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Age and Sex Characteristics of the Blood Cytokine Profile in Rats Subjected to Prenatal Stress

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Parameters of blood cytokine profile in male and female rats subjected to prenatal stress on the model of swimming in cold water (10°C, 5 min, days 10-16 of gestation) were studied. Prenatal stress had no significant effects on the blood levels of IL-6 and IL-10 cytokines. The blood concentration of proinflammatory cytokine TNFα in 60-day-old rats was higher than in age-matched controls. Stress led to a lower level of anti-inflammatory IL-4 in the blood of 30-day-old males compared to controls. In female rats subjected to prenatal stress, the concentration of IL-4 decreased on day 21, but increased by day 60 of postnatal ontogeny. Specific effects of prenatal stress on the blood cytokine profile in male and female animals at different periods of ontogeny were revealed. Different and even opposite changes in blood cytokine levels could be largely mediated by sex- and age-specific features of immune functions after prenatal stress.

Key Words: *male and female rats; prenatal stress; blood cytokines; periods of postnatal ontogeny*

Special attention of biomedical specialists is now paid to the influence of intrauterine development conditions on the features of postnatal ontogeny of mammals. Analysis of the influence of pathogenic factors on the embryo at different stages of embryogenesis revealed periods when the organism is most susceptible to the damaging agents. These are the initial stage of development, when the embryo is moving along the oviduct and the beginning of basic organogenesis (days 9-10 after fertilization). It was demonstrated that damages formed in the initial period before the completion of basic organogenesis (15-16 days of intrauterine development) lead to malformations, embryo death, congenital diseases, and anatomical abnormalities in the fetus [1].

There negative effect of stressors on the immune response parameters in mammals was convincingly demonstrated. Immobilization stress and forced swimming in animals are known to cause an increase in the blood levels of IL-4, IL-6, IL-10, and TNFα [2]. Stress during pregnancy induces a pronounced suppression of placental transcripts associated with immune processes such as T-cell regulation, cytokine signal transduction, and innate immune response in the maternal body [3].

Studies have shown that prenatal stress in a model of social confrontation of pregnant females reduces basal corticosterone levels, suppresses proliferation of leukocytes [4], neutrophils, monocytes, T and NK cells, and reduces the number of lymphocytes in the offspring [5]. Maternal stress has a modulating effect on the endocrine response of the offspring in adulthood to acute and chronic immobilization stress [6].

During the postnatal period in rats, the immune system continues to develop and undergoes significant functional and morphological changes. Histological maturation of the bone marrow and thymus is completed by day 14 of postnatal development, the mesenteric lymph node — by day 21, and the spleen — by day 42. However, the histological signs of maturity of

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the immune system components do not confirm its functional immunocompetence.

Despite considerable interest to the study of physiological functions during pregnancy, the dependence of the immune parameters of the offspring in the early stages of development on the intrauterine developmental conditions remains understudied. Age-dependent changes in immune system components in mammals of different sexes after adverse exposure during prenatal period have been insufficiently investigated.

Our aim was to study the age- and sex-specific cytokine profile in rats subjected to prenatal stress on the model of forced swimming in cold water.

MATERIALS AND METHODS

The study was performed in accordance with the requirements approved by the Ethical Commission of the P. K. Anokhin Research Institute of Normal Physiology (Rules of Work with Experimental Animals; protocol No. 1, September 3, 2005), and recommendations of the World Society for the Protection of Animals (WSPA), as well as the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasburg, 1986).

The experiments were performed on 96 rats obtained from adult Wistar animals (Stolbovaya Branch of Scientific Center of Biomedical Technologies of Federal Medical-Biological Agency of Russia). Before mating, sexually mature male (n=12) and female (n=28) rats were kept in the vivarium of the P. K. Anokhin Research Institute of Normal Physiology under standard conditions with free access to water and food under artificial light (12/12 h light/dark) at 20-22°C for 6 days.

Vaginal swabs were daily taken from females caged with males to obtain animals with known gestational age. Material for cytological examination was taken with a cotton-gauze swab previously soaked in physiological solution. Unstained smears were examined under a BIOMED-5 electron microscope at ×10 and 40 (Fig. 1). The day when spermatozoa were detected in the vaginal smears was considered the first day of pregnancy.

Pregnant females were randomized into two groups (14 rats in each group). Females of the control group were not subjected to stress and were kept in home cages throughout the experiment. Females of the experimental group were forced to swim in cold water (10°C) for 5 min from days 10 to 16 of pregnancy [7]. After swimming, the rats were dried and placed in their home cages.

