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Some outcomes and a hypothetical mechanism of combined lead and benzo (a)pyrene intoxication, and its alleviation with a complex of bioprotectors

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ABSTRACT

Rats were exposed 3 times a week during 6 weeks to repeated intraperitoneal injections of lead acetate solution in water (Pb) and/or benzo(a)pyrene solution in petrolatum oil (B(a)P) in various dose ratios. Towards the end of the period, the animals developed a moderate subchronic intoxication having some features characteristic of lead effects. The type of combined toxicity estimated with the help of isoboles constructed by the Response Surface Methodology was found to be varied depending on a particular effect, its level, and dose ratio. However, Pb and B(a)P in combination often displayed an additive or even superadditive action. In the group exposed to this combination compared with the group of rats exposed to B(a)P alone, its concentration in the organism was increased while the concentration of some B(a)P oxidative metabolism products was reduced. Such inhibition of B(a)P biotransformation, assumingly associated with impaired heme and, thus, cytochrome P450 synthesis induced by lead intoxication, can serve as an explanation for certain enhancement of the genotoxic effect of B(a)P. This effect was not present in the same combined intoxication if a complex of antitoxic bioprotectors was being administered in the background.

1. Introduction

Inorganic compounds of lead (Pb) and polycyclic aromatic hydrocarbons (PAH), including benzo(a)pyrene (B(a)P) belong to the most persistent and ubiquitous environmental pollutants. This fact accounts for a very high likelihood that these substances may occur in combinations impacting on large populations, particularly in industrially developed regions.

The problem of combined toxicity pertains to the theoretically most complex and practically important challenges in preventive toxicology [1–8]. Over a number of years, it has been a subject of extensive studies for our research team [9–15]. One of the least studied aspects of this problem is the possibility that inorganic toxicants (lead compounds in particular) may have some effect on the biotransformation of organic ones and thus on their toxicokinetics and toxicodynamics.

Oxidative transformations as a result of which a molecule of an organic substance acquires reactive nucleophylic groups (C-hydroxylation, N-hydroxylation, etc.) mainly take place in cells on the smooth endoplasmic reticulum in the liver and other organs. These transformations are controlled by so-called microsomal mixed-function oxidases. The central place in this poly-enzyme complex belongs to hemoprotein enzymes, among which cytochrome P450 plays a major role [16–18].

For a number of organic substances, including B(a)P and other PAHs, some primary products of microsomal oxidation may be more toxic, and also genotoxic, than the parent substance. However, during this and subsequent stages of oxidation, other products of PAH biotransformation may be formed which (unlike the parent substance) are capable of entering into various reactions of conjugation (glucuronide, sulfate, glutathione, glutamic, as well as methylation and acetylation) resulting in water- and fat-soluble compounds (conjugates), which are more easily eliminated with urine and, partly, bile [19–23].

As is well known, lead intoxication impairs porphyrine metabolism and thus inhibits heme synthesis. It may be expected that lead

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intoxication reduces, by the same mechanism, the enzyme activity reserve of hemoproteins, including cytochrome P450. Therefore some alterations in the toxicokinetics and toxicodynamics of the PAHs are quite likely to happen when they occur along with lead intoxication. Indeed, explicit changes in naphthalene biotransformation (i.e. the simplest analogue of PAH) were demonstrated by us previously [23] under combined intoxication with naphthalene and lead. We also found noticeable shifts in the toxicokinetics of B(a)P administered to rats against the background of subchronic intoxication with a combination of substances including lead acetate [22].

In the available literature there are some references to a combined environmental pollution with lead and some PAHs (e.g., [24]). However, the binary combined effects of B(a)P and lead on the organism have not been the focus of direct studies in the previous works of ours or of anyone else judging by the literature known to us. Data derived in just this kind of study have made up the subject of this paper.

2. Materials and methods

The experiment was performed on outbred white male rats with an initial body mass of around 260 g, 12 animals in each group. All these rats were housed (6 animals per a cage) in conventional conditions, breathed unfiltered air, and were fed standard balanced food and bottled artesian water. The experiments were 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) and were approved by the Ethics Committee of the Ekaterinburg Medical Research Center for Prophylaxis and Health Protection in Industrial Workers.

The rats were injected intraperitoneally (i.p.) during 6 weeks, three times a week, lead acetate solution in water at a dose of (by substance) 220 mg/kg of body mass and/or B(a)P solution in petrolatum oil at a dose of 10 mg/0.5 mL per animal, or the same substances in half-doses.[1] The control animals were injected i.p. water or petrolatum oil in corresponding volumes.

Over the entire experimental period, a half of the rats in the control group and in the group exposed to the toxic combination were given orally a complex of bioprotective substances listed in Table 1. The bioprotective effects of these substances in various intoxications, including lead-induced, has been demonstrated by us many times [10,22,23,25–27].[2] The same publications provide general and specific theoretical premises for such «biological prophylaxis».

