MEDICAL AND BIOLOGICAL ASPECTS OF THE ASSESSMENT OF THE RISK FACTORS

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INDICATORS WHICH ARE APPLIED WHEN ASSeSSING EFFECTS ON A BODY EXERTED BY NITRATES AND N-Nitrosodimethylamine INTRODUCED WITH DRINKING WATER

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The authors comparatively assessed N-Nitrosodimethylamine (N-NDMA) contents in blood samples taken from children who consumed drinking water with increased nitrates and N-NDMA concentrations and in blood samples taken from children who consumed drinking water which fully corresponded to the existing hygienic standards; the article dwells on the results of this comparative assessment. We detected authentic discrepancies ($p<0.005$) in N-NDMA contents between blood samples taken from children from the focus group (0.0045 ± 0.0009 mg/dm^3) and the reference one (0.003 ± 0.0006 mg/dm^3). We revealed that free-radical oxidation mechanisms were activated in children from the focus group who were exposed to N-NDMA. Lipids hydroperoxidation content in blood serum was proved to be 1.6 times higher in children from the focus group than in those from the reference one. When N-NDMA was detected in blood of children from the focus group, they ran 1.73 times higher risks of damages to their cells membranes.

Our assessment of antioxidant protection revealed that glutathione-S-transferase became less active, B12 vitamin content went down, and glutathione peroxidase increased in children from the focus group against those from the reference group; all these parameters were 1.2–1.7 times different between the groups ($p = 0.000–0.030$). The children from the focus group also ran 2.91 times higher risks of an increase in glutathione peroxidase content.

We detected an authentic cause-and effect relation between an increase in IgG to N-NDMA and growing N-NDMA concentrations in blood ($R^2 = 0.958$, at $p = 0.001$). Risk of changes occurring in this parameter of humoral immunity was 1.3 times higher in the focus group.

The results of the experimental research allowed us to reveal an increase in fetal proteins (S-CEA and CA 199) contents detected in blood serum of children from the focus group against those from the reference one; the contents were 2.7 and 3.9 times higher correspondingly ($p = 0.010–0.023$). This increase could be a sign of ongoing processes which characterized tissue proliferation; it could also become a mechanism of uncontrolled cellular proliferation.

The performed research allowed us to substantiate and fix the following biological markers of the effects: an increase in IgG to N-NDMA and in glutathione peroxidase, ASAT activity, and total bilirubin level which can be applied in risk assessment and in giving grounds for permissible concentrations of these toxic compounds in blood.

Key words: *nitrates, N-nitrosodimethylamine, drinking water, exposure indicator, indicator of an effect, odds ratio, specific sensitization.*

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Non-organic compounds are a significant chemical factor that causes population health risks in the RF due to contamination of drinking water supply sources. Thus, consumption of drinking water with high nitrates concentrations that constantly tend to increase[1] [1], is hazardous because when they penetrate a body, endogenous synthesis makes them turn into highly toxic N-nitrosoamines[2] [2]. N-nitrosoamines are widely used in industry; their synthesis in natural reservoirs as well as in a human body is quite possible. They are stable, dissolve easily, and can penetrate drinking water in multiple ways; all the above said means drinking water is one of the main sources of N-nitrosoamines introduction into a human body[3].

A vital aspect in any hygienic assessment is to reveal a relationship between effects produced by drinking water contamination and the consequent biological effects [3–5]. To detect hazardous effects produced on human health by chemical factors related to drinking water, experts apply epidemiologic, instrumental (laboratory), and clinical techniques that allow to assess exposure to chemical factors.

Biomarkers of effect and their determination is one of basic instruments applied to detect persistent cause-and-effect relationships between health disorders and exposure to environmental chemical factors [6]. Pathogenetic mechanisms related to negative effects produced by nitrogen-containing substances (nitrates and nitrosoamines) consumed with drinking water still remain an unsolved task of contemporary hygiene and human ecology.

