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Combined subchronic fluoride-lead intoxication and its attenuation with the help of a complex of bioprotectors

B.A. Katsnelson, L.I. Privalova, Ye. P. Kireyeva, O.S. Yeremenko, M.P. Sutunkova, I.E. Valamina*, A.N.Varaksin**, V.G. Panov**, J.I. Kazmer

The Medical Research Center for Prophylaxis and Health Protection in Industrial Workers
* The Urals State Medical Academy
** The Institute of Industrial Ecology of the Russian Academy of Sciences. (the Urals Branch) Ekaterinburg, Russia

Key words
Fuoride; lead; combined toxicity; bioprotectors

Parole chiave
Fluoro; piombo; tossicità combinata; bioprotettori

Summary
Background: *Combined toxicity of lead and fluoride has been studied insufficiently, and there is no known information about attempts to inhibit it with any bioprotectors.* **Methods:** *Lead acetate and sodium fluoride, administered separately or in combination, were injected i.p. to rats at isoeffective sublethal doses 3 times a week for 6 weeks. Some of the rats were exposed to the same combination against the background of oral administration of a bioprotector complex (BPC) comprising pectin, glutamate, and multivitamin/multimineral preparations. Following exposure, functional and biochemical indices and histopathological examinations of the femur of exposed and control rats were evaluated for signs of toxicity.* **Results:** *We have shown that with regard to a number of effects on the organism level the combined toxicity of lead and fluoride may be evaluated as additive or even superadditive, but lead reduces fluoride accumulation in the bone, and pathological changes in the bone tissue proved to be less marked for combined exposure compared with separate exposures. The BPC has been demonstrated to attenuate a range of the combined harmful effects of lead and fluoride, including those on the bone tissue.* **Conclusions:** *In spite of the fact that fluoride and lead may reciprocally attenuate their harmful effects on the bone tissue in case of combined exposure, they prove to be more toxic for soft tissues just in combination than when administered separately. The development of combined intoxication may be substantially inhibited by means of the tested set of innocuous biologically active agents.*

Riassunto
«Intossicazione subcronica da piombo e fluoro combinati ed attenuazione degli effetti per mezzo di un complesso di bioprotettori». **Introduzione:** *La tossicità combinata di piombo e fluoro non è stata finora studiata in modo adeguato e non vi sono informazioni sulla possibilità di inibirla con la somministrazione di bioprotettori.* **Materiali e Metodi:** *Acetato di piombo e fluoruro di sodio, somministrati separatamente o in combinazione, sono stati*

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Corrispondenza: Professor Boris A. Katsnelson, MD, DSc, Dept. of Toxicology and Biological Prophylaxis, Medical Research Center for Prophylaxis & Health Protection, 30 Popov Str, Ekaterinburg, 620014 Russia - Tel. 7- 343- 3718-721 - Fax: 7- 343- 3718-740 - E-mail: bkaznelson@ymrc.ru; honored_sci@yahoo.com

iniettati per via intraperitoneale a ratti a dosi subletali tre volte alla settimana per 6 settimane. Ad un gruppo di questi animali è stato dato per via orale un bioprotettore contenente pectina, glutammato ed una miscela multivitaminica e multiminerale. Nei ratti trattati, ed in quelli di controllo, sono stati determinati numerosi indicatori biochimici e funzionali e si è proceduto anche allo studio istologico del femore così da poter valutare la tossicità indotta. **Risultati:** *Si è potuto osservare come l'effetto combinato di fluoro e piombo possa essere considerato come additivo e talora anche superadditivo e come il piombo riduca il livello di fluoro nell'osso ed infine come le alterazioni patologiche nel tessuto osseo fossero meno evidenti nell'esposizione combinata in confronto a quella dei singoli metalli somministrati separatamente. L'aggiunta di bioprotettori (BPC) è stata capace di attenuare una serie di effetti del fluoro e del piombo, compresi quelli riscontrabili nel tessuto osseo.* **Conclusione:** *Malgrado fluoro e piombo siano in grado di ridurre reciprocamente i loro effetti nocivi sul tessuto osseo quando somministrati in combinazione, questi si sono dimostrati più lesivi per i tessuti molli quando dati in combinazione piuttosto che separatamente l'uno dall'altro. Lo sviluppo di una intossicazione dei due xenobiotici, iniettati contemporaneamente, può essere inibita in modo sostanziale dal bioprotettore testato.*

The exposure of urban populations to toxic environmental pollutants resulting from the operation of nonferrous metallurgy and chemical industry is, as a rule, of a multicomponent nature. Amongst toxic environmental combinations typically present in a range of urban areas in the Urals (Russia) are those of fluorine compounds (due, first of all, to emissions from electrolytic aluminium and superphosphate production facilities) and inorganic lead compounds (due to primary and secondary metallurgy of lead, copper and alloys of these metals and to persistent environmental contamination with lead accumulated over a long period of automotive transport's operation on leaded gasoline). Besides, a combined lead-fluoride pollution of workroom and ambient air is possible in the ceramic industry using sodium silicofluoride along with lead glazes.

Both of these elements are characterized by high toxicity, affecting adversely a lot of systems in the organism, often with similar targets of toxic action (6, 7, 9, 22, 27). In particular, one should note the relationship between the toxicodynamics of both elements and calcium metabolism and the toxic effects of both elements on the thyroid gland and on the bone tissue.

However, there are very little factual data on the combined toxicity of lead and fluorides in the scientific literature. This issue attracted the attention of some US authors in connection with the old discussion about the benefits and risks of water treatment with fluoride (as a method of preventing caries) because in a number of cities in the eastern states there are still sections of water supply piping made of lead. An evaluation of lead content of the blood in more than 280 000 children in the state of Massachusetts revealed that water treatment with fluoride raises this index as well as the related prevalence of neuropsychiatric disorders (18). In an experiment on rats it was shown that when lead is added to drinking water in combination with fluoride the concentration of this metal rises in both blood and teeth, whereas lead and fluoride combination did not influence the accumulation of fluoride in the same tissues (25). A reduced learning ability was discovered in the offspring of female rats exposed to a combined effect of lead and fluoride, in comparison with the action of lead only or of fluoride only (26). In the same offspring, exposure to this combination produced the greatest reduction in the glutamate content of the brain (hippocampus), glutamate being the principal mediator of excitation in the central nervous system and playing an important role in the learning processes.