At the end of the exposure period, we estimated more than 50 physiological, cytological and biochemical indices describing the condition of the organism, including:

- Weighing of the body
- Estimation of the CNS ability to evoke temporal summation of subthreshold impulses (a variant of the withdrawal reflex and its facilitation by repeated electrical stimulations in an intact, conscious rat) (e.g., [10,11,29–31])
- Recording of the number of head-dips into the holes of a hole-board (which is a simple but informative index of exploratory activity frequently used for studying the behavioral effects of toxicants and drugs) (e.g. [10,11,29,30,32,33])
- Collection of daily urine with the help of metabolic cages for assessing its output (diuresis), specific gravity (density), and total coproporhyrin content.

Table 1
Method of administration and dosage of bioprophylactic complex components.

Bioprotector	Estimated dose per rat and method of administration
N-acetylcysteine	With food, 30 mg
Glycine	With food, 12 mg
Sodium glutamate	With drink, 160 mg (1.5 % solution instead of water)
Iodine	With food, 4 mkg
Iron	With food, 1 mg
Calcium	With food, 160 mg
Vitamin C	With food, 4.5 mg
Vitamin D3	With food, 1.7 μg
Pectin	With food, 200 mg
Fish oil rich in Omega 3 PUFA and vitamin A	Orally, 1 drop

- Sampling of capillary blood from a notch on the tail for examining the hemogram, hemoglobin 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 the rats were killed by decapitation under light ether narcosis and the whole available volume of blood was collected by exsanguination. The liver, spleen, kidneys, testes and brain were weighed. The biochemical indices determined from the blood included reduced glutathione (GSH), total serum protein, albumin, globulin, bilirubin, alkaline phosphatase, alanine- and asparate-transaminases (ALT, AST), catalase, gamma glutamyl transferase (GCTP), SH-groups, urea, uric acid, creatinine and homocysteine.

Tissue touching imprints were made from the surfaces of freshly cut liver, kidneys, spleen and mesenteric lymph nodes on a glass slide, which were dried at room temperature and stained by Leishman's stain. The cell composition and signs of cell damage were estimated under a binocular light microscope, Carl Zeiss Primo Star with a USCMOS imaging camera at 100x and 1000x. Microscopy involved counting 100 cells from each lymph node imprint and 300 cells from the imprints of other organs.

All the above-mentioned clinical laboratory tests on blood and urine were performed using well-known techniques described in many manuals (for instance [34]).

The Random Amplification of Polymorphic DNA (RAPD) Test. For this assay, we analyzed totally 36 blood samples, each sample in three replications. The samples were collected into special vessels cooled to −80 °C. These were then promptly delivered in cryocontainers to a specialized laboratory. To isolate DNA from the cells, we used a GenElute (Sigma) set of reagents in accordance with the manufacturer's guidelines for use. The DNA content of the samples was determined spectrophotometrically (Ultraspec 1100 pro, Amersham Biosciences, Ltd., Amersham, UK), then they were freezed and stored at −84 °C in a kelvinator (Sanyo Electric Co., Ltd., Moriguchi, Japan) till the beginning of the implementation of the RAPD (Random Amplified Polymorphic DNA) test performed as described by us earlier (e.g. [29]). This technique allows one to define quantitatively the degree of DNA fragmentation as an estimate of the genotoxicity of harmful agents and the protective effects of the bioprotectors studied. The method is based on the fact that, unlike a fragmented DNA, which, in the agarose gel in electrophoresis, forms the so-called comet tail, a non-fragmented DNA has a very low degree of migration and virtually stays in the same place (comet head), the degree of migration being directly related to the degree of DNA fragmentation. DNA amplification was carried out using specific primers and tritiated nucleotides. To characterize the degree of damage to DNA we used the "coefficient of fragmentation" i.e. the ratio of total radioactivity of all tail fractions to that of the head.

For chromatographic determination of benzo(a)pyrene and its metabolites contents of the blood, urine, feces, and liver homogenate,

[1] In the context of studies like ours, it is important to induce experimental intoxications of moderate severity which might be comparable with those typical of modern occupational human exposures. respective experimental concentrations were found empirically.

[2] Recently some other researchers (e.g, [28]) demonstrated anti-lead hepatoprotective effect of the lemon juice in combination with flaxseed protein.

Table 2
Some functional indices characterizing the health condition of rats exposed to subchronic impact of the studied toxic agents or their combinations ($x \pm s_x$).