Our research goal was to substantiate indicators of negative effects in children under chronic exposure to nitrates and N-nitrosodimethylamine (N-NDMA) consumed with drinking water. The substantiation was based on modeling and assessment of "exposure indicator – indicator of an effect" relationship.

Data and methods. To achieve our goals, we applied a set of sanitary-hygienic, epidemiologic, and statistical techniques. Hygienic assessment of drinking water quality on examined territories was performed on the basis of monitoring data obtained from "Center for Hygiene and Epidemiology" of Perm Center for Hydrometeorology and Environmental Monitoring and data provided by the Federal Scientific Center for Medical and Preventive Health Risk Management Technologies.

Water samples were examined in respect of nitrates content[4] and N-NDMA content[5] on the examined territory and the reference one; the results were assessed with regard to maximum permissible concentrations according to the hygienic standard 2.1.5.1315-03[6].

To substantiate indicators of effects, we performed an in-depth examination of two groups of children who lived in the same region with the same social-economic and geochemical features. The focus group was made up of children exposed to nitrates and N-NDMA consumed with drinking water; the reference group included children who weren't exposed to these chemicals. Children were examined in full conformity with the obligatory compliance with the ethical standards fixed in Helsinki Declaration adopted in 1975 and supplemented in 1983.

Examinations of biological media taken from children included determination of N-NDMA in blood and nitrates in urine. Parameters detected in the reference group were applied as assessment criteria for nitrates content in urine and N-NDMA content in blood. We examined 153 children aged

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[1] Loginova E.V., Lopukh P.S. Hydroecology: course of lectures. Minsk, Belarus State University Publ., 2011, 300 p. (in Russian).

[2] Drinking water quality guidelines. *The World Health Organization*, 2004, Vol. 1. Available at: http://www.who.int/water_sanitation_health/dwq/gdwq3rev/ru/ (access date 01.06.2018).

[3] Arustamov E.A., Barkalova N.V., Levakova I.V. Ecological grounds for use of natural resources. The 5th edition., revised and complemented. Moscow, 2008, 320 p. (in Russian).

[4] State Standard 31867-2012. Drinking water. Determination of anions content with chromatography and capillary electrophoresis. *KODEKS: an electronic fund of legal and reference documentation*. Available at: http://docs.cntd.ru/document/1200097406 (access date 26.08.2018).

[5] MG 4.1.1871-04. Gas chromatography determination of N-nitrosodimethylamine (NDMA) in drinking water and water reservoirs. *GOSTRF.COM.* Available at: http://www.gostrf.com/normadata/1/4293855/4293855338.htm (access date 26.08.2018).

[6] HS 2.1.5.1315-03. Maximum permissible concentrations (MPC) of chemicals in water objects for communal and drinking water supplies. *KODEKS: an electronic fund of legal and reference documentation.* Available at: http://docs.cntd.ru/document/901862249 (access date 26.08.2018).

4-10 who attended schools and pre-school children facilities (53% were girls and 47% boys) and lived on territories with increased nitrates concentrations in drinking water (up to 1.2 MPC, 66.9±12.92 mg/dm^3, the focus group). To perform a comparative analysis, we examined 100 children of the same age (the reference group) who consumed drinking water without any excessive nitrates contents, their average concentration being 0.2 MPC (10.9±2.7 mg/dm^3).

Blood samples were analyzed with capillary gas chromatography technique on a gas chromatographer with N-nitrosoamines specific thermionic detector and analytical column of DB-624-30m*0,32mm*1,8μm series [7]. To prepare blood samples, we applied an automated solid phase extraction system (SPE) to concentrate and extract an analyte (N-NDMA) out of a biological medium matrix [8, 9]. Urine samples with respect of nitrates concentration were examined with capillary electrophoresis[7].

