+$	%	15.0 (12.25–15.75)	12.0 (9.25–18.25)	11.05 (7.0–14.25)	
	10^9/l	0.67 (0.59–1.0)	0.45 (0.34–0.82)	0.50 (0.32–0.75)	
$CD3^+CD4^+$	%	28.0 (18.0–39.0)	26.0 (21.0–41.75)	35.5 (29.75–42.5)	
	10^9/l	0.87 (0.53–1.58)	1.17 (0.43–0.91)	1.79 (1.49–2.19)	$p_{1-3}=0.010$
$CD3^+CD8^+$	%	23.0 (20.75–24.5)	16.5 (13.25–21.8)	13.5 (11.0–17.25)	$p_{1-3}=0.010$
	10^9/l	1.8 (1.32–1.62)	0.50 (0.19–0.8)	0.75 (0.55–1.3)	$p_{1-3}=0.010$
$CD3^-CD16^+56^+$	%	25.0 (23.0–28.5)	12.0 (3.0–23.0)	11.0 (3.5–17.5)	$p_{1-2,\ 1-3}=0.010$
	10^9/l	1.34 (1.03–1.60)	0.25 (0.09–0.73)	0.37 (0.20–0.92)	$p_{1-2,\ 1-3}=0.010$
CD4/CD8		1.57 (1.33–1.62)	2.11 (1.63–2.74)	2.45 (1.78–2.42)	$p_{1-3}=0.017$
$CD3^+CD95^+$	%	11.0 (9.0–12.0)	3.0 (2.0–4.5)	3.0 (1.0–4.5)	$p_{1-2,\ 1-3}=0.001$
	10^9/l	0.34 (0.28–0.38)	0.11 (0.04–0.18)	0.13 (0.06–0.18)	$p_{1-2,\ 1-3}=0.001$
$CD4^+IFN\text{-}\gamma^+$, %	Spont.	3.26 (2.36–3.85)	2.44 (1.44–3.21)	3.28 (1.22–5.88)	
	Ind.	5.58 (4.25–8.8)	2.35 (1.2–3.17)	4.97 (3.24–7.38)	$p_{2-3}=0.04$
$CD4^+IL\text{-}4^+$, %	Spont.	1.98 (1.22–2.0)	2.85 (1.41–2.86)	3.18 (2.18–4.83)	$p_{1-3,\ 1-2}=0.001$
	Ind.	3.35 (2.94–5.77)	1.45 (1.13–2.43)	1.84 (1.08–4.45)	$p_{1-3}=0.001$
Polarization Index, standard units	Spont.	1.81 (1.52–3.2)	1.22 (0.84–2.4)	0.84 (0.52–2.2)	$p_{1-3}=0.025$
	Ind.	1.45 (1.06–2.58)	1.31 (1.23–2.11)	0.96 (0.57–1.4)	
IFN-γ, pg/ml		12.75 (9.56–13.25)	5.13 (1.11–6.25)	3.12 (1.36–4.44)	$p_{1-3}=0.001$
IL-4, pg/ml		0.38 (0.18–0.59)	2.1 (2.01–2.85)	2.93 (2.2–3.65)	$p_{1-3}=0.001$
IFN-γ/IL-4, standard units		59.1 (19.45–87.0)	2.2 (0.54–3.16)	1.16 (0.5–1.77)	$p_{1-3}=0.001$

$p_{1-3,\ 2-3,\ 1-2}$ – level of significance of differences between groups (criterion Mann–Whitney). spont., spontaneous level; ind., induced level.

Table 3. Populations and subpopulations of lymphocytes and parameters of spontaneous and induced production of intracellular cytokines of children with ELBW in PCA, Me (LQ-UQ).

