MEDICAL AND BIOLOGICAL ASPECTS RELATED TO ASSESSMENT OF IMPACTS EXERTED BY RISK FACTORS

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Research article

MODIFICATION OF IMMUNOCYTES VIABLE PARAMETERS IN CHILDREN ASSOCIATED WITH COMBINED EXPOSURE TO CHEMICAL TECHNOGENIC AND EXTREME CLIMATIC FACTORS

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The article dwells on results obtained via immunologic parameters of immunocytes death among children living and attending educational establishments in regions beyond the Polar circle where industry was developing rather intensely.

Our research goal was to examine early disorders in immunologic profile as per immunocytes death among children living in Polar Regions under combined exposure to adverse chemical technogenic and extreme climatic factors.

75 children took part in the research (a test group); they lived and attended educational establishments in regions beyond the Polar circle where industry was developing rather intensely. Benzpyrene is a priority chemical exogenous factor in this region and a climatic one is extremely low temperature in winter (average temperature is -33.8 ^oC in this season). A reference group was made up of children (n = 35) who lived and attended educational facilities in Polar Regions where there was no exposure to technogenic chemical factors. The authors analyzed several immunogram parameters including $CD3^+CD95^+$, Annexin-V presenting cells, TNFRα, $CD3^+HLA\text{-}DR^+$, bax and p53. Cell death parameters were examined with fluorescent analysis via flow cytometry. Also, the authors assessed specific sensitivity of IgG to benzpyrene via allergosorbent testing with enzyme marker.

The research revealed hyperexpression of lymphocytes-cellular profile parameters in children from the test group in comparison with the reference one. They had 1.4 times higher expression of immunocytes stained with AnnexinV and a number of cells stained with PI (Propidium Iodide) was considerably higher than a number of cells stained with AnnexinV as well as the same parameters in the reference group(by 1.5 times) thus indicating that immunocytes predominantly die due to necrosis. There was hyperexpression of $HLA\text{-}DR^+$ receptor on lymphocytes (both its relative and absolute quantity in 12.4–13.7 % children). Expression of $CD95^+$ receptor (a membrane marker of immunocytes apoptosis) was 1.3 and 1.4 times higher (relative and absolute value accordingly).The authors detected an authentically elevated contents of tumor necrosis factor receptor (TNFR) as well as intracellular anti-tumor antigen p53, and antiapoptotic protein bax that were by 1.5, 1.2 and 1.3 times higher accordingly ($p < 0.05$) against the reference group. There was a significant difference in production of IgG specific to benzpyrene in children from the test group since its expression was 2.4 times higher than in children from the reference group ($p < 0.05$). The authors detected elevated risks of excessive expression both for membrane factors of cellular death TNFR (RR = 12.17), $CD3^+CD95^+$(RR = 5.42), HLA^-DR^+ (RR = 4.80) that were apoptosis affectors and for intracellular transcription factors bax (RR = 4.55) and p53 (RR = 3.71) that modulated apoptogenic signals. This risk was associated with combined exposure to chemical tehcnigenic and extreme climatic conditions.

It wasestablished that children living in the Polar Regions under combined exposure to chemical technogenic and extreme climatic conditions had imbalance in the immune status that became apparent via excessive expression of membrane ($HLA\text{-}DR^+$, $CD95^+$, TNFR) and intracellular (p53, bax) parameters with cell death program shifting towards necrosis (as opposed to the reference group that was exposed only to extreme climatic factors). These parameters indicate there is immune deficiency and a significant probability of viral infections and their complications.

Key words: *cell death, immunogram, children, extreme climatic conditions, benzpyrene.*