Chronic introduction of nitrates and N-NDMA with drinking water results in an increased N-NDMA concentration in blood. To perform criterial assessment of effects, we profoundly examined and assessed body reactions, namely deviations in biochemical and immunological parameters. Laboratory examinations of biological media taken from children included the following:

1. *Biochemical research* ((lipids hydroperoxides, superoxide dismutase (SOD), nitrogen oxide in blood serum; 8-hydroxi-2-deoxyguanosin in blood and urine, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), albumin, cholesterol, conjugated bilirubin, crude bilirubin, malonic dialdehyde (MDA), AOA (anti-oxidant activity) in blood plasma, crude protein, dextrose, creatinine, urea, alkaline phosphatase, gamma-glutamyl transferase (GGT), calcium, phosphor, iron, ratio of apolipoprotein A1 to B100 (Apo A1/Apo B100); methemoglobin in whole blood; B12).

2. *General clinical research* ((erythrocytes, hemoglobin, thrombocytes, leucocytes, lymphocytes, reticulocytes, eosinophils, neutrophils, and color index).

3. *Immunologic research* (IgG to nitrosoamines, cancer antigen 199 (CA 199) and carcinoembryonic antigen (S-CEA) in blood serum).

Immunologic and biochemical parameters were examined with unified procedures in Immune Biological Techniques Department (headed by O.V. Dolgikh, Doctor of Medical Sciences) and in Biochemical and Cytogenetic Techniques Department (headed by the M.A. Zemlyanova, Doctor of Medical Sciences) of the Federal Scientific Center for Medical and Preventive Health Risk Management Technologies[8, 9] [10–12].

Laboratory research was performed with an automated hematologic analyzer, biochemical automated analyzer, immunoassay analyzer, photoelectric photometer, and flow cytofluorimeter.

Indicators of effects were substantiated as per odds ratio (OR) calculation; this parameter characterized a correlation between N-NDMA concentration in blood and biochemical parameters of a response. OR>1 condition was considered to be a criterion showing the correlation existed [13].

We determined parameters of OR dependence on N-NDMA concentration in blood with building up a regression model in a form of an exponential function OR=ea0-a1x, where OR is odds ratio; x is N-NDMA concentration in blood, mg/dm3; a0, a1 are parameters of a model determined with regression analysis.

We assessed validity of an obtained model on the basis of one-factor dispersion analysis as per Fischer test (F>3.63). Discrepancies in the results were considered to be statistically significant at $p \leq 0.05$.

We processed data obtained during the research and assessed parameters of the models with Statistica 6.0 applied software and specific software products [14].

Results and discussion. Results of our research on determining nitrates and N-NDMA concentrations in water on the examined territories allowed us to reveal that nitrates concentration was 4.7 times higher, and N-NDMA concentration, 2.5 times higher, than in water consumed by children from the reference group (Table 1).

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[7]Organizational Standard M 26-2017. A procedure for measuring mass concentrations of nitrate-ions in urine with capillary electrophoresis / A certificate of accreditation given to a measuring procedure No. 88-16207-030-RA.RU.310657-2018

[8]Kamyishnikov V.S. A reference book on clinical–biochemical research and laboratory diagnostics. Moscow, MEDPress-inform Publ., 2004, 920 p. (in Russian).

[9] Tkachuk V.A, Clinical biochemistry. Edited by academician V.A. Tkachuk, – the 3rd edition, amended and supplemented. Moscow, GEOTAR-Media Publ., 2008, 462 p. (in Russian).

Table 1

Nitrates concentration in water and urine, N-NDMA concentration in blood of children from the focus and the reference group

Drinking water, mg/dm^3, ($p \leq 0.005$)			
Nitrates concentration		N-NDMA concentration	
Reference group	Focus group	Reference group	Focus group
10,9 ± 2,7	66,9 ± 12,9	0,0065 ± 0,0013	0,016 ± 0,003
Biological media, mg/dm^3			
Nitrates concentration in urine		N-NDMA concentration in blood	
Reference group (n=100)	Focus group (n = 153)	Reference group (n = 100)	Focus group (n =153)
43,7 ± 8,74	78,3 ± 15,66	0,003 ± 0,0006	0,0045 ± 0,0009