The day of birth was considered the first day of postembryonic development. Until day 21 of life, the offspring were left with the female. After weaning and transition to independent feeding, the conditions of the animal maintenance did not change throughout the experiment.

Male and female suckling (day 21), infantile (day 30), and juvenile (day 60) animals [8] were divided into groups (8 animals per group) according to the experimental conditions (control/experiment). Animals were decapitated, blood serum samples were obtained using S-Monovette serum separator systems (Sarstedt); the tubes were allowed to stay for 30 min, centrifuged on a CM-6M benchtop laboratory centrifuge (ELMI) at 4°C for 10 min at 3000 rpm, frozen in liquid nitrogen, and stored at -70°C.

The serum concentrations of proinflammatory (IL-6, TNFα) and anti-inflammatory (IL-4, IL-10)

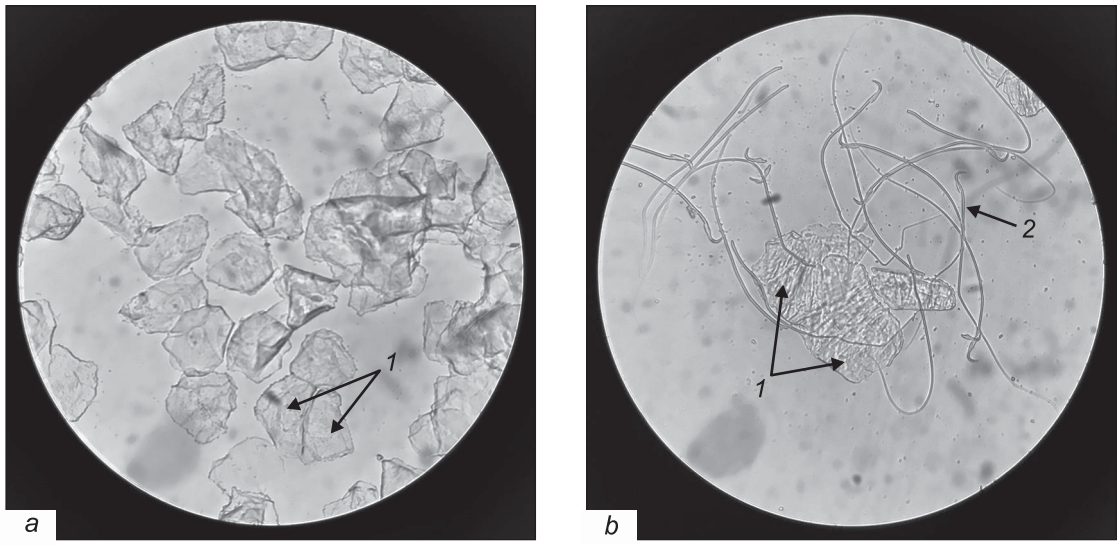

Fig. 1. Cytological composition of vaginal smears of the nonpregnant (*a*) and pregnant (*b*) rats, ×40. *1*) Cell scales, *2*) spermatozoa.

cytokines were determined by ELISA using Interleukin-4-EIA-BEST, Interleukin-10-EIA-BEST, Interleukin-6-EIA-BEST, Alpha-TNF-EIA-BEST kits (Vector-Best). All kit components and serum were prepared prior to ELISA testing according to the manufacturer's instructions. The optical density of the solution was measured on an ImmunoChem-2100 microplate reader (HTI).

The data were statistically processed using Statistica 10.0 software (StatSoft, Inc.). Since the distribution of the obtained data differed from normal (Shapiro–Wilk test), statistical processing of the results was performed using nonparametric Mann–Whitney *U* test. The differences were considered significant at $p<0.05$.

RESULTS

At the first stage of the study, we analyzed the blood levels of proinflammatory cytokines IL-6 and TNFα in the offspring of control and prenatally stressed rats (Table 1). Age-dependent fluctuations in the IL-6 levels in male control rats were insignificant. In females on day 30 of postnatal ontogeny, IL-6 concentration was 84.3% ($p<0.05$) lower than on day 21. In control rats, sex differences in the blood concentration of this cytokine were found on day 21 of life: the concentration of IL-6 was significantly higher in females than in males ($p<0.05$).

The results supplement the available data on this problem. In particular, it was shown that under normal conditions, the level of cytokine IL-6 production in Wistar rats and, accordingly, its blood concentration on day 10 of life were higher than in newborn animals, but decreased in sexually mature animals [9].