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Introduction. Population health is greatly influenced by living conditions, state of immune protection and an extent to which a body can adapt to adverse environmental conditions. Climatic conditions in Polar Regions are uncomfortable for people and social conditions there are much worse than those in central and southern regions in the country. Large industrial complexes located in the northern Siberia contaminate the environment and create threats of adverse impacts on human health, in particular for children population groups that are the most sensitive to changes in the environment [1–4]. There are available data in literature on changes in both levels of morbidity and its structure in different children groups who live under adverse technogenic exposure [5, 6]. A huge diversity of chemicals that are haptens in their essence occurs in the environment. Carcinogens are able to interact with each other, become active in favorable chemical conditions, transform and/or persist in any organic or non-organic medium over a long time [5, 7]. Aromatic and polyaromatic hydrocarbons have a significant place among toxicants that are ambient air pollutants. They are contained in exhaust gases, emissions from industrial enterprises and from large and small heating systems. Benzpyrene is a widely spread carcinogen in northern regions in Russia. Benzpyrene can enter a body via different ways and inhalation is among them. The substance is chemically and thermally stable, able to accumulate in biological media, and becomes carcinogenic in a concentration that is higher than 0.1 μg/100 m³. Apart from carcinogenic effects, benzpyrene produces mutagenic, embryotoxic, and hematotoxic ones [6].

In Polar Regions combined exposure to climatic and exogenous environmental factors exerts negative influence on a human body, induces grave stress responses, and creates additional difficulties for a developing (child) body. This set of factors influences the children's immune system significantly resulting in deadaptation, a decrease in reserve capabilities and a growth in morbidity. The immune system is highly sensitive to many environmental factors. In case ambient air is contaminated heavily, changes in health often become apparent via occurring secondary immune deficiency that makes for prevalence of sensitization [8–10].

Studies on health of population living in industrially developed Polar Regions in Russia are especially vital; such studies should concentrate on determining peculiarities of effect markers that characterize early disorders in immunologic health of children under climatic conditions existing beyond the Polar circle.

Our research goal was to examine early changes in immunologic profile indicators (apoptosis markers) among children living in Polar Regions under combined exposure to negative chemical technogenic and climatic factors.

Data and methods. The research involved examining 75 children (a test group) who lived and attended educational establishments on a territory with intensely developing industry located beyond the Polar circle (Krasnoyarsk region). Benzpyrene is a priority chemical exogenous factor in this region and a leading climatic factor is extremely low temperature in winter (average temperature is –33.8 °C). A reference group was made up of children (n = 35) who lived and attended educational establishments on territories beyond the Polar circle where there was no exposure to technogenic chemical factors.

An examination of children's immune state included analyzing the total leucocytes number, and relative and absolute lymphocytes quantity with conventional clinical analysis procedures accomplished with Drew-3 (D3) Drew Scientific hematologic analyzer (Great Britain, USA). Membrane and transcriptional indicators of lymphocytes cell death as well as quantity of IgG antibodies specific to benzpyrene were examined in children's peripheral blood.

The research included determining quantity of the following lymphocytes subpopula-

tions: activated T-lymphocytes $CD3^+CD95^+$, $CD3^+HLA\text{-}DR^+$. Levels of lymphocytes cell death were determined via staining with *Annexin V-FITC* (USA) and simultaneously with *Propidium Iodium PI* (USA); it allowed identifying cells at early stages in apoptosis. To assess a system of programmed cell death, we examined intracellular expression of Bax protein, intracellular apoptosis marker p53-portein, and surface expression of tumor necrosis factor receptor (TNFR1). Lymphocytes were extracted from peripheral blood via centrifuging in Ficoll-Urografin gradient solution. Gradient density for human blood corpuscle fractioning is equal to 1.077 g/cm^3. These indicators were selected basing on a hypothesis that programmed cell death was modified due to combined exposure (extreme climatic temperatures and contamination with exogenous chemical haptens) occurring under combined impacts exerted by various risk factors.

Lymphocytes immunophenotyping was performed via using monoclonal antibodies to surface differential antigens on immune system cells; it was done via flow laser cytofluorometry with *BDFACS Calibur flow cytofluorometer* (USA). Lymphocytes suspension was added with monoclonal antibodies (MAT) to a certain lymphocytes marker with these MAT being stained with fluorochrome. Then these antibodies bound to lymphocytes that expressed a marker relevant to a specific monoclonal antibody. Cell suspensions were washed via centrifuging in *Cell Wash solution* (BD, USA) thus removing antibodies that remained unbound to cells.

IgG specific to benzpyrene was identified with using reagins that were conjugated with peroxidase; it was done via allergosorbent testing procedure with an allergen being sorbed on cellulose substrates. Photometric measuring of optical density was performed with *Sunrise ELISA reader* (Tecan, Austria).