Table 2

Comparative analysis of biochemical and immunological parameters ($p<0.05$)

Parameter	Focus group			Reference group			Discrepancies between groups as per averages (p)
	M ± *m*	Frequency of samples deviating from the physiological standards, %		*M* ± *m*	Frequency of samples deviating from the physiological standards, %		
		higher	*lower*		*higher*	*lower*	
Lipid hydroperoxides, µmol/dm^3	259,8 ± 51,3	68,8	6,2	163,8 ± 39,1	0,0	73,7	0,010
B12, pmol/dm^3	116,6 ± 12,4	8,3	75,7	139,0 ± 12,5	38,6	12,6	0,030
Glutathione-S-transferase, ng/ml	103,7 ± 22,5	0,0	85,7	170,7 ± 28,8	81,0	9,5	0,000
Glutathione peroxidase,ng/ml	43,4 ± 3,8	50,0	25,7	40,2 ± 2,4	0,0	46,7	0,010
ASAT, E/dm^3	29,9 ± 1,5	50,9	35,7	27,3 ± 0,87	23,1	57,5	0,002
Alkaline phosphatase, E/dm^3	447,0 ± 41,4	50,0	25,0	382,5 ± 23,2	10,7	57,3	0,010
Crude bilirubin, µmol/dm^3	11,4 ± 1,2	41,1	47,3	9,7 ± 0,7	22,4	60,8	0,005
IgG to N-NDMA, g/dm^3	0,29 ± 0,09	51,7	41,4	0,16 ± 0,03	12,1	67,3	0,010
S-CEA, ng/ cm^3	1,2 ± 0,5	11,0	32,0	0,5 ± 0,04	0,0	30,2	0,010
CA-199, units/ml	15,3 ± 5,2	38,9	33,3	8,2 ± 3,5	3,0	85,1	0,023

Our research revealed that long-term exposure to nitrates and N-NDMA consumed with drinking water caused increased N-NDMA concentration (1.5 times higher) in blood of children from the focus group against children from the reference group ($p \leq 0.005$). Performed chemical and analytical research allowed us to reveal increased nitrates concentrations in urine of children from the focus group (1.5 times higher against the reference group).

Increased N-NDMA concentration in blood substantiates indicators of negative effects in a body. Comparative analysis of biochemical and immunological parameters in children from the focus group and the reference group was the next stage in our research on cause-and-effect relationship. The results are given in Table 2.

Increased lipids hydroperoxides concentration in blood plasma is known to be a signal that oxidation processes are activated at cellular membranes level. The performed research revealed that lipids hydroperoxides level in blood serum of children from the focus group (259.8±51.3 µmol/dm^3) was authentically 1.6 times higher than the same parameter in children from the reference group (p=0.01). Increased lipids peroxides concentration was detected in 68.8% of samples taken in the focus group. There were no similar samples in the reference group (p=0.01).

We assessed antioxidant protection in children from the focus group and revealed a 1.2 times decrease in glutathione-S-transferase concentration and a 1.7 times lower vitamin B12 level than in the reference group (p=0.000–0.030). We also detected that decreased levels of glutathione-S-transferase and vitamin B12 were registered in 75% and 85% of samples; as for the reference group, the share of such samples amounted to 12.6% and 9.5% correspondingly. Children from the focus group had 1.2 times higher glutathione peroxidase concentration than children

from the reference group. Increased glutathione peroxidase concentration was registered in 50% of samples taken in the focus group but there were no such samples in the reference group (p=0.01).

An increase in free radical oxidation processes leads to disorders in penetrability and functional properties of cellular membranes, in particular, hepatocytes [6]. It is also proved by an increase in ASAT and alkaline phosphatase activity in blood serum of children from the focus group (the parameter was up to 1.2 times higher than in the reference group, p=0.002–0.01). A number of samples with increased activity of these enzymes amounted to 51% and it was 2.21 times higher than in the reference group (23 %) (p=0.002–0.01).

