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FEATURES OF IMMUNOGENETIC STATUS IN CHILDREN RESIDING IN NITRATE GEOCHEMICAL PROVINCE

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The authors assessed immunological health of children under excessive intake of nitrates with drinking water, formation of its immunological and genetic status being determined by living conditions in a particular biogeochemical province of the Perm Krai (Region).

This work aims to study the features of the immunogenic status in children living in a nitrate geochemical province.

Materials and methods. The cell differentiation markers (CD3+CD95+) were determined with flow cytometer. The level of protein expression controlling apoptosis processes – Bcl-2, p53 and surface expression of receptor to tumor necrosis factor TNRF were determined with an appropriate MCAT and simultaneous negative isotypic control procedure. The genetic features were determined using a real-time polymerase chain reaction and allele discrimination based on the diagnosis of single-nucleotide polymorphisms.

Results. The changes in cellular component of the immune system (inhibition of T-cell receptors CD95+, and intracellular proteins Bcl2), TSH deficiency, the increased expression of tumor markers associated with the growth in N-nitrosodimethylamine concentration in the blood, significant both in relation to the reference level and the comparison group, were found. An increase in specific IgG to N-nitrosodimethylamine ($p<0.05$) was detected. The study results of the gene polymorphism frequency revealed its features in children of nitrate province according to the criterion of minor allele of genes: MTHFR, PPARA, CPOX, CYP1A2, BRCA1, which characterizes the eventual violations of detoxification and cancer proliferation functions.

Conclusions. Revealed disorders of immune reactivity and genetic polymorphism indicate the immunogenic status peculiarities in population living in the nitrate technogenic chemical province of Perm region, and might be used as markers of pathological health disorders associated with tumor processes and cellular immunodeficiency inducing the identified imbalance of cellular and humoral immunity (deficiency of proapoptotic factors and hyperproduction of IgG to N-nitrosoamines).

Keywords: cell-mediated immunity, a genetic polymorphism, *BRCA1* gene rs3950989, N-nitrosamines.

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Introduction. Under long-term exposure to various toxic substances in concentrations that do not cause an externally detectable effect there can still be hidden changes in a number of physiological, neurohumoral, immunological and biochemical parameters of individual organs and systems. These substances include nitrates and their metabolites – N-nitrosamines. N-nitrosodiethylamine (NDMA) belongs to the super mutagen class and has a high toxicity, teratogenic and embryotropic action, as well as an immunosuppressive effect. What is more, it leads to malignant tumors growth and is a weak allergen [1]. Nitrates may lead to endogenous nitrotyrosine and the formation of N–nitrosamines that are very likely to be a carcinogenic factor for humans (group 2A) [3, 5]. N–nitrosamines metabolism through microsomal oxidation system using cytochrome P-450 leads to the formation of methyldiazonium ion (direct carcinogen), which can methylate the DNA cell, inducing malignant tumors of the lung, stomach, esophagus, liver and kidneys. The choice of indicators/markers for assessing the relationship between the biological system and potentially hazardous chemical factors is relevant [1, 4].

Materials and methods. The study focused on children aged 4-8 years (n=111): 57 people (monitoring group) living in conditions of high nitrate content (exceeding hygienic standard by 1.1 times) in water used for drinking purposes, and 54 people (comparison group) living in the area of relative sanitary-epidemiological well-being (water with standard content of nitrates). Children attended pre-school educational institutions for children in the Perm Territory.

The N-nitrosodimethylamine content in drinking water samples was evaluated via gas chromatography with a thermionic detector. The technique enables determining N-nitrosodimethylamine in drinking water in the concentration range of 0.005 - 0.1 mg/dm^3 with an error of ± 20 % when a confidence level amounts to 0.95. The blood tests on the N-nitrosamines content were carried out with gas chromatography-mass spectrometry method. The N-nitrosamines concentrations ranged from 0.002 to 0.1 mg/dm^3 with an error of ±27 % at a confidence level of 0.95.

The examination of 95 children involved studying cell differentiation markers (CD3+CD95+). The level of proteins expression controlling apoptosis – p53 and surface expression of the receptor to tumor necrosis factor (TNFR) was determined by flow cytometer FACSCalibur using appropriate MCAT ("BD", USA) and simultaneous negative isotypic control procedure. The mediator of intercellular immune regulation of tumor necrosis factor (TNF) and indicators of

proliferative reactions of carcinoembryonic antigen (CEA) and CA199 were analyzed with ELISA test on the "Elx808IU" analyzer.