No significant changes in the level of IL-6 in the peripheral blood serum of male and female rats subjected to prenatal stress compared to the control group were revealed. No significant age-related changes in the IL-6 level in prenatally stressed males were found. However, in prenatally stressed females, the concentration of this proinflammatory cytokine on day 60 was 111.1% ($p<0.01$) higher than at day 21.

The TNFα content in the peripheral blood of control juvenile male rats (day 60 of life) was 74.4% ($p<0.001$) and 46.6% ($p<0.01$) lower than that of suckling (day 21 of life) and infantile (day 30 of life) animals, respectively. In intact females, blood levels of TNFα on days 30 and 60 were lower than on day 21 (by 69.4 and 62.9%, respectively; $p<0.01$).

Sex differences in TNFα concentration were also revealed in infantile control animals (day 30): this parameter in females was 46.3% ($p<0.05$) lower than in males.

In male and female rats subjected to prenatal stress, the blood concentration of TNFα on day 60 of life was higher than in controls by 200.8 and 91.7%, respectively ($p<0.05$).

Similar fluctuations of IL-6 and TNFα content were revealed earlier in the model of physical stress in rats under chronic sciatic nerve compression [10]: the level of the analyzed cytokines significantly increased in comparison with the control (sham-operated animals).

It should be noted that in our previous experiments on models of acute stress caused by immobilization of rats in individual plastic boxes [11] or restriction combined with subthreshold electrocutaneous stimulation [12], we revealed a decrease in the content of proinflammatory cytokines in the peripheral blood. The contradictions observed at first glance can be due to the use of different types of stressors, sex and age peculiarities of the animals, as well as other factors.

TABLE 1. Concentration of Proinflammatory Cytokines in Peripheral Blood Serum of Wistar Rats (pg/ml; Me (Q1; Q3))

Group		IL-6	TNFα
Control (males)	day 21 of life	11.37 (10.06; 27.56)$^{+}$	124.20 (68.16; 240.22)
	day 30 of life	35.49 (35.44; 39.37)	59.50 (52.65; 122.47)$^{+}$
	day 60 of life	8.98 (8.31; 10.93)	31.79 (23.86; 41.32)$^{ooo xx}$
Prenatal stress (males)	day 21 of life	9.19 (8.31; 15.71)	59.50 (52.58; 72.23)
	day 30 of life	14.44 (9.18; 24.06)	56.04 (54.31; 69.03)
	day 60 of life	16.19 (4.81; 24.06)	63.83 (54.31; 70.76)$^{*+}$
Control (females)	day 21 of life	37.62 (28.51; 41.31)	104.26 (59.56; 134.59)
	day 30 of life	5.92 (4.81; 6.55)^{o}	31.92 (30.06; 73.38)^{oo}
	day 60 of life	32.81 (3.93; 108.94)	38.72 (35.26; 54.31)^{oo}
Prenatal stress (females)	day 21 of life	12.64 (7.85; 13.59)	44.78 (30.77; 75.68)
	day 30 of life	13.56 (3.94; 20.56)	58.64 (52.58; 65.56)
	day 60 of life	26.69 (15.31; 36.31)^{oo}	74.22 (70.76; 88.94)^{*xx}

Note. $p<0.05$ in comparison with *control, $^{+}$females; $^{o}p<0.05$, $^{oo}p<0.01$, $^{ooo}p<0.001$ in comparison with day 21; $^{xx}p<0.01$ in comparison with day 30.

Prenatally stressed females demonstrated a tendency to an increase in TNFα content in the peripheral blood with age: on day 60 of life, this parameter was higher than on day 30 by 26.6% ($p<0.01$). Under these conditions, sex differences in TNFα concentration were found in juvenile animals: this parameter in females was higher than in males by 16.3% ($p<0.05$).

At the next stage, we studied changes in the levels of anti-inflammatory cytokines IL-4 and IL-10 in the blood of rats after prenatal stress (Table 2). The IL-4 content in control juvenile males (day 60 of life) was lower than in suckling (day 21 of life) and infantile (day 30 of life) rats by 84.9% ($p<0.01$) and 78.8% ($p<0.05$), respectively. In control females, the concentration of this cytokine on days 30 and 60 was lower than on day 21 by 55.4 and 80.1%, respectively ($p<0.01$).

In contrast to the age-dependent decrease in the IL-4 level in the control group rats detected by us, opposite changes were observed in other works. For example, the article [9] showed that both male and female rats had increased level of this cytokine produced by the spleen by the age of puberty. Age-related fluctuations in IL-4 concentration can be due to the fact that in animals, starting from day 19-21 of postnatal development, a T-cell immune response is already possible [13]. Opposite changes in IL-4 content in peripheral blood seem to be associated with the unequal degree of involvement of this cytokine in the formation of immune reactions in mammals at different stages of ontogeny.