The purpose of our study was, on the one hand, to explore the type of combined fluoride-lead intoxication over a sufficiently broad range of indices, and on the other hand, to try and inhibit its development by means of a complex of bioprotectors selected in accordance with the general principles of "the biological prophylaxis", a research field that

our team has been developing and implementing in practice for a long period of time (11-14, 23). Rather than considering this issue in connection with water treatment with fluoride and lead contamination of drinking water, we consider it in terms of industrial contamination affecting a lot of compartments in environment and, thereby, the various paths of toxic exposure of the working and common population. In our experiments, therefore, we have thought it reasonable to abstract from the role of this or that path and take advantage of the "artificial" model of intoxication caused by repeated intraperitoneal injections, a model widely adopted in toxicology. One of its essential advantages is the possibility of exact dosage of toxicants to which the interior milieu is exposed.

Materials and methods

This experiment was carried out on outbred white female rats (from our own breeding colony) with the initial body weight of 180-190 g, 15 animals in each exposed and control group. All rats were housed in conventional conditions, breathed unfiltered air and were fed standard balanced food and clean bottled water. The study was planned and implemented in accordance with the "International guiding principles for biomedical research involving animals" developed by the Council for International Organizations of Medical Sciences (1985).

The toxicants used were sodium fluoride and lead acetate. The bioprotector complex (BPC) included: sodium glutamate, apple pectin ("Promavtomatika", Russia), Complivit Calcium D3 multivitamin-multimineral preparation (containing the full range of vitamins plus iron, manganese, calcium, and selenium) and "Complivit Calcium D3" as an additional source of calcium («Farmstandart-UfaVITA», Russia). We have described both the theoretical rationales for choosing these bioprotectors and the extensive positive experience from their trials under various isolated and combined intoxications, including those due to lead, in a list of publications (10-15, 23).

The model of subchronic intoxication was created by repeated intraperitoneal injections of the salts under study to rats 3 times a week for 6 weeks (totally, 18 injections). The dosage of the salts corresponded to 0.025 LD_{50} and amounted to 1.45 mg/kg for sodium fluoride, and 5.5 mg/kg for lead acetate. Animals in the control group were administered normal saline in the same volume (0.5 ml per rat).

Sodium glutamate was administered instead of drinking water as 1.5% solution (in terms of glutamic acid). The average daily volume of the solution drunk by a rat was 10-12 ml. The other preparations included into the BPC were ground and given to the animals five times a week mixed with food, whereby pectin (in terms of 1 g per 1 kg of body mass) was mixed with a portion of food separately from the multivitamin preparations. The doses of vitamins and mineral supplements were calculated allowing for the physiological need of rats for them, which was increased 2-3 times considering the presumably raised demand for antioxidant vitamins under toxic exposure to fluoride and lead (vitamins and minerals in the standard food were also taken into account). The dosage of «Complivit Active» was 1 tablet per 10 rats, and the dose of «Complivit Calcium D3» was 1 tablet per 45 rats.

After the exposure period, the following procedures were performed for all rats:

- weighing;
- estimation of CNS ability to the temporal summation of sub-threshold impulses - a variant of withdrawal reflex and its facilitation by repeated electrical stimulations in intact, conscious rat (24);
- recording of the number of head-dips into holes and number of crossed squares on a hole-board, which is frequently used for studying behavioral effects of toxicants and drugs (e.g.) (1, 4);
- collection of daily urine for analysis of its density, urine output as well as lead, fluoride, coproporphyrin, delta-aminolevulinic acid (δ-ALA), and creatinine contents;
- sampling of capillary blood from a notch on the tail for examining the standard hemogram, reticulocytes count, haemoglobin content, and for cytochemical determination of succinate

dehydrogenase (SDH) activity in lymphocytes (by the reduction of nitrotetrazolium violet to formazane, the number of granules of which in a cell is counted under immersion microscopy).

Then rats were killed by decapitation and blood was collected by exsanguination. Biochemical indices determined from serum included calcium, total protein, albumin, globulin, bilirubin, cholesterol, glucose, ceruloplasmin, malonyldialdehyde (MDA), alkaline phosphatase, alanine- and asparate-transaminases (ALT, AST), gamma glutamine transferase, amylase, and cholinesterase.

The fluoride content of the urine and of the bone tissue (after pyrohydrolysis) was determined potentiometrically with the help of a Shimadzu atomic absorption spectrophotometer.

The thyroid hormones and the thyrotropic hormone contents was determined in the blood serum by the ELISA method on a Multiskan EX Microplate Photometer with on-board software.

The bone marrow smears from the femur were fixed with methanol and stained by the Pappenheim method for counting the number of micronuclei per 1000 polychromatophilic erythrocytes.

The femurs released from the muscle sheath were fixed in 10% neutral formalin and then were decalcified in Trilon B. For making tissue specimens, bone fragments were passed through a set of alcohols of increasing concentration and then were embedded in wax. Microsections were prepared with longitudinal orientation of preparations and were stained with hematoxyiline-eozine and with picro-fuchsine by the van Gieson method. For comparative assessment of changes in different groups, we used semiquantitative criteria characterizing the condition of the bone tissue in the diaphysis, metaphysis and epiphysis areas and the maturity of the growth cartilage against a 4-point scale: 0 = the feature is absent; 1 - the feature is expressed weakly; 2 - the feature is expressed moderately; 3 - the feature is expressed markedly. We also measured the thickness of the bone plate of the diaphysis using an ocular micrometer, whilst the number of osteocytes/osteoblasts in the bone diaphyses and the proportion of bone trabeculae in the metaphysis preparation were estimated with the help of Avtandilov grid (2).

