MEDICAL AND BIOLOGICAL ASPECTS OF THE ASSESSMENT OF THE RISK FACTORS

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ASSESSMENT OF VIOLATIONS OF THE PROTEOMIC PROFILE IN BLOOD PLASMA IN CHILDREN BEING UNDER INHALATION EXPOSURE TO FINE DUST CONTAINING VANADIUM

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The results of a research and evaluation of the protein profile in child blood plasma exposed to vanadium-containing fine dust in the impact area of metallurgical production plants are presented below. It was found that under conditions of poor air quality in the residential area due to vanadium pentoxide dust content at the level up to 1.2 mean daily MAC (34 RfC_{chr}), by the suspended solids – up to 0.6 mean daily MAC (1.2 RfC_{chr}), there is vanadium concentration in blood of the exposed 4-7 aged children, that exceeds up to 6 times the reference level. The technology of the proteomic analysis showed that children with high content of vanadium in blood have changes in proteomic profile in blood plasma in the type of increase of the relative volume of acid glycoprotein alpha-1; reduction of clusterin, apolipoprotein A-IV, alpha-2-HS-glycoprotein, that are associated with vanadium concentration in blood. In the absence of timely primary and secondary prevention and the preservation of vanadium sustained exposure the revealed cell-molecular abnormalities allow us to predict further development of functional disturbances on tissue and organ levels as the early development of osteoporosis and osteoarticular pathology, atherosclerotic vascular changes, autoimmune allergic processes on the background of disorders of immune regulation, oncology diseases.

***Key words**: proteomic profile, blood plasma, vanadium, molecular markers, inhalation exposure*

Background. Vanadium, a chemical found in fine dust, has been identified as a hazardous air pollutant in the residential areas located in close proximity to the metallurgical plants specialized in the processing of vanadium-containing ores.

Vanadium and fine dust are listed as air pollutants in the WHO Air Quality Guidelines for Europe developed jointly with the International Programme on Chemical Safety expert group and the EC in 2000.

Vanadium consumed with aerosol intake as part of the condensation or disintegration (fine dust) aerosol is more toxic as opposed to elementary vanadium and its alloys with other metals including ferrovanadium, vanadium carbide. Due to high reactivity, it can easily cross blood brain barrier and cumulate in intracellular and tissue structures. In addition to the general resorptive effect, it has specific irritant and systemic effects on the organs and target systems. It affects mainly the respiratory organs (toxicological profiles by the

U.S. ATSDR, 2009) [9], but can also have genotoxic and mutagenic effects [17]. Adverse vanadium effects are identified at the cellular-subcellular level. This occurs as a free radical process resulting from the interaction with the protein complexes I, III and IV of the respiratory chain. This leads to oxidative stress, irreversible modifications of nucleic acids and proteins, and as a result, the disturbance of their structure and func-

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tional activity at the molecular level [11].

To expand the evidence of adverse health effects and improve the effectiveness of measures for early diagnosis and prevention, it is necessary to study the proteomic profile of blood plasma using proteomic analysis and target proteins. The changing of the spectrum of such proteins is associated with exposure to the fine dust containing vanadium [3].

The present study continues the series of works on the organization and administration of studies of protein profile disorders in child blood plasma under exposure to heavy metals conducted by the Federal Scientific Center for Medical and Preventive Health Risk Management Technologies.

Materials and Methods. A hygienic assessment of ambient air quality in terms of the content of fine dust and vanadium pentoxide was conducted as exemplified by the air pollution situation in an area of close proximity to vanadium-processing facilities. We studied the results of long-term monitoring observations for 2010-2015 (data by the Perm center for Hydrometeorology and Environmental Monitoring and Perm office of Rospotrebnadzor) and field research (data by the Federal Scientific Center for Medical and Preventive Health Risk Management Technologies). The study took into account the estimates for the dispersion of pollutants in the ambient air from stationary sources made with the help of spatio-temporal analysis in ArcView (version 3.2). Concentrations of pollutants in the ambient air of populated areas were estimated on the basis of a comparative analysis with the maximum permissible concentrations and reference concentrations for chronic inhalation exposure (MPC d.av. and RfCchr) [1, 5]. The zone of the source influence included the area where the surface concentrations exceeded 0.05 MPC d.av. [6].