The following data were obtained when studying the effect of prenatal stress on IL-4 content in peripheral blood of rats of different age and sex. We have found that prenatal stress in the model of swimming in cold water leads to the decrease of IL-4 concentration in the blood of 30-days old males by 65% ($p<0.05$) compared to the control animals. Under these conditions, IL-4 content in females decreased by day 21 of life (by 79.6%; $p<0.05$), but increased by day 60 of postnatal ontogeny (by 115.5%; $p<0.05$) in comparison with the controls.

In rats subjected to prenatal stress, age-dependent fluctuations in IL-4 levels were found only in males but not in females. In male rats at the juvenile age (day 60 of life), the blood concentration of IL-4 was 60.4% lower ($p<0.05$) than in the suckling period (day 21 of life).

After prenatal stress, sex differences in IL-4 content were revealed on day 60 of life: this parameter was significantly higher in females than in males ($p<0.05$).

In males of the control group, the serum IL-10 level was minimum on day 60 of life and was lower than on days 21 and 30 by 99.3 and 98.5%, respectively ($p<0.01$). A similar pattern was observed in intact females: IL-10 concentration in juvenile (day 60 of life) was lower than that in suckling (day 21 of life) and infantile (day 30 of life) periods by 99.5 and 97.7%, respectively ($p<0.05$).

Sex differences in IL-10 content in the blood of intact rats were revealed only on day 60 of life: this parameter was 18.6% higher in females than in males ($p<0.05$).

No significant changes in IL-10 levels in the blood of animals subjected to prenatal stress were detected compared to controls. Age- and sex-dependent

TABLE 2. Concentration of Anti-Inflammatory Cytokines in Peripheral Blood Serum of Wistar Rats (pg/ml; Me (Q1; Q3))

Group		IL-4	IL-10
Control (males)	day 21of life	45.00 (34.85; 123.82)	269.05 (230.95; 566.66)
	day 30 of life	31.96 (24.03; 48.15)	128.57 (54.76; 256.50)
	day 60 of life	6.78 (3.40; 18.19)^{ooxx}	1.93 (1.78; 2.08)$^{+ooxx}$
Prenatal stress (males)	day 21of life	14.56 (11.18; 33.76)	57.14 (26.58; 141.66)
	day 30 of life	11.18 (9.15; 17.26)*	88.09 (30.95; 92.86)
	day 60 of life	5.76 (2.38; 12.52)^{o}	30.95 (11.90; 88.09)
Control (females)	day 21of life	76.63 (54.45; 108.94)	482.82 (294.50; 623.81)
	day 30 of life	34.18 (15.91; 39.16)^{oo}	101.33 (40.77; 167.70)
	day 60 of life	15.23 (10.50; 22.00)^{oo}	2.29 (2.09; 2.38)^{ox}
Prenatal stress (females)	day 21of life	15.61 (8.82; 34.18)*	130.34 (60.68; 263.68)
	day 30 of life	14.56 (9.82; 26.06)	69.05 (30.95; 88.09)
	day 60 of life	32.82 (16.59; 40.26)*	59.52 (50.00; 123.50)

Note. $p<0.05$ in comparison with *control, $^{+}$females; $^{o}p<0.05$, $^{oo}p<0.01$, $^{ooo}p<0.001$ in comparison with day 21 of life; $^{x}p<0.05$, $^{xx}p<0.01$ in comparison with day 30 of life.

differences in the content of this cytokine in prenatally stressed animals were also not revealed.

The data obtained complement the information on the nature of the effect of stress on some cytokine profile parameters in mammals. In particular, the experiments on the model of chronic swimming in cold water in rats showed a significant increase in the relative degree of IL-4 and IL-6 gene expression in the thymus tissues, but a decrease in the hypothalamus [14]. Moreover, the activity of proinflammatory cytokine IL-6 gene was several times higher than that of anti-inflammatory cytokine IL-4. According to the authors, these changes could be caused by fluctuations in the activity of the hypothalamic–pituitary–adrenal complex and the immune system. These data, as well as the results of our studies, suggest a change in the balance and disturbance of signaling interactions between pro- and anti-inflammatory cytokines [15].

The presented results illustrate the specific effect of prenatal stress on the physiological state of the offspring, in particular, on the indicators of the cytokine profile of biological tissues in different periods of postnatal ontogeny. The unequal and even multidirectional fluctuations of cytokine concentrations in the blood of male and female rats are largely mediated by sex-specific changes in immune parameters in different periods of life after prenatal stress exposure.

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