The statistical significance of differences between the mean values of indices was estimated by means of multiple comparisons with Bonferroni correction (19). Also, recursive linear discriminant analysis was applied to find minimum sets of features enabling 100 % correct classification of an object (in our case, classification of a rat into either of the two groups compared) (16).

Results

Table 1 presents data relevant for juxtaposing systemic effects of the Fluoride – Lead combination with those of either Fluoride or Lead separately (and each of respective group of rats - with the control one). In table 2 data pertaining to combined exposure and control groups are repeated for comparing them with those obtained when rats were exposed to the same Fluoride – Lead combination on the background of the bio-protective complex (BPC). To avoid data redundancy in this table, it does not show the results obtained for the group of rats that were administered the BPC without any toxic exposure, because a statistically significant difference from the control group was observed for 5 indices only: SDH activity of blood lymphocytes (760.0±5.9), blood creatinine (88.85±5.62), thyroxinee (27,13±1,34), bone fluoride (9.38±0.98), bone lead (4.28±1.12) (the dimensional units of the values are the same as in tables 1 and 2).

The mean scores of the histological characteristics of the bone tissue are given in Table 3, and quantitative morphometric characteristics in Table 4. For illustrating histopathological changes in the femur, we have had to restrict ourselves to 5 microphotographs demonstrating the condition of bone trabeculae, typical for the metaphyses of the rats in the groups compared (figures 1–5).

Discussion

As can be seen from table 1, both toxicants as well as their combination caused changes (in comparison with the control group) in a large number

Table 1 - Some indices to the condition of rat organism after the exposure period (for comparing effects of separate *vs* combined exposures to Fluoride and Lead) (x±s.e.)

Index	Control group	Groups exposed to:		
		Fluoride	Lead	Fluoride and Lead
Body mass, g	236.25±4.27	230.77±4.27	219.17±6.21	221.67±3.16
Temporal summation of sub-threshold impulses, sec.	12.42±0.79	11.94±1.26	14.50±1.17	18.34±0.56*•
Number of head-dips into holes during 3 min	6.80±0.51	6.20±1.21	4.60±0.81	3.70±0.6
Number of squares crossed over within 3 min	13.90±1.62	17.20±4.45	11.80±2.09	5.70±1.27•
Haemoglobin, g/l	96.01±2.62	105.72±4.56	78.67±2.07*•	78.35±2.80*•
Erythrocytes, 10^{12} g/l	6.48±0.22	6.59±0.15	5.29±0.14*•	5.20±0.11*•
Reticulocytes, ‰	16.67±2.55	23.10±5.94	63.70±8.96*•	86.67±12.34*•
Lymphocytes, %	73.40±5.96	66.90±2.21	61.11±2.95*	57.20±3.87*
Segmented neutrophils, %	15.50±1.59	21.50±2.06	29.67±2.17*	34.70±3.10*•
Monocytes, %	4.50±0.99	6.60±1.33	6.22±1.18	5.20±1.17
Eosinophils, %	4.50±0.75	4.20±0.68	2.50±0.50	2.67±0.56
Succinate dehydrogenase activity, number of formazane granules per 50 lymphocytes	705.5±4.1	590.3±7.6*	599.9±7.3*	579.8±9.9*
ALT activity in blood serum, mM/h*l	0.53±0.08	0.47±0.07	0.67±0.06	0.55±0.05
AST activity in blood serum, mM/h*l	0.62±0.10	0.51±0.12	0.66±0.09	0.57±0.10
De Ritis coefficient	1.22±0.15	1.12±0.24	1.10±0.19	1.11±0.19
MDA in blood serum, nmol/l	4.17±0.28	4.67±0.26	5.31±0.32*	5.00±0.35
Ceruloplasmin in blood serum, mg/l	25.50±1.32	24.64±2.33	34.77±2.07*•	32.97±2.40•
Coproporphyrin in urine, nM/l	83.23±37.71	105.04±18.62	453.10±72.42*•	479.78±86.45*•
Coproporphyrin, in urine, nM/day	1.88±0.70	3.06±0.59	13.71±3.00*•	17.54±2.02*•
δ–ALA in urine, μmol/l	13.66±2.79	14.38±1.64	152.03±14.00*•	164.83±14.25*•
δ–ALA in urine μmol/day	0.38±0.07	0.42±0.05	4.33±0.58*•	6.47±0.68*•▪
Total protein content of blood serum, g/l	79.67±3.61	83.78±1.95	71.31±2.35•	69.75±2.50•
Albumins content of blood serum, g/l	39.50±0.78	39.61±0.70	32.18±0.37*•	32.31±0.93*•
Alkaline phosphatase in blood serum, nmol/(s*l)	102.42±8.55	99.94±12.28	127.15±6.94•	170.90±11.81•▪
Activity of γ-glutamintransferase in blood serum, nmol/(s*l)	3.34±0.27	3.33±0.37	4.27±0.44	3.87±0.27
Calcium in blood, mmol/l	2.91±0.04	2.36±0.08*	2.39±0.15*	2.17±0.08*
Creatinine in blood serum, μmol/l	72.30±0.03	93.78±4.91	95.21±16.37	80.90±7.18
Cholinesterase in blood serum, units/l	970.40±111.23	990.00±84.44	298.83±22.40*•	255.18±23.86*•
Micronuclei per 1000 polychromatophilic erythrocytes	0.63±0.26	1.88±0.52	2.63±0.65*	1.29±0.42
Thyrotropic hormone of hypophysis in blood serum, Mmol/l	0.20±0.04	0.16±0.02	0.18±0.01	0.12±0.02
Thyroxine in blood serum, pmol/l	31.99±1.59	30.15±1.71	39.51±2.59•	40.21±3.02•
Triiodothyronine in blood serum, pmol/l	2.88±0.27	3.05±0.28	2.97±0.38	2.15±0.39
Fluoride in urine, μg/day	22.07±2.48	36.47±2.21*	26.26±2.65	51.99±4.41*•▪
Fluoride in bone, mg/kg	12.57±0.53	45.00±4.94*	10.25±0.94•	33.5±3.48*▪
Lead in urine, μg/day	0.20±0.06	0.24±0.02	5.03±1.33*•	5.90±1.26*•
Lead in bone, mg/kg	1.98±0.29	4.64±0.73	305.54±9.53*•	279.13±17.46*•

Note: *=is a statistically significant difference from the "control" group; •=the same from the "Fluoride" group; ▪=the same from the "Lead" group by multiple comparison test with Bonferroni correction, $p \leq 0.05$

Table 2 - Some indices to the condition of rat organism after the exposure period with or without bio-protection (x±s.e.)