A comprehensive in-depth study included a survey of 44 children aged 4-7 living in a residential building located in the area of influence of vanadium-containing fine dust. The dust entered the atmosphere with emissions from the vanadium-processing facilities. To conduct a comparative analysis, 38 children of the same age living in a residential area far from the area of influence were selected. The groups were comparable in terms of the social and living conditions, lack of aggravated family history or occupational exposures, and the quality of drinking water. Written informed consent of the parents of the children included in the sample was obtained prior to the survey following the ethical standards set out in the WMA Declaration of Helsinki (as amended in 2008). The research quality was ensured due to the participation in the federal and international quality assessment systems.

The chemical analysis of the vanadium content in child blood was conducted with the help of inductively coupled plasma mass spectrometry following the guidelines MUK 4.1.3230-14 [4]. The registered vanadium levels were compared against the reference levels (RfL) [7].The children with a vanadium level higher than the reference level (C_{Va}>RfL_{Va}) were included in the observation group. The children with a vanadium level below or equal to the reference level ($C_{Va} \leq RfL_{Va}$) were included in the comparison group.

To study the changes in the protein composition (proteome), a thermally stable blood plasma fraction (prepared in accordance with the recommended procedures) was used [2, 10, 15, 16]. Proteomic analysis included fractionation of blood plasma proteins by two-dimensional polyacrylamide gel electrophoresis, performed using a set of equipment for two-dimensional electrophoresis (Bio-Rad, USA) and in accordance with the recommended procedures [13, 14]. Photographs of the gel were made using GelDoc XR imaging systems (Bio-Rad, USA) with a resolution of 254 dpi. As a result of fractionation of thermally stable blood plasma fraction by two-dimensional electrophoresis, proteomic maps were obtained. They contained information on specific proteins and their characteristics (molecular weight and isoelectric point) required for subsequent protein identification. Proteomic map analysis (gel distribution by groups, gel normalization, spot identification, calculation of the relative volume of spots, identification of the intergroup differences) was performed using PDQuest 8.0 (Bio-Rad, USA). For each of the protein spots, relative amount was calculated expressed as a percentage of the total volume of significant spots on the gel (V%). The average relative amount of spots was calculated for each group. As a result of two-dimensional electrophoresis of plasma samples, proteomic maps were generated in which the area and intensity of protein spots were proportional to the amount of protein in blood plasma. Evaluation of the occurrence and relative volume of the protein spots in the proteome maps of children with elevated vanadium levels was carried out on the basis of a comparative analysis with the results in the children with the vanadium content at the reference level.

The protein spots were prepared for identification with the help of HELC-MS/MS method by excising the protein spots from the gel and washing of the silver by using sodium thiosulfate and potassium hexacyanoferrate (III) solution. Then trypsinolysis of the proteins in the gel was performed while exposed to dimethylated trypsin (Sigma manufacture) following the recommended procedures [12, 18], and lyophilization of the resulting protein hydrolyzate in a centrifugal vacuum concentrator CentriVap (Labconco, USA).

Chromatography of the protein hydrolyzate after its preliminary dissolution in 5% acetonitrile with addition of 0.1% formic acid (FA) was made in selected optimal conditions: Mobile phase: A) water, 0,1% FA; B) acetonitrile, 0,1 FA; gradient: equilibration (5% B) - 15 min; 5-80% B - 140 minutes; 80% B - 20 min; flow rate: 200 NL / min; Sample volume: 20 l; Column T ° C: 25.0 ° C; T ° C Sample: 5.0 ° C.

Mass spectrometry of the obtained samples was performed during a data-dependent experiment on a tandem mass spectrometer 4000 QTRAP (AB Sciex, Canada) with NanoSpray ion source. The obtained spectra were used to build an amino acid sequence in the peptide, and then the peptide sequence was searched for in the protein composition using available databases. Protein identification was made by daughter ion spectra in the SwissProt database using Mascot database search engine (Matrixscience, UK). Peptide identification with a statistical significance of $p \leq 0.05$ was used as a reliability criterion.

Identification and evaluation of the relationship between the relative volume of the selected protein spots in the proteomic map and the blood concentration of vanadium are made by calculating the odds ratios (OR) and its confidence interval (DI) [8]. The criterion for the interconnection "vanadium concentration in the blood is an indicator of the effect" was OR≥1.

Results. Assessment of the ambient air quality in the residential areas impacted by the emissions from the metallurgical plants has indicated an unsatisfactory situation for the last 5 years in terms of the content of vanadium pentoxide dust at a level of up to 1.2 MPCd.av., suspended solids – up to 0.6 MPCd.av., fine dust fractions PM2.5 and PM10 - 0,58-0,73 MPCmax.single The excess of the reference levels for chronic inhalation exposure to vanadium totaled 34 RfCchr, suspended solids - 1,2 RfCchr. The area polluted with vanadium pentoxide dust (at a level of 1 to 2.2 MPCd.av.), which is home to about 20 thousand people, including 4000 children aged 0-14, extends from the metallurgical plants to a residential area in the south, east, north and north-east directions.