We assessed excretory function of the bile-excreting tracts and detected a 1.2 times increase in crude bilirubin level in blood serum of children from the focus group against the same parameter in the reference group (p=0.005).

We examined immune regulation parameters and detected an authentic 1.8 times increase in level of IgG specific to N-NDMA against the same parameter in the reference group (p=0.01). Increased IgG to N-NDMA level was detected in 51% of samples taken in the focus group and it was 4.2 times higher than in the reference group (12 %, p=0.01).

Increased S-CEA concentration was detected in blood serum of 11% children from the focus group. We detected authentic deviations in fetal proteins concentrations from the same parameter in the reference group: S-CEA level was 2.4 times higher (p=0.01); CA-199, 1.9 times higher (p=0.023).

Detected cause-and-effect relationships within "N-NDMA concentration in blood – immunologic and biochemical parameters" system allowed us to determine regularities related to changes in immunologic and biochemical parameters of blood; these regularities confirmed that the toxicant exerted specific and non-specific impacts on the immune and digestive system [15]. Models and parameters that describe "N-NDMA concentration in blood – IgG to N-NDMA concentration in blood" are given in Table 3.

Assessment of a correlation between specific sensitization to N-NDMA as per IgG criterion revealed that increased N-NDMA concentration in blood of children from the focus group led to increased level of IgG to N-NDMA and was a linear relationship (Figure 1).

Table 3

Parameters and criteria of the model for "N-NDMA concentration in blood – IgG to N-NDMA concentration in blood" relationship

Model equation	Model parameters		Fischer test, F	Validity of model, p	Determination coefficient, R^2
	b_0	b_1			
y = 0,0094+10,76x	0,00944	10,76	1202,19	0,001	0,958

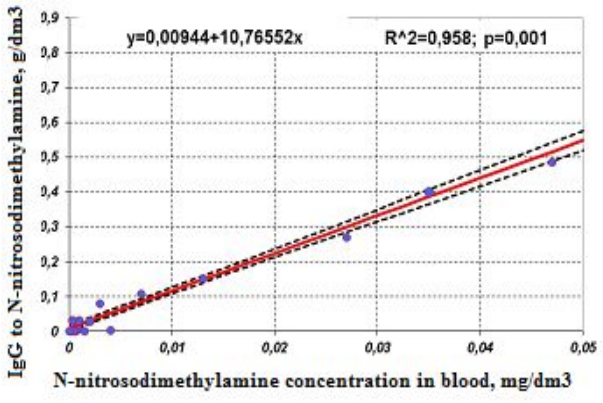

Figure 1. Model of linear "N-NDMA concentration in blood – IgG to N-NDMA concentration in blood" relationship

As we detected an increase in average group N-NDMA concentration in blood of the examined children from the focus group, we also detected an authentic (p=0.001) increase in concentration of IgG specific to N-NDMA that was confirmed by the obtained model of linear "N-NDMA concentration in blood – IgG to N-NDMA concentration in blood" relationship described with the following equation: y=0.0094+10.76x (Table 3, Figure 1).

Concentration of IgG specific to N-NDMA deviated from average values; a share of explained dispersion of these deviations was related to a factor parameter of N-NDMA concentration and amounted to 96%.

Models and parameters that described "N-NDMA concentration in blood – CA-199 concentration" relationship and "N-NDMA concentration in blood – S-CEA concentration" relationship are given in Table 4.

Table 4

Parameters and criteria of the model for "N-NDMA concentration in blood – CA-199, S-CEA concentration in blood" relationship

Model equation	Model parameters		Fischer test, F	Validity of model, p	Determination coefficient, R^2
	b_0	b_1			
y = 1,072+34,92x	0,611	3,090	12,170	0,0008	0,135
y = 0,611+3,09x	1,072	34,915	21,137	0,0001	0,203

Mathematical modeling allowed us to obtain authentic relationships for "N-NDMA concentration in blood – CA-199, S-CEA concentration in blood" that were described with the following equations: y=0.611+3.09x and y=1.072+34.92x accordingly (p=0.0001–0.0008). The detected linear relationships showed that an increase in average group concentrations of fetal proteins (CA-199 an S-CEA) in blood of the examined children from the focus group was related to N-NDMA concentration in blood. These linear relationships are shown on Figures 2 and 3.

