MEDICAL AND BIOLOGICAL ASPECTS OF THE ASSESSMENT OF THE RISK FACTORS

UDC 613.64: 616.717 – 057

NEUROTRANSMITTER SYSTEM OF IMMUNE REGULATION AS A MARKER OF IMMUNOLOGICAL DISORDERS IN PUPILS IN THE CONDITIONS OF INCREASED ENTRY OF STRONTIUM WITH DRINKING WATER

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The evaluation of immunological markers in schoolchildren exposed to strontium is performed. It is shown that under the conditions of increased administration of strontium with drinking water the indication of spontaneous and induced levels of neurotransmitters in vitro allows to detect early functional disorders of the immune system. It was found that the following markers of specific hypersensitivity and mediators of intercellular immune regulation (IgG specific to strontium, cytokines IL-6, IL-10, IL-12, IL-17, α-TNF, GM-CSF, spontaneous and specifically stimulated, RANKL, OPG(may be proposed for the identification of health risk as early markers of immune disorders in school children living in areas of strontium geochemical provinces.

Key words: *strontium, cytokines, markers.*

Numerous studies clearly indicate that there is a relationship between hygienic factors and the immune status of the population [1,2,3]. Changes in the state of the immune system is one of the indicators of a healthy person's adaptation to the environmental chemical pollution, including metals. Stable strontium is listed among the chemicals with immunotropic and mutagenic activity (toxicological profiles of the Agency for Toxic Substances and Disease (ATSDR) 2004, 2008).

Strontium ions are similar to calcium ions and can replace the latter in the body, which is the main type of action of the compounds of this element [6,7,8]. Strontium biologically competes with calcium. It was demonstrated that strontium ions that have a different diameter than the calcium ions are capable of blocking the ionic channels of the latter, which can determine the inhibitory effect of strontium on the immune response, registered in natural killer cells, and the effects on other cells, particularly skeletal system [9 ,10]. T- and B-lymphocyte recognition of small molecules, including metals, can be explained by the hapten hypothesis which postulates that haptens become antigenic by binding covalently to endogenous proteins and peptides. Adaptation reactions occur at the level of different systems, primarily regulatory, which lately has been considered a single functional system that determine the body balance control [1,3].

Regulatory and effectory systems of the adaptive branch of the immune system are among the major components of the immune system that perform the immune response to the environmental factors including chemical factors. Therefore, an in-depth study of the state of the immune response to toxicants requires the use of specific immunity parameters produced by T- and B-lymphocytes and cytokines as indicator diagnostic sensitization criteria, as well as various humoral mediators [11,12,13].

In the course of an immune reaction mediators are liberated: cytokines, endogenous regulators and effectors of the immune system [14,15]. Cytokines are secreted signaling proteins that participate in intercellular and intersystem interactions, cell growth, differentiation and activation; regulate cytotoxic (anti-tumor and anti-viral), humoral, cell-mediated (Th1 or Th17) or allergy (Th2) immune responses, the result of which is determined by the balance of cytokines produced from pro- or anti-inflammatory properties also play an important role in the regulation of bone metabolism processes [4,

5.16]. Indication of spontaneous and induced levels of mediators reveals functional adaptive reserves of the immune system in the context of environmental exposure of antigens, which are mainly metals [1].

The purpose of this study is to identify marker indicators of immunodisorders in secondary school students exposed to heightened intake of strontium with drinking water .

Materials and methods.

In the course of an in-depth study of health of Perm secondary school students, we conducted genetic and immunological testing of 113 children aged 7-9 residing permanently in an endemic area with elevated levels of strontium in underground waters (12 MAC). The control group was composed of 57 children residing in an area with the standard quality of water in terms of strontium content. The observation and control groups were matched in terms of ethnic, gender, and age of the participants, somatic morbidity and social status. The sample was adequate for reliable determination of intergroup differences.