Показатели		Newborns with ELBW conceived using ART (subgroup 1, $n = 24$)	Newborns with ELBW conceived naturally (subgroup 2, $n = 25$)	Full-term newborns conceived naturally (subgroup 3, $n = 15$)	p
Parameters	10^9/l	5.5 (4.25–6.2)	4.4 (3.2–4.5)	12.47 (10.11–14.01)	$p_{1-3,\ 2-3,\ 1-2} = 0.001$
Lymphocytes	%	69.0 (59.0–80.0)	83.0 (74.0–86.0)	39 (33.5–47.5)	$p_{1-3,\ 2-3,\ 1-2} = 0.010$
	10^9/l	3.1 (1.88–3.52)	3.78 (3.44–5.48)	4.9 (4.25–5.85)	$p_{1-3,\ 1-2} = 0.014$
$CD3^+$	%	60.0 (44.0–66.0)	58.0 (52.0–63.0)	60.0 (37.5–64.25)	
	10^9/l	1.86 (0.83–2.32)	2.68 (1.94–3.54)	2.79 (1.94–3.21)	$p_{1-3,\ 1-2} = 0.014$
$CD19^+$	%	17.0 (6.0–22.0)	25.0 (19.0–32.0)	11.05 (7.0–14.25)	$p_{2-3} = 0.010$
	10^9/l	0.53 (0.11–0.77)	1.36 (0.65–1.74)	0.50 (0.32–0.75)	$p_{2-3,\ 1-2} = 0.010$
$CD3^+CD4^+$	%	17.0 (16.0–41.0)	33.0 (27.0–39.0)	35.5 (29.75–42.5)	$p_{1-3,\ 2-3} = 0.001$
	10^9/l	0.53 (0.3–1.44)	1.39 (1.08–2.06)	1.79 (1.49–2.19)	$p_{1-3,\ 1-2} = 0.010$
$CD3^+CD8^+$	%	26.0 (26.0–44.0)	21.0 (16.0–32.0)	13.5 (11.0–17.25)	$p_{1-3} = 0.01$
	10^9/l	0.92 (0.49–1.36)	0.87 (0.74–1.28)	0.75 (0.55–1.3)	
$CD3^-CD16^+56^+$	%	18.0 (13.0–47.0)	12.0 (8.0–14.0)	11.0 (3.5–17.5)	$p_{1-3,\ 2-3} = 0.010$
	10^9/l	0.56 (0.46–0.89)	0.47 (0.38–0.72)	0.37 (0.20–0.92)	$p_{1-3} = 0.012$
CD4/CD8		0.62 (0.39–1.58)	1.6 (1.19–1.71)	2.45 (1.78–2.42)	$p_{1-3,\ 2-3,\ 1-2} = 0.013$
$CD3^+CD95^+$	%	9.0 (7.0–10..0)	5.0 (4.0–7.0)	3..0 (1.0–4.5)	$p_{1-3} = 0.012$
	10^9/л	0.18 (0.05–0.2)	0.085 (0.065–0.18)	0.13 (0.06–0.18)	
$CD4^+IFN\text{-}\gamma^+$, %	Spont.	4.1 (3.49–4.79)	3.93 (0.8–4.45)	3.28 (1.22–5.88)	$p_{1-3} = 0.010$
	Ind.	6.67 (6.31–7.77)	5.26 (4.31–6.87)	4.97 (3.24–7.38)	$p_{1-3} = 0.010$
$CD4^+IL\text{-}4^+$, %	Spont.	1.21 (0.99–2.06)	2.66 (2.0–4.54)	3.18 (2.18–4.83)	$p_{1-3} = 0.010$
	Ind.	2.71 (2.25–3.18)	0.84 (0.52–1.21)	1.84 (1.08–4.45)	
Polarization Index,	Spont.	3.25 (1.57–4.84)	2.22 (1.71–2.94)	0.84 (0.52–2.2)	$p_{1-3} = 0.010\ p_{2-3} = 0.03$
standard units	Ind.	2.42 (2.15–2.76)	2.42 (1.63–3.17)	0.96 (0.57–1.4)	$p_{1-3,\ 2-3} = 0.010$
IFN-γ, pg/ml		7.5 (6.5–8.75)	4.25 (1.31–7.79)	3.12 (1.36–4.44)	$p_{1-3} = 0.010$
IL-4, pg/ml		2.17 (2.05–2.29)	2.97 (2.07–3.41)	2.93 (2.2–3.65)	
IFN-γ/IL-4, standard units		3.82 (3.65–6.5)	1.85 (0.63–2.29)	1.16 (0.5–1.77)	$p_{1-3} = 0.010$

$p_{1-3,\ 2-3,\ 1-2}$ – level of significance of differences between groups (criterion Mann–Whitney). spont., spontaneous level; ind., induced level.

compared to the rates of infants from 1st and 3rd subgroup 2.6 and 3.2 times. Premature infants born from induced pregnancy remained high content of cytotoxic T lymphocytes and NK-cells compared with the control group rates. The content of activated cells expressing the CD95 + receptor in infants from the 1st subgroup, increased at birth, by PCA of 38–40 weeks decreased to the rates of full-term infants. We observed the increase of the relative content of $CD16^+CD56^+$ populations in infants from the 2nd subgroup. Spontaneous and induced level of IFN-γ^+-producing cells in 1st subgroup infants by the gestation age of 38–40 weeks significantly exceeds the rates of the comparison group. The production of intracellular IL-4 in the spontaneous test remained low.

Polarization Index of cytokine-producing cells in all ELBW infants exceed similar parameters of the 3rd comparison subgroup, and was shifted towards Th1-dependent immune response.

In the content of serum cytokines, we observed significant difference in infants born from induced pregnancy with the comparison group; in this group the level of IFN-γ was 1.7 and 2.4 higher than the rates of ELBW infants from spontaneous pregnancy and the comparison group; it resulted in an increased ratio of the main pro- and anti-inflammatory cytokines.

Discussion

The results of recent studies on the increase of efficiency of assisted reproductive technologies (ART) confirm that ART-induced pregnancies are associated with an increase in the incidence of gestational complications associated with the manifestations of endothelial dysfunction. Our study demonstrated that over 50% of women who delivered ELBW infants were diagnosed with placental insufficiency.

The induction of superovulation in *in vitro* fertilization and embryo transfer programs is associated with significant exogenous hormonal load on the woman's body.