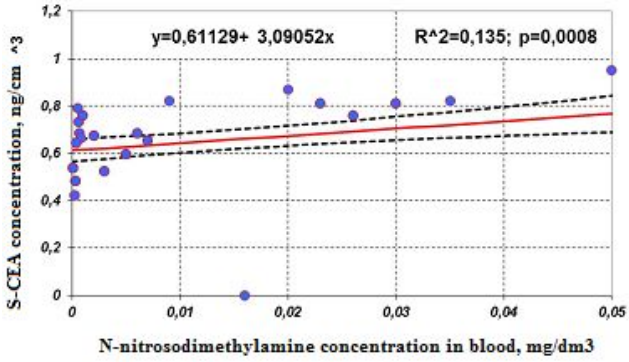

Figure 2. Model of linear "N-NDMA concentration in blood – S-CEA concentration in blood" relationship

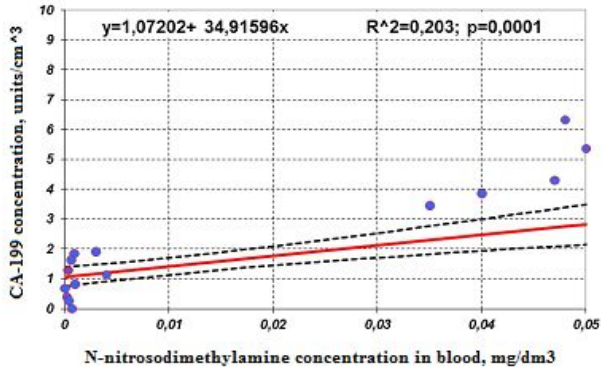

Figure 3. Model of linear "N-NDMA concentration in blood – CA-199 concentration in blood" relationship

Concentrations of CA-199 and S-CEA in blood deviated from average values; shares of explained dispersion of these deviations related to N-NDMA factor parameter amounted to 14% and 20% correspondingly, the determination coefficient being authentic.

Basing on the obtained regularities, we detected that if N-NDMA concentration in blood increased by 1 mg, CA-199 and S-CEA concentration increased on average by 0.2 units/ml and 0.002 units/ml correspondingly. This correlation can be considered a signal parameter showing a proliferation process occurs under such level of exposure to nitrates and N-NDMA in drinking water. When concentrations of nitrates and N-NDMA in drinking water increase, fetal proteins concentrations in blood will go up too.

Indicators of an effect and parameters of models that describe dependences of deviations in biochemical blood parameters on N-NDMA concentration in blood and characterize occurrence of negative effects in children are given in Table 5.

Assessment of parameters that characterize oxidation processes activity reveals that free radical damage to cellular membranes is intensified. An authentic correlation between increased lipids hydroperoxides concentration in blood serum and N-NDMA content (R^2=0.73; F=27.97; p=0.000) was an evidence that free-radical oxidation processes became more active.

Our research revealed that if N-NDMA concentration in children's blood increased, glutathione peroxidase, an intra-cellular enzyme, authentically (p=0.000) became more active (R^2=0.93; F=39.99).

Statistically authentic cause-and-effect relationships between a decrease in glutathione-S-transferase (R^2=0.49; F=88.99; p=0.000) and increased N-NDMA concentration in blood also proves there was strain in a body antioxidant protection as a response to more active free-radical processes. Liver enzymes, ASAT and alkaline phosphatase, probably became more active due to lytic impacts exerted by highly toxic N-NDMA on

Table 5

Parameters and criteria of models for "N-NDMA concentration in blood – biochemical blood parameters" relationship