Results were estimated with Statistica, Statsoft, Inc. (USA), a universal software package for data analysis. Significance of differences was estimated with Student's t-test; differences between groups were considered valid at $p < 0.05$.

Results and discussion. Having performed immunocytes phenotyping, we revealed the following in the test group: hyperexpression of absolute and relative lymphocytes levels of activation phenotypes such as activated T-killers $CD3^+HLA\text{-}DR^+$, $CD3^+CD95^+$ lymphocytes (relative value), TNFR1 receptor 1, as well as p53, an intracellular protein that induced apoptosis. Values that deviated from reference ones were detected in 15.3 %, 12.1 %, 47.9 % and 18.8 % children accordingly.

Examined children from the test group had 1.4 times higher activation of cells stained with *Annexin V* against the reference group ($p < 0.05$). Cells stained with *PI* occurred in much greater quantities than phenotypes stained with *Annexin V* as well as the same parameters in the reference group (1.5 times higher). It is due to cells reaching the later stage in cell death faster under stress or due to them being already dead (necrosis). Cells lose cellular membrane integrity at later stages in apoptosis and absorb *PI* [11, 12]. However, this indicator remained within reference levels.

The research also revealed elevated $HLA\text{-}DR^+$ expression on lymphocytes in the test group since it was by 12.4–13.7 % higher than in the reference one. Expression of later activation marker $HLA\text{-}DR^+$ indicates how intense an immune response is. Excessive T-lymphocytes expression can occur due to many diseases that involve chronic inflammation (autoimmune diseases, hepatitis C, pneumonia, etc.) [12, 13]. We also established elevated levels of immunocytes $CD95^+$ apoptosis receptor that was 1.3 and 1.4 times higher than in the reference group (relative and absolute value accordingly). This marker is expressed on all cells in the immune system and plays an important role in control over the immune system functioning (immunocytes life cycle). An increase in a number of lymphocytes that

Table

Immunologic profile parameters of children from the examined groups

Parameter	Reference level	Test group, $M \pm m$, $n = 75$	Reference group, $M \pm m$, $n = 35$
$CD3^+HLA^-DR^+$, %	8–20	$28.347 \pm 1.199^{*/**}$	$27.359 \pm 2.242^{*}$
$CD3^+HLA^-DR^+$ lymphocytes, abs.,$10^9/dm^3$	0.1–0.5	$0.775 \pm 0.042^{*/**}$	$0.548 \pm 0.052^{*}$
$CD3^+CD95^+$ lymphocytes, %	15–25	$27.658 \pm 0.953^{*/**}$	20.200 ± 2.263
$CD3^+CD95^+$ lymphocytes, abs., $10^9/dm^3$	0.4–0.7	$0.765 \pm 0.038^{**}$	0.605 ± 0.087
Annexin V-$FITC^+7AAD^-$, %	0.5–1.0	$0.828 \pm 0.128^{**}$	0.609 ± 0.109
Annexin V-FITC+$7AAD^+$, %	5.0–7.0	$7.730 \pm 1.237^{**}$	5.090 ± 0.494
TNFR1, %	1–1.5	$6.246 \pm 0.430^{*/**}$	$4.118 \pm 0.384^{*}$
Bax, %	5–9	$8.318 \pm 0.645^{**}$	6.312 ± 0.998
p53, %	1.2–1.8	$5.570 \pm 0.448^{*/**}$	$4.808 \pm 0.425^{*}$
IgG specific to benzpyrene, arb.units	0–0.2	$0.198 \pm 0.029^{**}$	0.081 ± 0.008

Note: * means there is statistically authentic difference from reference level as per independent Student's t-test at $p < 0.05$; ** means there is statistically authentic difference from reference group as per independent Student's t-test at $p < 0.05$.

present $CD95^+$ is a natural sign that the immune system has been activated and it is typical for an immune-inflammatory process [14–16]. Also, there was an authentic increase in tumor necrosis factor receptor (TNFR) and cellular tumor antigen p53 in blood of children from the test group, by 1.5 and 1.2 times accordingly against the reference group. We also noted that there were no significant differences in pro-apoptotic protein Bax contents from reference levels but this indicator was significantly (by 1.3 times) higher in the test group than in the reference one. Exposure to cold is known to induce significant shifts in a body that become apparent via changes in a quantity of proteins, re-distribution of their fractions, and an increase in quantities of protein metabolism products [17].