According to published data, the maintenance of induced pregnancy with high doses of hormones has a direct effect on the endothelial function and the regulation of angiogenesis in the formation of the fetoplacental complex [13,14]. Our results show that the 1st trimester of all women delivered ELBW infants was characterized by the imbalance of vasoconstrictors and vasodilators (an increase in the endothelin-1 level against the background of a general reduction of nitrite and nitrate). In case of ART-induced pregnancy, these changes are accompanied by more prominent endothelin-1 production, reduction of endogenous nitrite, dysregulation of angiogenesis: an increase in the concentration of proangiogenic (VEGF) and a decrease in the level of anti-angiogenic (sVEGF-R1 and endoglin) factors.

Complications of pregnancy: threatened miscarriage at different gestational age, infertility, and ART failures in particular, are also associated with the increase in the content of natural killer cells and the increase in the number of T-cytotoxic lymphocytes and B cells [15,16].

The studies showed that the immune status of women in the early stages of pregnancy following ART is characterized by an increase in the absolute number of leukocytes, lymphocytes, and the number of B and NK-cells. The number of basic lymphocyte subpopulations in women who delivered preterm infants after spontaneous pregnancy did not differ from that of the comparison group, except for significantly increased percentage of T-helper cells and a tendency of decrease in cytotoxic T-lymphocytes, while the level of immunoregulatory index was significantly higher than that in women with induced pregnancy.

Assessing intercellular and serum cytokines we established an increase in the relative number of T-helper cells producing IFN-γ and IL-4 and decreased serum IL-4 in ART-induced pregnancy. Meanwhile, the polarization index of cytokine-producing cells was not significantly different, but the concentration of Th-1 and Th-2 cytokines ratio (IFN-γ/IL-4) in blood serum of patients after ART indicated the predominance of cell-mediated immune

response in early pregnancy, which is consistent with the literature. Women who delivered premature infants after spontaneous conception displayed increased production of IFN-γ by CD4+-cells in both spontaneous and stimulated tests.

The factors determining the clinical condition and the immune response of infants were the high incidence of obstetric and somatic diseases in women conceived via ART; endothelial dysfunction and angiogenesis disorder revealed at early stage of pregnancy, as well as the peculiarities of immune regulation under hormonal support.

Infants delivered following ART-induced pregnancy had significantly lower anthropometric measures, lower Apgar scores, and a higher risk of inflammatory infections.

The immune status of infants delivered after induced pregnancy was characterized by a more severe leukopenia, relative lymphocytosis, an increase in the number of cytotoxic cells ($CD8^+$ and $CD16^+CD56^+$), and susceptibility of T-lymphocytes to apoptosis coupled with the decrease in the absolute number of $CD4^+$ lymphocytes at birth; these deviations remained by the full-term PCA. The reduction of spontaneous production of regulatory intracellular and serum cytokines (IL-4) as well as the increased level of IFN-γ in umbilical cord blood in these infants indicated a shift of the cytokine balance (IFN-γ/IL-4) in the direction of Th-1-dependent immune response. However, higher concentration of $CD4^+IL\text{-}4^+$-cells in the stimulated test in children delivered after induced pregnancy showed significant functional reserve of the immune system in terms of the synthesis of regulatory cytokines. By the PCA of 38–40 weeks, the spontaneous expression level of $IL\text{-}4^+$ by T-helper cells in ELBW infants born from ART-induced pregnancy, remained significantly low; while there was increased production of intracellular and serum IFN-γ. However, the content of IL-4 in peripheral blood reached the level of full-term infants.

The absolute number of leucocytes in umbilical cord blood in ELBW infants from spontaneous pregnancy was significantly higher than in full-term infants. By the full-term PCA, those infants still had leukopenia and relative lymphocytosis; the number of subpopulations of T-cells did not change, the percentage of B-cells was increased. There was no significant difference in the number of cytokine-producing cells and in serum cytokines compared with the rates of full-term infants during the whole observation period, except for the polarization index ($CD4^+IFN\gamma^+/CD4^+IL\text{-}4^+$) that was increased at PCA of 38–40 weeks in case of stimulated cells.

Thus, the risk factors of very early preterm delivery are the following: threatened miscarriage, chronic placental insufficiency, endothelial dysfunction, increased spontaneous production of intracellular cytokines ($CD4^+IFN\gamma^+$ and $CD4^+IL\text{-}4^+$).

ELBW infants from ART-induced pregnancy, in contrast to ELBW infants from spontaneous pregnancy, are characterized by lower anthropometric measures, Apgar scores, higher level of inflammatory infections (pneumonia), grade II intraventricular hemorrhage, movements disorders in the form of lower paraparesis and long hospital stay.

Immune system activation signs, such as the increase in number of $CD8^+$- and $CD3^-CD16^+CD56^+$-lymphocytes, the expression level of Fas-receptor by T-cells, the increased production of intracellular, and serum IFNγ against the decrease in the number of $CD4^+$-cells suggest enhancing of cytotoxic effector potential and proinflammatory orientation of cell responses in ELBW infants born after assisted reproductive technology. The revealed changes in cellular and humoral immunity during the nursing period, in ELBW infants after *in vitro* fertilization, of course, require further observation in respect of the formation of the immune system later in life.

Declaration of interest

The authors report that they have no conflicts of interest.

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