The study of the metal-content of biological media (mg/dm3) was performed by the use of a mass spectrometer with inductively coupled plasma.

The content of strontium-specific IgG was determined by testing allergo-sorbent enzyme label. The tested chemical compound was conjugated with protein on nitrocellulose membrane, using 0.5% sodium chloride solution as a control. Later we performed the incubation of the tested serum with antigenic complexes, adsorbed on a nitrocellulose membrane, and the binding of the Fab-fragment-specific human antibodies. At the second stage of enzyme immunodetection, for the formation of the classical “sandwich”, we added to the test and control samples monoclonal antibodies conjugated with horseradish peroxidase to the Fc-fragment of human IgG, followed by competitive binding of specific antibodies to the chemical substance with specific portions of the variable domains of monoclonal Fab-antibody fragments. After washing the wells with phosphate buffer, and color development by adding a chromogenic substrate, we stopped the reaction with stop-reagent and removed the used membranes, carried out photometric measurement and recording of the optical density in the test and control samples. We matched them with the optical density of standard samples with known concentrations of immunoglobulin G to the protein of chicken eggs. For such standard samples, we manufactured calibration discs for conjugation of the samples with known content of human Ig G antibodies to the protein of chicken eggs. We used standard IgG samples, a conjugate, a chromogenic substrate, and a stop-reagent from the reagent kit for the determination of total lgG (see "Instructions for the general lgG reagent kit – IFA-BEST." And 8662, ZAO "Vector-Best", Novosibirsk, Russia), specific lgG (see: "Instructions. A set of reagents for qualitative determination of allergen-immunosorbent lgG antibodies in serum" "Immunoteks", Russia, Stavropol). Analysis of the cellular regulation markers - IL-6, IL-10, IL-17, System α-TNF, GM-CSF, VEGF in serum, as well assessment of secretion of cytokines by activated mononuclear cells ex vivo whole blood with the use of strontium were performed with the help of polarization fluoroimmunoassay using test systems for the determination of cytokines and "cytokine-STIMULUS-BEST" by "Vector-Best" (Novosibirsk) on the analyzer «Elx808IU». The impact of the mitogens and strontium complex (Sr 0.01 mg/ml) on the cytokine production by immunocompetent blood cells was determined by the use of the effect index, which is calculated as the ratio of cytokine production by blood cells stimulated by these activators, and the level of spontaneous production.

The markers of bone metabolism RANK and OPG were measured by polarization fluoroimmunoassayusing with the use of «ampli-sRANKL» and «Osteoprogerin» test systems by Biomedica (Austria). IL-12 content was measured using enzyme immunoassay kits «IL-12 + p40» (BioSource Europe S.A., Belgium) that allow for the determination of total IL-12 levels by measuring bioactive heterodimer p35-p40 homodimer antagonist and p40-p40; the standards in the kits are calibrated by the International Standard 95/544 for human IL-12 (NIBSC, Herfordshire, UK, EN6 3QG). The statistical analysis of the obtained data was performed using Statistica (V.6.0). The study results were processed using the methods of variation statistics including the calculation of the arithmetic mean and its standard error. The significant difference was assessed with the help of t test. Differences were considered significant at $p \leq 0.05$. Correlation analysis was performed (a system of coupled linear mathematical models) dependency "hapten - specific immune response." In the absence of the normal distribution of sample data, and availability of a small number of observations, the

nonparametric Mann-Whitney criterion was used. The study of statistical relationships was performed by calculating the Spearman correlation coefficient (rs). The nature of the statistical distribution of the samples was established by the criterion of consent - $\chi 2$. Qualitative data was presented as absolute or relative (%) frequencies, quantitative features were presented as M ± m (arithmetic mean ± error of the mean). Differences between the groups were considered significant at $p < 0.05$. The testing of statistical hypotheses was performed at $p \leq 0.05$ critical level.

Results and discussion.