There was an authentic difference between two groups regarding antibodies to benzpyrene since production of specific IgG was 2.4 times higher among children from the test group than among those from the reference one ($p < 0.05$). When polycyclic hydrocarbons enter a human body, they create an epoxy-compound under impacts exerted by enzymes; this compound enters a reaction with guanine and it disrupts DNA synthesis, leads to disorders or mutations that make for oncologic diseases development including such types of cancer as carcinoma and sarcoma [16]. Stress-dependent protein p53 inhibits a change in cellular cycle phases as a response to DNA damage thus inducing cell apoptosis [18–20]. In this research expression of phosphoprotein p53, as well as expression of tumor necrosis factor receptor TNFR, was established to be higher than the upper reference level in blood of children from the test group and was authentically higher than the same parameter in the reference one. Therefore, we can conclude that extreme climatic conditions and excessive quantities of exogenous hapten benzpyrene (evidenced by hyperexpression of specific IgG) act together as apoptosis stimulators making for excessive quantities of proteins that speed up immunocytes death in children living in industrially developed areas beyond the Polar circle.

Having assessed relative risks (RR)[1] of disorders in apoptosis development under exposure to excessive contamination with

[1] Rukovodstvo po otsenke riska dlya zdorov'ya naseleniya pri vozdeistvii khimicheskikh veshchestv, zagryaznyayushchikh okruzhayushchuyu sredu [The Guide on assessing population health risks under exposure to chemicals that pollute the environment]. Moscow, The Federal Center for State Sanitary Epidemiologic Surveillance of the RF Public Healthcare Ministry Publ., 2004, 143 p. (in Russian).

benzpyrene, we established that elevated benzpyrene concentrations in blood of the examined children led to elevated risks of excessive expression, both regarding membrane cell death factors such as TNFR (*RR* = 12.17), $CD3^+CD95^+$ (*RR* = 5.42), and HLA^-DR^+ (*RR* = 4.80) that were apoptosis affectors, and intracellular transcription factors that modulated apoptogenic signals such as Bax (*RR* = 4.55) and p53 (*RR* = 3.71). And here there was no risk of disrupted effector structures of apoptotic scenario that were bound to *Annexin V*.

Therefore, an additive effect produced by extreme climatic conditions together with exogenous hapten stimulation leads to an imbalance in immune state of the examined children living in Polar Regions; this imbalance becomes apparent via hyperexpression of cell death indicators such as an increase in oncosuppression transcription factors Bax and p53 as well as membrane factors TNFR, $CD3^+$HLA-DR^+, and $CD3^+CD95^+$ responsible for induction and transcription stages in apoptosis scenario.

It should be noted that this research focuses on analyzing peculiarities of programmed cell death modification in children under combined exposure to climatic (extremely cold temperatures) and technogenic (exogenous chemical) adverse factors and its effects on apoptosis; the research results allow predicting risks of early health disorders.

Conclusion. Peculiarities of immunocytes viability parameters in children living under combined exposure to technogenic and extreme climatic factors are hyperproduction of IgG specific to benzpyrene as well as excessive expression of cell death indicators such as transcription and oncosuppression factors Bax and p53 and membrane phenotypes $CD3^+$HLA-DR^+, $CD3^+CD95^+$, and TNFR. The latter are responsible for apoptosis realization, both at a stage when it is induced and at a stage of intracellular modulation of its program that is modified negatively under exposure to haptens in the environment (benzpyrene) and it predetermines immune deficiency in children living under these conditions. Having assessed relative risks (*RR*) of disorders in apoptosis under exposure to excessive contamination with benzpyrene, we revealed that elevated benzpyrene concentration in the examined children's blood resulted in elevated risks of excessive expression, both regarding membrane cell death factors TNFR (*RR* = 12.17), $CD3^+CD95^+$ (*RR* = 5.42), and HLA^-DR^+ (*RR* = 4.80) that were apoptosis affectors, and intracellular transcription factors Bax (*RR* = 4.55) and p53 (*RR* = 3.71) that modulated apoptogenic signals.

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