Index	Control group	Groups exposed to:	
		Fluoride and Lead	Fluoride and Lead on the background of bio-protectors
Body mass, g	236.25±4.27	221.67±3.16*	215.83±4.21*
Temporal summation of sub-threshold impulses, sec.	12.42±0.79	18.34±0.56*	17.00±0.57*
Number of head-dips into holes during 3 min	6.80±0.51	3.70±0.68	5.80±1.67
Number of squares crossed over within 3 min	13.90±1.62	5.70±1.27*	9.40±2.13
Haemoglobin, g/l	96.01±2.62	78.35±2.80*	80.41±2.24*
Erythrocytes, 10^{12} g/l	6.48±0.22	5.20±0.11*	5.46±0.22*
Reticulocytes, ‰	16.67±2.55	86.67±12.34*	42.86±6.40♦
Lymphocytes, %	73.40±2.38	57.20±3.87*	62.60±2.35*
Segmented neutrophils, %	15.50±1.59	34.70±3.10*	26.20±1.97♦*
Monocytes, %	4.50±0.99	5.20±1.17	7.30±0.84
Eosinophils, %	4.50±0.75	2.67±0.56	4.14±0.86
Succinate dehydrogenase activity, number of formazane granules per 50 lymphocytes	705.5±4.1	579.8±9.9*	678.6±13.7♦
ALT activity in blood serum, mM/h*l	0.53±0.08	0.55±0.05	0.64±0.09
AST activity in blood serum, mM/h*l	0.62±0.10	0.57±0.10	0.69±0.09
De Ritis coefficient	1.22±0.15	1.11±0.19	1.20±0.09
MDA in blood serum, nmol/l	4.17±0.28	5.00±0.35	4.08±0.34
Ceruloplasmin in blood serum, mg/l	25.50±1.32	32.97±2.40*	33.94±1.86*
Coproporphyrin in urine, nM/l	83.23±37.71	479.78±86.45*	432.60±67.13*
Coproporphyrin, in urine, nM/day	1.88±0.70	17.54±2.02*	14.18±2.10*
δ–ALA in urine, μmol/l	13.66±2.79	164.83±14.25*	131.96±8.04 *
δ–ALA in urine μmol/day	0.38±0.07	6.47±0.68*	4.48±0.45♦*
Total protein content of blood serum, g/l	79.67±3.61	69.75±2.50*	70.93±1.22
Albumins content of blood serum, g/l	39.50±0.78	32.31±0.93*	33.83±1.05*
Alkaline phosphatase in blood serum, nmol/(s*l)	102.42±8.55	170.90±11.81*	194.65±21.19*
Activity of γ-glutamintransferase in blood serum, nmol/(s*l)	3.34±0.27	3.87±0.27	5.08±0.51*
Calcium in blood, mmol/l	2.91±0.04	2.17±0.08*	2.68±0.09♦
Creatinine in blood serum, μmol/l	72.30±2.42	80.90±7.18	89.90±7.68
Cholinesterase in blood serum, units/l	970.40±111.23	255.18±23.86*	242.75±15.86*
Micronuclei per 1000 polychromatophilic erythrocytes	0.63±0.26	1.29±0.42	1.00±0.29
Thyrotropic hormone of hypophysis in blood serum, Mmol/l	0.20±0.04	0.12±0.02	0.12±0.02
Thyroxine in blood serum, pmol/l	31.99±1.59	40.21±3.02*	40.65±1.54*
Triiodothyronine in blood serum, pmol/l	2.88±0.27	2.15±0.39	2.30±0.24
Fluoride in urine, μg/day	22.07±2.48	51.99±4.41*	44.92±2.36*
Fluoride in bone, mg/kg	12.57±0.53	33.5±3.48*	24.94±1.69♦*
Lead in urine, μg/day	0.20±0.02	5.90±1.26*	2.50±0.31♦
Lead in bone, mg/kg	1.98±0.29	279.13±17.46*	253.13±12.99*

Note: *=is a statistically significant difference from the "control" group; ♦=the same from the "Lead and Fluoride" group by multiple comparison test with Bonferroni correction, $p \leq 0.05$

Table 3 - The scores for qualitative features of the femur condition, (x±s.e)