The polymorphism (SNP) of genes CYP1A1_3, MTHFR, CPOX, SULT1A1, SOD, system piroxicam genes PPAR, FAS, FOXP3, VEGF, APO-E, NO-synthase, MMP9, p53, NR3C1 was studied in all examined persons. The genetic material was isolated from peripheral blood using a DNA extraction kit from the clinical material "DNA-Sorb-B" (NextBio, Russia). The polymorphism was genotyped using "SNP-screen" kit (Synthol, Russia) on CFX96 Real Time System. The frequencies of gene polymorphisms for correspondence are estimated according to the Hardy-Weinberg equilibrium. The analysis of the mean values relative to normal levels is presented in diagrams as x±SE. The results with p<0.05 were considered as significant. The Bonferoni correction was also used for paired comparisons. It sets the significance level at p<0.008 (less than 1%).

Results and discussion. Increased levels of nitrates and N-nitrosodimethylamine in water (4.7 times and 2.5 times respectively) towards the comparison territory were determined (Table 1). It was found that long-term exposure to nitrates and N-nitrosodimethylamine in drinking water within concentrations range of 45-51.7 mg/dm^3 and 0.01-0.016 mg/dm^3 respectively leads to the fact that N-nitrosodimethylamine concentrations in the blood of children in the observation group is 1.5 times more (0.0045±0.0014 mg/dm^3) relative to the comparison group (0.003±0.0009 mg/dm^3) (table 1). The chemical analysis enables to determine nitrates in the urine of children from the observation group at the level of 66.6 mg/dm^3, which is 1.5 times more than in the comparison group (43.7 mg/dm^3).

The level of membrane activation marker CD3+CD95+ (in 100% of children), transcription factor p53 (in 87% of children) and TNFR receptor (in 100.0% of children) responsible for apoptosis (p<0.05) was significantly reduced in relation to the norm (Tab. 2). The methodological technique of estimating the odds ratio changes of immunological tests while increasing contaminant concentrations in biological media enabled to determine significant (p<0.05) reduction of CD3+CD95+ when the N-nitrosodimethylamine concentration in the blood (R2=0.91 by p<0.05) went up. The tumor necrosis factor content was significantly reduced by 3 times in relation to the control group. The elevated level of fetal protein CA 199 was recorded in the serum in 2 % of children living in the observation area. There was a significant (p<0.05) increase in the concentration of CA 199, CEA with growth in the N-nitrosodimethylamine concentration in the blood (R2=0.74 at p<0.05). A high level of specific sensitization to N-nitrosodimethylamine by the IgG criterion (93% of the examined persons in the observation group, p<0.05) was found with a significant difference towards the comparison group (excess 2.2-times, p<0.05).

The results of genetic analysis revealed key genes whose polymorphism frequency differed significantly from the regional average prevalence of polymorphic deviations (Table 3). The polymorphism of detoxification and oxygenation genes changed: methylenetetrahydrofolate reductase gene MTHFR, and piroxicam proliferator genes (*PPARA, PPARG, PPARGC1A*) associated with the 2nd phase of the detoxification of xenobiotics – conjugation (the frequency of the mutant allele was 1.5 times higher than in the control group).

Increase of the T allele frequency of the methylenetetrahydrofolate reductase gene (*MTHFR* rs1801133) and the A allele frequency of the oncoproliferation gene *BRCA1* rs3950989, as well as increased by 1.5-2,0 times the frequency of occurrence of variant alleles of metalloproteinase 9 (*MMP9* rs17576), cytochrome (*CYP1A2* rs2069522), and coproporphyrinogenoxidase (*CPOX* rs1131857) genes in the group of children, that consume water with a high content of nitrates are characterized as a risk group for the development of processes associated with the folate cycle, homocysteine and collagen metabolism (violation of vascular endothelium function), detoxification and metabolism of haptens, cancer processes.

An increased frequency of the minor allele of the BRCA1 gene enzyme rs3950989 is significantly associated with a deficit in the level of tumor necrosis factor (TNFR) which indicates the formation of additional risk factors of carcinogenesis under geochemical provinces (Table 3).