The analysis of the quality of drinking and household water used at childcare facilities in the area under study (Kungur) revealed a 7-times higher concentration of strontium as compared to the control area (Siva) (7,49 ± 0,38 mg / l and 0.91 ± 0.15 mg / L, respectively).

In the blood of the children residing in the areas with a high concentration of strontium, we determined the rate of excess of this element relative to the comparison group to 3.9 times (0.125 ± 0.021 g/cm³ and 0,031 ± 0,003 g/cm³; reference interval 0.01 - 0.077 g/cm³ (P.Heitland, 2006)). Conducted clinical and laboratory studies revealed the presence of pathological changes in the immune system of schoolchildren residing in the area under study: a higher level of specific sensitization to strontium for IgG was registered compared with the age norm (the concentration of specific IgG to strontium- 0.142 ± 0.03 provisional units at the rate of <0.10) (Table. 1).

Table 2 - Cytokine production, spontaneous and induced by peripheral blood cells of schoolchildren, the median (25-75% percentile) pg/cm3 (experiment)

Table 1 – The level of specific IgG to strontium in the blood of schoolchildren, provisional units

Indicator	Reference level	Control group	Observation group
IgG specific to strontium	<0,10	0,107 ±0,014	0.142±0.01*
Note – * the difference is significant as compared to control group (p<0.05)			

The in-depth study of strontium effects on cytokine-producing activity of immune cells in the dynamics of immunocompetent blood cells of children detected a difference in the rate of production of mediators in the conditions of preliminary strontium sensibilization and the lack thereof (Table. 2).

The analysis of induced production of proinflammatory IL-6, a cytokine involved in bone remodeling, showed that in the surveyed group, strontium-induced production of IL-6 was significantly lower than in the control group. Strontium stimulates the secretion of cytokines in both groups. At the same time, in the surveyed group of schoolchildren, the change in the value of stimulation (201) is higher than in the control group (143). The index of strontium effect on the synthesis of IL-6, as an early mediator, manifesting a pronounced reaction in the activated conditions, exceeds the rest of the studied cytokines.

Cytokine		IL-6	IL-10	IL-12	IL-17
Spontaneous production	Control	637,5 (262,5−957,5)	5,765 (4,075-12,42)	48,84 (43,12-67,95)	2,26 (1,8575-2,62)
	Observation	415 (144-997,5)	4,45 (3,67-7,08)	18,53 (7,89-29,01)*	2,41 (1,33-2,8)
Strontium-induced production	Control	101500 (81000-166750)	5,61 (4,1625-8,91)	37,41 (25,0-81,69)	2,47 (2,20-2,55)
	Observation	52500 (39100-86325)*	5 (4,05-11,22)	24,38 (13-45,36)	1,45 (1,18-1,84)*
Effect index					
Strontium	Control	143,38 (93,58-530,92)	0,90 (0,54-1,51)	1,03 (0,61-1,42)	1,04 (0,89-1,39)
	Observation	201,96 (55,04-423,89)	1,09 (0,79-2,00)	1,29 (0,85-1,77)	0,79 (0,37-1,36)*
Note – * The difference is significant as compared to control group at p<0,05					

The effect of strontium on the synthesis of strontium antiinflammatory IL-10 was not determined; spontaneous values did not have significant differences; however in the experimental group, the level of the cytokine was 1.3 times higher than in the control group.

A spontaneous concentration of proinflammatory IL-12, involved in the differentiation of Th0 towards Thl and in cellular defense, is lower in the experimental group as compared to the control group ($p<0.05$). Strontium induced production in both groups had no significant difference compared to the spontaneous, but more significant changes in cytokine production were found in the experimental group: IV strontium in the experimental group - 1.29, in the control group - 1.03.

We did not detect standby capacity of IL-17 in the group of children with strontium pre-sensitization; we did observe inhibition of IL-17 expression after incubation with the metal ($p <0.05$).