Feature	Control group	Groups exposed to:			
		Fluoride	Lead	Fluoride and Lead	Fluoride and Lead on the background of bio-protectors
Condition of periosteum					
Irregularity of bone outer contour	0.60±0.24	2.80±0.20*	1.20±0.20	2.40±0.24	0.40±0.24♦
Irregularity of osteogenic layer of periosteum	1.40±0.24	2.20±0.20	2.20±0.20	1.60±0.40	1.20±0.45
Prominence of vascularisation	1.40±0.24	2.20±0.20	2.60±0.24	2.00±0.32	1.20±0.45
Condition of bone tissue in diaphysis area					
Matrix staining non-uniformity	0.60±0.24	2.80±0.20*	2.80±0.20*	2.20±0.20	0.80±0.37
Matrix staining intensity	2.40±0.24	0.80±0.20	1.60±0.24	1.80±0.38	2.0±0
Regularity of bone plates	2.80±0.20	0.20±0.20*	0.60±0.24*	1.00±0.32	2.40±0.24
Wide lacunas	0.80±0.20	2.80±0.20	1.80±0.20	1.80±0.37	1.0±0
Condition of bone in metaphysis area					
Presence of chondroblasts in trabeculae	0.20±0.20	2.80±0.20*	0.40±0.24•	1.60±0.51	1.00±0.32
Prominence of osteoid in trabeculae	0.80±0.20	3.00±0.00*	0.20±0.20	2.20±0.49▪	0.80±0.37
Prominence of periosteal osteoid	0	2.40±0.24*	0.40±0.24	0.80±0.37	0.20±0.20•
Thickness of trabeculae	1.80±0.20	2.60±0.24	1.0±0	1.80±0.37	1.80±0.20
Anastomoses of trabeculae	1.80±0.20	2.40±0.24	0.60±0.24	1.40±0.24	1.60±0.24
"Packaging density" of trabeculae	2.0±0	2.60±0.24	0.60±0.24	1.40±0.24	1.60±0.24
Condition of growth cartilage					
Prominence of cartilage plate	1.0±0	2.80±0.20	1.20±0.37	2.20±0.37	1.40±0.24
Presence of vesicular chondrocytes	2.4±0.24	0.40±0.24	0.40±0.24	0.40±0.24	1.20±0.20

Note: comparisons of these groups are obtained by the same procedure as for tables 1, 2 with Bonferroni correction. The notation for statistically significant difference ($p \leq 0.05$) is the same as in previous tables

Table 4 - Morphometric characteristics of femur (x±s.e.)

Feature	Control group	Groups exposed to:			
		Fluoride	Lead	Fluoride and Lead	Fluoride and Lead on the background of bio-protectors
Thickness (mm) of diaphysis bone wall (x±s.e.)	0.57±0.015	0.82±0.025*	0.66±0.025•	0.85±0.042*	0.60±0.012♦
Number of osteoblasts/osteocytes in a square of Avtandilov grid	16.28±0.57	32.70±0.83*	23.82±0.64•	24.70±0.61	16.86±0.52♦
Specific proportion of bone trabeculae in metaphysis (as %% of area)	45.06±1.26	55.88±1.70*	31.74±0.76*•	48.18±1.62•▪	39.70±1.27*♦

Note: comparisons of these groups are obtained by the same procedure as for tables 1, 2 with Bonferroni correction. The notation for statistically significant difference ($p \leq 0.05$) is the same as in previous tables

of indices. Many of the observed changes may be categorized as nonspecific ("integral") features of intoxication characterizing the disturbance of homeostasis on organism level which is observed virtually in any chronic intoxication. In our case, such features are, for example, weight loss; disbal-

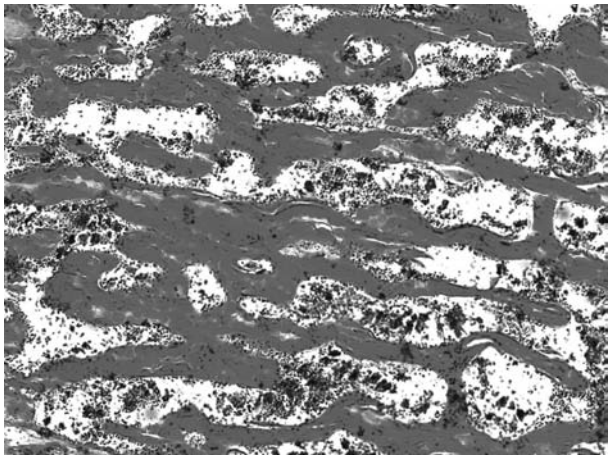

Figure 1 - Bone trabeculae in femur metaphysis of rats in control group. Staining by van Gieson, magnification x 100

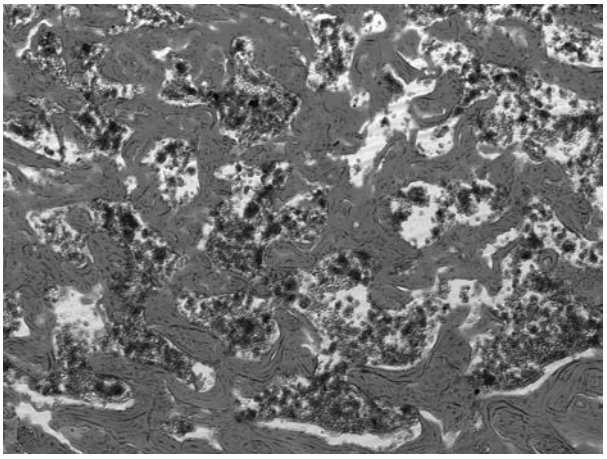

Figure 3 - Bone trabeculae in femur metaphysis of rats in group exposed to fluoride. Staining by van Gieson, magnification x 100

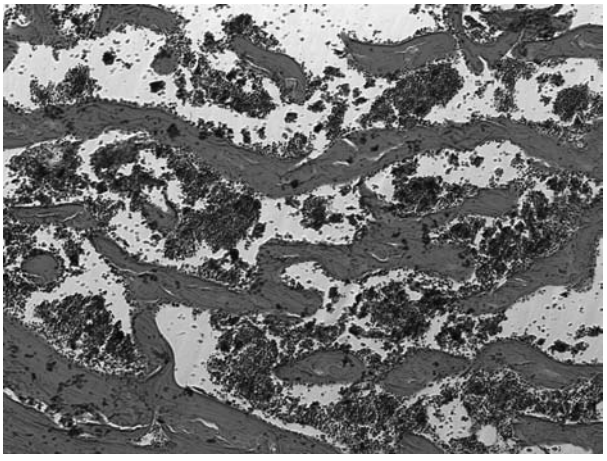

Figure 2 - Bone trabeculae in femur metaphysis of rats in group exposed to lead. Staining by van Gieson, magnification x 100