In-depth studies have shown that the blood of children suffered from prolonged inhalation exposure to fine vanadium-containing dust, and higher vanadium concentrations were recorded. It was found that the average blood concentration of vanadium was 0.00081 ± 0,00018 mg/dm3 which by 6,1-6,2 times exceeded the values in the comparison group (0,000132 ± 0,00001 mg/dm3, $p = 0.0001$) and the reference level. The average concentration of vanadium in the blood of children served as a marker of chronic vanadium exposure. This was confirmed by the presence of reliable direct correlation ($r = 0.75$, $p = 0.0005$) between the vanadium concentrations in blood and the average vanadium concentrations in the ambient air. This can be described by a linear equation: $y = 0,00078 + 21,95h$ where y is the vanadium concentration in the blood, mg/dm3; and x - average vanadium concentration in the ambient air, mg/m3.

A comparative analysis of the relative volume of the protein spots in the proteome maps of the child blood plasma in the observation group and comparison group revealed significant differences in the relative volume of a number of protein spots. The data on the average relative volumes of protein spots with distribution groups are presented in Table 1, Figure 1 below.

A comparative proteomic analysis of the blood plasma samples showed that the children with high blood vanadium levels had an increased relative volume of protein spots 0805 (an average of 1.84); reduced relative volume of spots 2304 (1.35 times), 2504 (1.85 times), 1706 (1.61-fold) as compared to the comparison group. As a result of mass spectrometric detection of different proteins, daughter peptide ion spectra were obtained, see Figure 2 for example.

Identification of proteins with altered relative volume has shown that a change in the protein profile of blood plasma in children with high vanadium concentration in blood is characterized by a significant ($p \leq 0.05$) increase in the relative volume of the alpha-1 acid glycoprotein, and decreased relative volume of clusterin, apolipoprotein A-IV, alpha-2-HS-glycoprotein. Assessment of the causality "vanadium concentration in blood as an effect indicator" revealed that in the observation group, the probability of changes in the identified plasma proteins is 2.52-3.71 times (DI = 1,87-4,78; p = 0,0001-0,002) higher than in the comparison group (Table 4).

Table 1

The results of densitometry measurements of the relative volume of blood plasma of the protein spots in children in the observation group and the comparison group ($p \leq 0.05$)

Number of protein spot	Average value of the relative volume of a protein spot (relative spot)		Criterion of differences between the groups (M_2/M_1)
	Comparison group (M_1)	Observation group (M_2)	
2304	60184.3	44755.8	0.74
0805	9566.7	17628.6	1.84
2504	40175.2	21613.7	0.54
1706	91964.8	57265.7	0.62

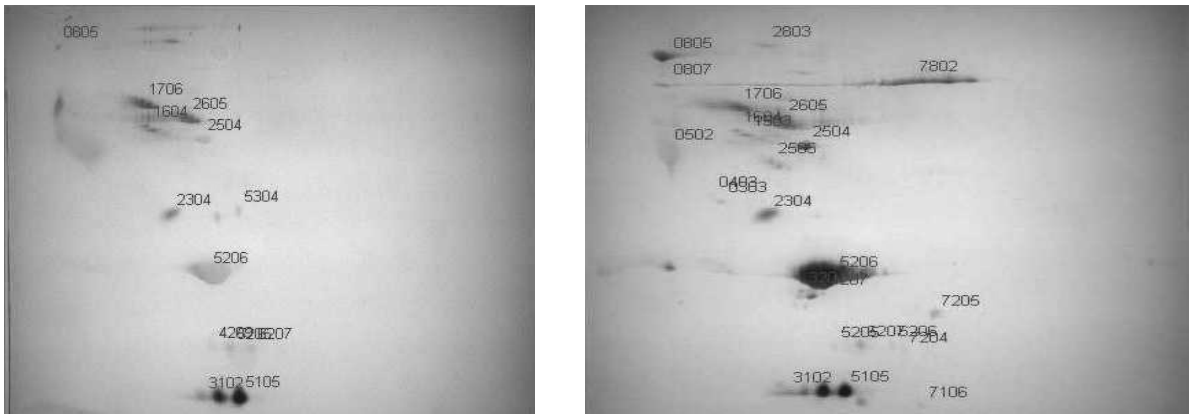

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Figure 1 - 2DE-gels of the child blood plasma: a) an individual from the comparison group; b) an individual from the observation group