Indicator of an effect	A change in a trend	Model parameters		Fischer test (F)	Validity (p<0,05)	Determination coefficient (R^2)
		a_0	a_1			
Lipid hydroperoxides	Increase	–1,80	8089,90	27,96	0,000	0,73
Glutathione-S-transferase	Decrease	0,28	4138,70	88,99	0,000	0,49
Glutathione peroxidase	Increase	–1,21	7927,64	39,99	0,000	**0,93**
ASAT	Increase	–0,38	81,89	85,66	0,000	**0,81**
Alkaline phosphatase	Increase	–1,39	1610,25	28,98	0,000	0,58
Crude bilirubin	Increase	–0,15	136,649	38,433	0,000	**0,84**
IgG to N-NDMA	Increase	–0,832	–24,497	85,465	0	**0,95**

Table 6

Results of research on cause-and-effect relationships between increased N-NDMA concentration in blood and biochemical blood parameters

Indicator	Response to an impact	Number of children		*OR*	95 % *DI*	Risk (R)	OR
		Risk factors occurrence	Absence of risk factors				
More active glutathione peroxidase	yes	18	13	8,92	3,28–24,25	0,49	2,91
	no	9	58				
More active ASAT	yes	70	43	4,67	2,67–8,19	0,50	1,78
	no	30	86				
Increased crude bilirubin	yes	93	58	11,55	5,52–24,16	0,59	1,65
	no	10	72				
Increased IgG to N-NDMA	yes	44	12	5,36	2,38–12,05	0,63	1,30
	no	26	38				

hepatocytes membranes and it could cause a risk of cytolysis syndrome. It was also proved by a detected statistically authentic dependence of an increase in activity of ASAT and alkaline phosphatase in blood serum on increased N-NDMA concentration in blood (R^2=0.58–0.81; 28.98≤F≤85.66; p=0.000).

We assessed excretory function of the bile-excreting tracts and proved that increased crude bilirubin level authentically depended on increased N-NDMA concentration in blood (R^2=0.84; F=38.43, p=0.000).

Results of our research on cause-and-effect relationships as per odds ratio (OR)[10] parameter are given in Table 6.

We verified a relationship between N-NDMA concentration in blood and glutathione peroxidase concentration (OR=8.92, DI=3.28–4.25) and it causes 2.91 times higher risk of greater strain in functional state of a body antioxidant protection system.

We also proved there was a correlation between increased penetrability of liver cells membranes (more active ASAT in blood serum) and increased N-NDMA concentration in blood (OR=4.67, DI is from 2.67 to 10.97). Accordingly, risk of increased liver enzymes levels is 1.78 times higher.

We assessed excretory-concentration function of the bile-excreting tracts and detected an authentic cause-and-effect correlation between increased crude bilirubin in blood serum and increased N-NDMA concentration in blood (OR=11.55, DI is from 5.52 to 24.16) (Table 6). Risk of a decrease in excretory function of the liver is 1.65 times higher.

We proved that concentration of IgG to N-NDMA depended on N-NDMA concentration in

[10] Chetyirkin E.M. Statistical forecasting techniques. Moscow, Statistices Publ., 1977, 356 p. (in Russian).

blood (OR=5.36, DI is from 2.38 to 12.05). Risk of changes in humoral immunity is 1.3 times higher.

Therefore, hygienic indication and criterial assessment of effects produced by chronic exposure to nitrates and N-NDMA consumed with drinking water allowed us to prove that changes in some biological regulation parameters (biochemical and immunologic ones) depended on increased N-NDMA concentration in blood. We substantiated indicators of effects produced by nitrates and N-NDMA consumed with drinking water on the basis of OR calculation and determination of cause-and-effect relationships between N-NDMA concentration in blood and indicators of responses. The substantiated indicators of effects are increased activity of glutathione peroxidase, increased ASAT activity, and higher levels of IgG to N-NDMA and crude bilirubin. These indicators of effects can be applied to assess risks of impacts on human health exerted by non-organic nitrogen-containing compounds consumed with drinking water; they can also be quite useful for prevention activities development.

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