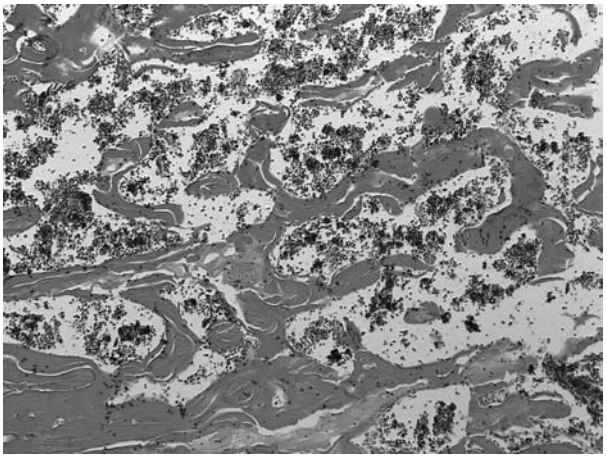

Figure 4 - Bone trabeculae in femur metaphysis of rats in group exposed to combined effect of lead and fluoride. Staining by van Gieson, magnification x 100

ance between excitation and inhibition processes in the central nervous system (judging by the temporal summation of sub-threshold impulses), motor activity (measured by the number of squares crossed), and exploratory behaviour (judging by the number of head dips into holes); general suppression of the energy metabolism showing itself by decrease in the blood lymphocyte SDH activity, while there was some enhancement of lipid peroxidation judging by the MDA content of the blood serum. It can be noted that, judging by some of these indices, lead is more toxic as compared with fluoride, although given in doses isoeffective in relation to their LD50.

At the same time, a number of changes may be classified as relatively specific for the effects of lead and/or fluoride. First of all, this applies to the typical indices for the effect of lead on red blood (reduction in the haemoglobin content and the number of erythrocytes with increased percentage of reticulocytes in them) and to the indices that reflect disturbances in porphyrin metabolism caused

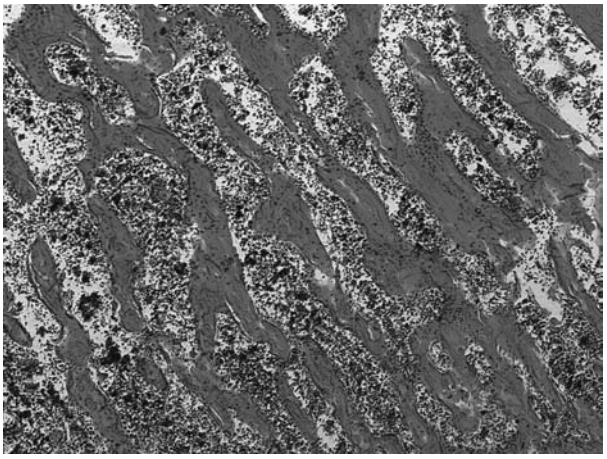

Figure 4 - Bone trabeculae in femur metaphysis of rats in group exposed to combined effect of lead and fluoride and administered bioprotectors. Staining by van Gieson, magnification x 100

by this metal (a sharp increase in the δ-ALA and coproporphyrin in urine). Characteristic of both lead and fluoride toxicities are disturbances of calcium metabolism which manifest themselves in a reduction in the calcium content of the blood. However, the activity of alkaline phosphatase, one of the key enzymes that control this metabolism, was increased in our experiment only under exposure to lead or its combination with fluoride. This is not quite consistent with the literature data, specifically, with the study (17), the authors of which related increase in the level of alkaline phosphatase in the blood serum in fluoride therapy of osteoporosis to the toxic effect of fluoride on both osteoblasts and osteoclasts. An increase in the serum level of both alkaline and acidic phosphatase was shown also to be present in rats administered fluoride with drinking water for 8 and 16 weeks (26).

Both fluoride and especially lead have displayed some mutagenic property judging by the increased number of micronuclei in the polychromatophilic erythrocytes of the bone marrow.

A comparison of values obtained for the groups of separate and combined exposure shows that for the majority of toxicodynamic indices the combined effect is more marked than the effect of fluoride only or lead only, and in some cases the difference of the combined effect from the effect of separate exposure is statistically significant. In cases where the effect under consideration due to separate exposure is observed for one of the toxicants only but is significantly enhanced in the presence of the second one, this combination may be deemed to act super-additively (potentiation, synergism). Examples of such synergism are shifts in all indices of nervous activity, reticulocyte count, excretion of δ-ALA, and alkaline phosphatase content of the blood serum. In other cases, in which this or that shift is provoked by both toxicants separately but is more marked in case of their combination, a marked effect of combined toxicity is also present but it would be difficult to determine the sign of possible deviation from simple additivity.

Earlier it was shown (for both hormonal shifts and histological changes in the thyroid gland) that lead has a thyrotoxic effect which can be attenuated by a complex of bioprotectors, in particular, by one with a iodine supplement (10). It is well known that fluoride, being a metabolic antagonist of iodine, also suppresses the hormonal function of the thyroid gland. This effect may be prevented by an increase in the calcium content of the rats' ration [for example, (27)]. In our experiment, both fluoride and lead caused a statistically insufficiently significant reduction in the thyrotropic hormone level, but under combined exposure this effect grew stronger and reached statistical significance. Neither fluoride nor lead produced a reduction in triiodothyronine level, but it was reduced under combined effect (i.e. obvious synergism took place). At exposure to lead separately or in combination with fluoride, the level of thyroxine was, on the contrary, raised.

The tendency towards an action of obviously antagonistic type, which is virtually absent if judged by all the used toxicodynamic indices, unexpectedly manifests itself when estimating combined mutagenicity: whereas fluoride and lead separately provoked a statistically significant increase in the number of micronuclei (3 and 4 times more than in controls, respectively), under combined exposure this increase in comparison with the control index was only 2-fold and statistically not significant.