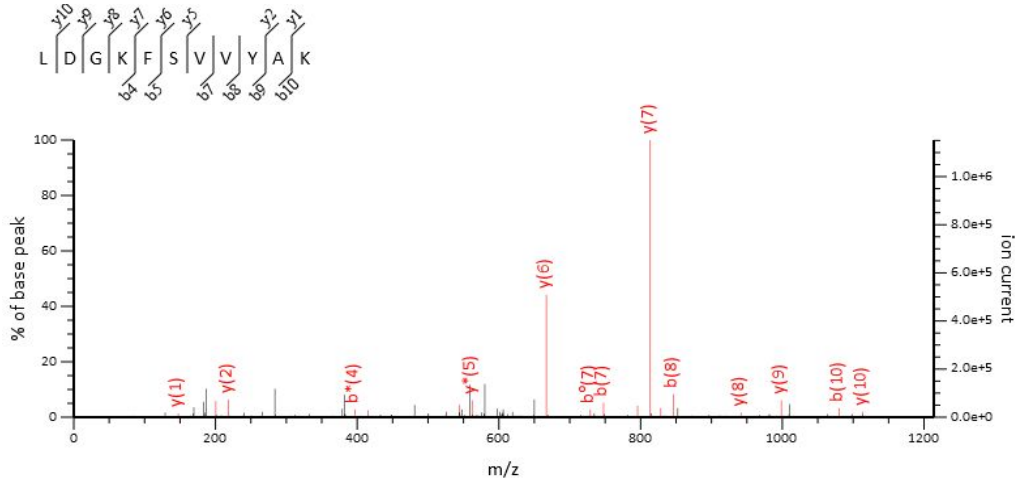

Figure 2 - Spectrum of the peptide LDGKFSVVYAK, fragment FETUA_HUMAN (alpha-2-HS-glycoprotein) (SwissProt data base) in the child blood plasma with high vanadium concentration in blood.

Table 4

Relationship between the changes in the protein profile of blood plasma and the vanadium concentration in blood

Effect indicator	Group	Response		OR	95 % *DI*
		yes	no		
Reduction of clusterin	Observation group	10	9	3.11	2.24–4.37
	Comparison group	5	14		
Increased alpha-acid glycoprotein 1	Observation group	9	10	2.52	1.87–3.44
	Comparison group	5	14		
Reduction of apolipoprotein A-IV	Observation group	12	7	2.94	2.10–3.95
	Comparison group	7	12		
Reduction of the alpha-2-HS-glycoprotein	Observation group	12	7	3.71	3.06–4.78
	Comparison group	6	13		

Conclusion. The study showed that children with high blood vanadium (up to 6 times higher than the reference level), as revealed by applying proteomic analysis, had significant changes in the proteomic profile of blood plasma. This is manifested in increased relative volume of the alpha-acid glycoprotein 1, reduced relative volume of clusterin, apolipoprotein A-IV, alpha-2-HS-glycoprotein. It proves the correlation between the changes in the proteome indicators and vanadium concentrations in blood. The revealed proteomic profile changes in the children with a high vanadium concentration in blood suggest the presence of adverse effects at the molecular level. This is characterized by dysregulation of cell proliferation and apoptosis, decreased anti-inflammatory and increased inflammatory activities, decreased bone mineralization, and dyslipidemia.

Thus, the results of the research revealed that poor air quality determined by a high content of vanadium-containing fine dust (vanadium pentoxide dust and 1.2 MPCd.av., suspended substances - 0.6 MPC d.av.) generates a high vanadium concentration in the blood of the exposed children aged 4-7, which is 6,1-6,2 times higher than that in the blood of non-exposed children and the reference level. A correlation between the changes in the proteomic profile of the blood plasma and the concentration of vanadium in it was confirmed. This is reflected in the changes at a molecular level in the form of a relatively increased alpha-acid glycoprotein 1, reduced relative volume of clusterin, apolipoprotein A-IV, alpha-2-HS-glycoprotein. The lack of preventive measures can promote further functional disorders at the tissue and organ levels, and result in early osteoporosis and bone and joint disease, atherosclerotic vascular changes, autoimmune allergic processes along with immune disorders and cancer. The use of the identified proteins as markers of adverse effects in children exposed to the vanadium-containing emissions from the metallurgical plants is appropriate in terms of expanding the evidence of adverse health effects required for hygienic examinations and investigations for an early detection and prevention of the risk of associated diseases.

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