Table 1

The results of the nitrate content in water and urine, N-nitrosodimethylamine in blood of children in the comparison and observation groups (one-time concentration of nitrate and N-nitrosodimethylamine in drinking water for the spring-summer period n=95)

Drinking water, mg/dm3 (R ≤0.005)			
Nitrate concentration		N-nitrosodimethylamine concentration	
MAC=45 mg/dm^3		MAC=0.01 mg/dm^3	
Arithmetic mean			
Comparison group	Observation group	Comparison group	Observation group
10.9±2.7	51.7±12.92	0.0065±0.001	0.016±0.003
Biological media, mg/dm^3			
Nitrate concentration in urine		N-nitrosodimethylamine concentration in blood	
Median		Arithmetic mean	
Comparison group	Observation group	Comparison group	Observation group
43.7	66.6	0.003±0.0009	0.0045±0.0014

Table 2

Parameters of immunity in children under exposure to nitrates (Perm krai)

Parameter	Norm	*Observation group, M±m*	*Comparison group,*
M±m	15-25	7,65±1,07*/**	12,833±2,28
CD3+CD95+-lymphocytes, relative., %	15-25	7.65±1.07*/**	12.833±2.28
p53, %	1.2-1.8	0.324±0.11*	0.522±0.10
TNFR, %	1-1.5	0.514±0.187**	1.418±0.301
CA-19-9, units/cm^3	0-35	18.07±2.2**	10.06±3.20
CEA (carcinoma embryonic antigen), ng/cm^3	0-2.9	6.35±0.08**	1.001±0.17
Tumor necrosis factor, pg/cm3	0-6	2.824±1.43**	8.199±3.85
Specific IgG to NDMA, c.u.	0-0.1	0.176±0.033**	0.115±0.022

* – the difference is significant relative to the reference level (p<0.05)
** – the difference is significant relative to the comparison group (p<0.05)

Table 3

Features of genetic polymorphism under human impact - BRCA1 gene associated with the expression of TNFR

Ген *BRCA1*/ ответ TNFR				
Genotype	Group	n	X (s.e)	OR (CI 95%)
G/G	Control	10	0.97(0.31)	0.00
G/A		12	0.77(0.16)	-0.17(-1.22-0.87)
A/A		6	1.61(0.35)	0.60(-066-1.86)
G/G	Study group	14	2.34(0.29)	0.00
G/A		30	1.20(0.28)	-1.16(-1.96-0.37)
A/A		8	0.98(0.49)	-1.38(-2.46-0.30)

According to the study results, it might be assumed that the hidden changes in immunological and biochemical parameters associated with the polymorphic genetic background are revealed under chronic exposure to nitrates in concentrations that do not cause acute effects. The studies carried out on the example of Perm region have confirmed that the excess concentrations of nitrates in water are metabolized to N-nitrosamines against the altered genetic background and lead to the immune-mediated mechanism of proliferative shifts in homeostasis which are characterized by an imbalance of membrane and transcription factors of apoptosis.

Conclusion. It was found out that the long-term exposure to nitrate with drinking water in a concentration range of 45-51.7 mg/dm^3 and N - NDMA at 0.01-0.016 mg/dm^3 creates an increased N-NDMA concentration in the blood of the children in the exposed group (0.0045±0.0014 mg/dm^3) relative to the comparison group (0.003±0.0009 mg/dm^3) by 1.5 times. There was a significant hyperproduction of specific IgG to N - NDMA ($p<0.05$) both in relation to the reference value and the comparison group. The following changes in the cellular component of immune system are revealed: inhibition of T-cell receptors CD3CD95+ of transcription factor p53 and TNFR receptor, an increased expression of tumor markers associated with elevated N-NDMA concentration in the blood, accurate in relation to the reference level and the comparison group. The results of gene polymorphism analysis revealed an excessive frequency of minor allele of *MTHFR* gene (*MTHFR*), the piroxicam proliferator genes and the first phase of detoxification of coproporphyrinogen oxidase (*CPOX*) and cytochrome (*CYP1A2*), cancer proliferation (*BRCA1*), characterizing genetic polymorphic variants of imbalance in metabolic processes. An increased frequency of the minor allele of the BRCA1 gene enzyme rs3950989 in children contaminated with N-NDMA is significantly ($p=0.016$) associated with a deficit in the level of tumor necrosis factor (*TNFR*), which indicates the formation of additional risk factors of carcinogenesis under nitrate geochemical province. The hidden changes in immunological and biochemical parameters associated with the polymorphic genetic background are revealed under chronic exposure to nitrates in concentrations that do not cause acute effects.

Conflict of interest. The authors state that there is no conflict of interest.

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