The daily excretion of fluoride with urine at the end of the combined exposure period was signifi-

cantly higher than for separate fluoride exposure. The fluoride content of the bone tissue for the combined exposure was, on the contrary, somewhat reduced in comparison with separate action of fluoride. Though this reduction is insufficiently significant statistically, one should not ignore the fact that a similar tendency to reduced background fluoride content of the bone under the effect of lead is observed for the separate exposure to the latter as well. Unfortunately, we do not have any data on the fluoride content of the blood, which does not allow us to confirm directly the logical hypothesis that lead (through its influence on calcium metabolism or by any other mechanism) interferes with the uptake of fluoride in the bone, thereby promoting an increase in its content of the blood as the central toxicokinetic pool, and hence not only in urine (as it was really discovered) but also in target organs which are sensitive to the toxic effect of fluoride. Circumstantial evidence for this hypothetical mechanism is the fact that, whereas the toxic effect of fluoride on these organs under combined exposure proved to be enhanced according to the indices considered above, its toxic effect on the bone, as is discussed below, was noticeably reduced. Similarly, at combined fluoride-lead exposure there was less lead in the bone than at separate lead exposure, although this difference (as well as a small enhancement of excretion of lead with urine) was not statistically significant. The same pattern was also observed with the attenuation of histopathological changes in the bone in comparison with the "lead only" group (see below).

If we assume that the action of toxic elements on the bone marrow is associated with their transition into the cellular microenvironment not only from the blood but also directly from the bone trabeculae, a reduction in their content in the bone tissue could explain the above-mentioned paradoxical antagonism in the effect of lead and fluoride on the formation of micronuclei in polychromatophilic erythrocytes.

If we compare the indices given in columns "Fluoride and Lead" and "Fluoride and Lead on the background of bioprotectors " in table 2 we can see that the administration of the BPC reduced (although not always statistically significantly) the increase in the temporal summation of sub-threshold impulses provoked by the toxic combination; and almost normalized the exploratory reflex and raised the motor activity of the rats, which was significantly inhibited under intoxication. At the same time, BPC produced a statistically significant decrease in the number of reticulocytes (sharply increased under intoxication without protection), produced a small and statistically insignificant normalizing effect on the haemoglobin content and the number of erythrocytes (reduced under this intoxication without protection). It also normalized the MDA level, which was non-significantly raised under intoxication without protection; reduced insignificantly the excretion of coproporphyrin and significantly - the excretion of δ-ALA, which were raised under exposure to lead in combination with fluoride without protection. Besides, BPC increased the concentration of calcium in the blood serum, which was more reduced under intoxication without protection. The reduction in the number of micronuclei in polychromatophilic erythrocytes of the bone marrow under the effect of BPC proved to be statistically not significant but quantitatively substantial. Finally, BPC brought about a significant reduction in the fluoride content of the bone and insignificant reduction in the lead content of it. It may be suggested that towards the end of the experimental period this reflects overall reduction in the toxic elements content of the organism as a result of protection of kidneys from the toxic damage and enhanced excretory function at the earlier stages of this period, so that by its end the excretion of both lead and fluoride with urine proved to be reduced in the group that had received their combination on the background of BPC administration.

Although a beneficial influence of the BPC on systemic indices of the combined F-Pb toxicity may be deemed rather moderate, the following is worth consideration.

First, if there were only 7 features showing statistically significant difference between groups exposed to fluoride and lead with or without bioprotectors, namely: reticulocytes count, segmented neutrophils count, succinate dehydrogenase activity (SDH), delta-aminolevulinic acid daily excretion

with urine, calcium concentration in blood, fluoride concentration in bone, lead daily excretion with urine, - none of these differences was not in favor of bio-protection.

Second, discriminant analysis provided 4 short sets of features by which a rat could be reliably (100% correctly) classified as falling into the group of combined intoxication which had developed without protection of the organism or under protection with BPC. Those sets are:

1. SDH plus daily excretion of coproporhyrin with urine. For this set, the squared Mahalanobis distance Δ^2 =14.5. The most informative feature is SDH: if it is excluded from the discriminant function, the percentage of correct classification diminishes by 40%, while exclusion of coproporhyrin diminishes it but by 6%.

2. The same two features plus Fluoride in bone. Adding the latter, we drastically increase the discriminability of the groups under comparison (Δ^2 = 26.3).

3. Again SDH and daily excretion of coproporhyrin, plus daily excretion of Lead with urine. This set is virtually as discriminative as the previous one (Δ^2=24.1).

4. SDH, plus Calcium in blood, plus Fluoride in bone. This set is more discriminative than the first but less discriminative as compared with either the 2nd or the 3rd one (Δ^2=18,7).

It is interesting that SDH activity (a generalized "energetic mirror" of the oxidation-reduction metabolism) proved a necessary member of any short set of features sufficient for a 100% correct classification. Thus, these groups are, highly probably, samples from different classes not only by individual specific features but also by the generalized functional status of the organism.

Microphotographs (figures 1 to 5) illustrate, using the trabecular structure of the femur metaphysis as an example, the general impression that one has when examining the histological structure of various areas of this bone. This impression is that under combined toxic impact pathological changes of the bone structure are rather attenuated in comparison with the corresponding effects of separate exposures, and that against the influence of BPC the histological pattern of the bone of rats exposed to combined exposure appears to be little different from normal. This impression is confirmed to be true by scoring (table 3) and quantitative (table 4) assessment of relevant histological characteristics of the bone.

As can be seen from table 3, the group of rats exposed to separate fluoride intoxication is statistically significantly different from the control group by 5 out of 15 features estimated using a score, and the group of rats exposed to separate lead intoxication - by only 3 of the same features. Irrespective of the statistical significance of the shift, according to the majority of features it has the same directionality in both groups. The shifts caused by lead or by fluoride have an inverse sign only by three out of 15 features, specifically: whereas fluoride caused a significant elevated prominence of osteoid in the bone trabeculae of the metaphysis, the tendency towards greater prominence of anastamoses between them and a significant increase in their "packaging density", lead provided a marked reduction in all these features. A tendency towards such reduction occurs in the group of combined fluoride-lead exposure as well, which is indirect evidence of the antagonistic character of the action with the prevalence of the effect of lead (with the prominence of osteoid in trabeculae and the "package density" of trabeculae being important exceptions, as these indices are clearly more influenced by fluoride).