Spontaneous production of α-TNF group was significantly lower than the control group. The level of strontium-induced production of this cytokine in the survey group was also lower than in the control group ($p <0.05$) (Table. 3).

Table 3 - Spontaneous and induced cytokine production by peripheral blood cells of schoolchildren, the median (25□75% percentile) pg / cm^3 (experiment)

Cytokines		α-TNF	GM-CSF	VEGF
Spontaneous production	Control	38,69(13,19-59,66)	1,25(0,85-2,38)	124,71(63,82-199,56)
	Observation	3,62(2,84-16,08)*	1,88(0,57-4,50)	84,11(28,16-170,72)
Strontium-induced production	Control	60,41(32,12-105,87)	23,75(4,66-44,56)	145,21(32,00-216,29)
	Observation	7,69(3,59-20,53)*	3,38(1,19-13,08)*	79,73(21,21-115,39)
Effect index				
strontium	Control	1,38(0,72-4,51)	26,26(4,21-52,43)*	0,84(0,57-1,13)
	Observation	1,26(0,86-3,63)	2,26(0,99-7,77)	0,62(0,44-1,66)
Note – * The difference is significant as compared to control group at p<0,05				

The concentrations of spontaneously secreted GM-CSF in both groups were not significantly different. In the context of strontium activation, the expression of the growth factor was stimulated; however, in the experimental group it was significantly lower.

The median concentration of spontaneous concentration of endothelial growth factor VEGF in children from the experimental group was lower than in the control group. Induced synthesis of the factor was significantly higher in the control group.

The conducted studies of strontium effects on osteometabolism by determining the concentration of RANKL mediators involved in bone remodeling, osteoprotegerin, IL-17, α-TNF, revealed in the children exposed to strontium an imbalance in the markers characterized by the RANKL / osteoprotegerin ratio, which is associated with a reduced ability to support the formation and activation of osteoclasts (Table. 3).

Table 3 - Comparative assessment of the content of bone remodeling markers in the blood of schoolchildren, the median (25-75% percentile), pg/cm³

Indicators	Experiment	Control
Ampli-sRANKL, pg / cm3	8,48(3,52-11,71)	5,53(1,29-13,15)
Osteoprotegerin, pg / cm3	20,80(19,05-48,55)	85,05(71,13-105,28)*
Ampli-sRANKL / Osteoprotegerin	0,16(0,13-0,32)	0,10(0,02-0,18)
Interleukin-17, pg / cm3	1,09±0,14	0,85±0,12
α-TNF, pg / cm3	1,57(1,45-2,245)	0,49(0,39-0,76)*
Note – * The difference is significant as compared to experimental group at p<0,05		

The ratio of RANKL / osteoprotegerin in the experimental group (0.16 (0.13-0.32)) was higher than the corresponding values in children residing in Siwa (0.10 (0.02-0.18)). Also we discovered changing concentrations of IL-17 in the bloodstream, a cytokine, one function of which, as a marker of immune regulation of osteometabolism. was induction of RANKL expression. The concentration of IL-17 in the observation group was 1.29 times higher than in the control group. In the bloodstream of the surveyed group, we registered a significant increase in the concentration of α-TNF as compared to the control group.

We identified a negative correlation ($r = -0.72$; $p < 0.05$) between the content of strontium and concentration of osteoprotegerin. The

connection between the strontium content and the level of RANKL / osteoprotegerin (r = 0,53; p <0,05) was positive. The ratio of RANKL / osteoprotegerin in the observation group (schoolchildren 0.11) was significantly higher than in the control group (0.06).

Conclusion

The studies found that the markers with specific hypersensitivity and mediators of intercellular immune regulation: strontium specific IgG, cytokines IL-6, IL-10, IL-12, IL-17, α-TNF, GM-CSF, spontaneous and specifically stimulated , IL-17, α-TNF in the bloodstream, RANKL, OPG may be used for the identification of health risks as early markers of immune disorders in schoolchildren living in the strontium-affected areas.

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