In this group of combined exposure, a statistically significant difference from the controls is also provided by none of 15 features, which confirms the impression of lead's counteraction against the osteo-toxic effect of fluoride. The mean scores of the features occupy, in most cases, an intermediate position between those for groups exposed to either fluoride or lead, while for three features (irregularity of the osteogenic layer of the periosteum, prominence of the vascularisation of the periosteum, and non-uniformity of the matrix staining) the score for the combined exposure is even lower than in both groups of separate exposure.

For the same combined impact of fluoride and lead but on the background of BPC, also no statistically significant difference from the controls was observed, but results are closer to the control in-

dices than to the indices for the other three groups. Moreover, for some of them there is no difference from the control group at all. Attenuation of combined exposure effects in the BPC group can be seen for 14 out of 15 indices, although for only one of them (irregularity of bone outer counter) the difference between the groups "combined impact" and «same with BPC» is statistically significant.

As can be seen from table 4, lead and, even to a greater extent, fluoride (alone or combined with lead) caused an increase in the thickness of the diaphysis osteal wall. Given the same direction of action of both toxicants for this index, the combined effect is actually determined by one of them and is not enhanced by the action of the second one, which may be interpreted as subadditivity of effects. With BPC administered, this index was practically normalized. The cellularity of the diaphysis is statistically significantly higher than in the control group for the separate action of both lead or (again to a greater extent) fluoride and their combination. However, in the combined exposure group, this index is somewhat lower than for exposure to fluoride alone, which, again, may be considered as a manifestation of lead-fluoride antagonism. At the same time, with BPC administered, this index, too, was completely normalized, being of the same value as for the controls (and so statistically significantly lower than under the same toxic exposure without bio-protection).

The effect of lead manifested itself in a decrease in the number of bone trabeculae in the metaphysis, while the effect of fluoride in an increase in them. With such contradirectionality of effects, combined exposure yielded a quasi-normalization of this index. Meantime, with BPC administered, it was significantly lower in comparison with its value in the control group and "fluoride" and "combined" groups but somewhat higher than in the "lead" group.

In the International Programme on Chemical Safety (IPCS) Environmental Health Criteria 227 (7) it is noted that "effects on the skeleton, such as inhibition of bone mineralization and formation, delayed fracture healing and reductions in bone volume and collagen synthesis, have been observed in a variety of studies in which rats received fluoride orally for periods of 3-5 weeks". Besides the sources of information given in that review, noteworthy is the study (3), in which it was shown that after repeated intraperitoneal injections of NaF in doses of 2.5 or 25 mg/kg daily for 3 months "the quantity of bone tissue increased and the quality diminished with increase in NaF levels administered". Our data point to a similar action even at lower fluoride exposure.

In the IPCS Environmental Health Criteria 165 (6), note was taken of biochemical mechanisms of probable influence of lead on bone tissue metabolism and, specifically, it was pointed out that it may contribute to the development of osteoporosis. At the same time it was stressed that "there has only been limited experimental study of these concerns to date". As far as we know, such experimental studies were not numerous or exhaustive in subsequent years as well. Thus, González-Riola et al. (5) observed, after a 50-day exposure of rats to lead acetate with food, only a reduction in the thickness of the growth cartilage plate of the femur with no change in bone length. After intragastric administration of lead acetate to rats at a dose of 100 mg/kg a day for 7 days and even after 7 days following the end of exposure, Jelea and Jelea (8) found an increase in the weight and length of the femur and tibia with enhanced bone mineralization. Monir et al. (20) discovered in the femur of mice given to drink lead-containing water for 4 months a decrease in the mineral/matrix ratio, collagen maturity and crystallinity.

Differences in methods of exposure, doses and duration of action in studies performed by different authors hinder comparison between the effects of fluoride and lead on bone tissue. Moreover, we have failed to find any data in the literature on the character of their combined action on the bone tissue. As for our results, they allow us to speak about various features of essential similarity in the pathological changes caused by these toxicants in the cortical bone tissue under appreciable differences in changes in the trabecular bone tissue, but in both cases the combined effect is mainly subadditive or explicitly antagonistic.

Nevertheless, pathological changes in the bone tissue even under seemingly antagonistic combined

exposure are serious enough to justify the imperative to attenuate their action by bioprotectors. It is all the more reasonable considering the fact that, by a great number of extra-skeletal intoxication indices, the combined toxicity of lead and fluoride has been proven to be of an additive or even superadditive character (synergism), and the BPC tested by us attenuates the corresponding effects. It is beyond doubt that this BPC protects against changes in the bone tissue as well.

Conclusion

Repeated intraperitoneal injections of sublethal doses of fluoride or lead to rats for 6 weeks were observed to produce intoxications, a number of features of which are characteristic of the effects of chronic exposure to the corresponding elements. With regard to their combined exposure, it has been shown that for a range of effects on organism or systemic level the combined toxic effect is additive or exceeds simple summation (i.e. synergism is observed). It is particularly noteworthy that under the effect of fluoride the adverse effect of lead on porphyrin metabolism (one of the key mechanisms underlying the toxicodynamics of this metal) is enhanced. At the same time, despite largely unidirectional character of respective changes, the combined toxic action of fluoride and lead on the bone tissue is dominated by subaddiitivity or even explicit antagonism of effects. At least, one of the mechanisms explaining such "discrepancy" in the combined toxicity of lead and fluoride is likely to be their reduced ability to accumulate in the bone with redistribution into soft tissue.

Against the background administration (with food and drink) of a complex comprising innocuous bioprotectors targeting some toxicodynamic and toxicokinetic mechanisms, an attenuation of many harmful effects of the lead-fluoride combination (especially of those on the bone tissue) has been observed.

No potential conflict of interest relevant to this article was reported

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