Indices	Groups of rats by type of exposure [+]								
	Combi-ned cont-rol	B(a)P 0.5	B(a)P 1.0	Pb 0.5	Pb 1.0	B(a)P 0.5 + Pb 0.5	B(a)P 0.5 + Pb 1.0	B(a)P 1.0 + Pb 0.5	B(a)P 1.0 + Pb 1.0
Initial body mass, g	258.75 ± 5.12	264.17 ± 4.68	266.25 ± 5.04	263.33 ± 4.14	264.17 ± 4.60	265.00 ± 4.17	260.83 ± 3.88	260.83 ± 3.93	258.75 ± 4.44
Final body mass, g	327.08 ± 9.34	329.58 ± 10.12	324.58 ± 8.82	327.50 ± 11.31	312.50 ± 10.83	316.67 ± 9.68	295.83 ± 8.34*	325.42 ± 9.40	287.27 ± 7.02*
Body mass gain, %	26.30 ± 2.07	24.63 ± 2.63	21.96 ± 2.80	24.04 ± 2.65	18.09 ± 2.75*	19.43 ± 2.86*	13.35 ± 2.38*	24.76 ± 3.06	12.33 ± 3.2*1
Mass of left testis, g/100 g of body weight	0.52 ± 0.02	0.48 ± 0.01	0.45 ± 0.02*	0.53 ± 0.02	0.48 ± 0.03	0.48 ± 0.03	0.41 ± 0.04*	0.49 ± 0.02	0.40 ± 0.03*
Mass of right testis, g/100 g of body weight	0.53 ± 0.03	0.46 ± 0.03	0.44 ± 0.02*	0.52 ± 0.03	0.47 ± 0.02	0.48 ± 0.03	0.40 ± 0.04*	0.47 ± 0.02	0.40 ± 0.03*
Liver mass, g/100 g of body weight	3.17 ± 0.13	3.33± 0.14	3.51 ± 0.15	3.44 ± 0.17	3.63 ± 0.22	3.74 ± 0.23*	4.01 ± 0.16*	3.93 ± 0.20*	3.80 ± 0.10*
Spleen mass, g/100 g of body weight	0.18 ± 0.01	0.21 ± 0.01*	0.23 ± 0.02*	0.21 ± 0.01*	0.27 ± 0.01*	0.25 ± 0.01*	0.33 ± 0.02*	0.23 ± 0.01*	0.38 ± 0.03*
Kidney mass, g/100 g of body weight	0.56 ± 0.01	0.55 ± 0.01	0.58 ± 0.02	0.63 ± 0.02*	0.68 ± 0.02*	0.66 ± 0.03*	0.69 ± 0.02*	0.61 ± 0.01*	0.69 ± 0.03*
Brain mass, g/100 g of body weight	0.63 ± 0.02	0.63 ± 0.03	0.65 ± 0.03	0.61 ± 0.02	0.64 ± 0.02	0.62 ± 0.01	0.66 ± 0.03	0.63 ± 0.02	0.68 ± 0.01
Number of head dips into holes within 3 min	7.75 ± 1.13	7.75 ± 1.70	5.67 ± 0.64	5.92 ± 1.37	4.33 ± 1.02*	4.50 ± 1.19	3.00 ± 0.56	6.00 ± 1.58	3.25 ± 0.62
Number of squares crossed within 3 min	8.08 ± 1.10	11.00 ± 1.87	8.67 ± 1.39	5.25 ± 1.38	6.58 ± 1.47	6.00 ± 1.35	4.50 ± 1.24*	5.92 ± 1.72	3.00 ± 0.79*
Temporal summation of sub-threshold impulses, sec	20.80 ± 1.04	25.00 ± 1.04	20.20 ± 1.04	21.20 ± 1.04	15.35 ± 0.85	14.88 ± 1.08	13.39 ± 0.86	14.80 ± 1.04	23.70 ± 1.04
Leukocytes, 10^3/ μL	9.20 ± 1.48	11.25 ± 0.79	9.78 ± 1.26	15.35 ± 1.88*	18.08 ± 3.10*	14.02 ± 1.46*	14.87 ± 1.89*	12.10 ± 1.36	20.12 ± 3.08*
Granulocytes, %	5.65 ± 1.88	6.91 ± 1.35	6.46 ± 1.57	8.64 ± 2.10	6.71 ± 1.71	7.11 ± 2.70	9.03 ± 2.48	7.99 ± 3.24	11.50 ± 2.51
Eosinophils, %	2.00 ± 0.46	1.75 ± 0.37	2.50 ± 0.53	2.25 ± 0.41	3.00 ± 0.71	2.00 ± 0.19	2.00 ± 0.41	2.63 ± 0.46	1.75 ± 0.25
Band cells, %	1.25 ± 0.16	1.13 ± 0.13	1.13 ± 0.13	1.00 ± 0.00	1.63 ± 0.26	1.38 ± 0.18	1.22 ± 0.15	1.13 ± 0.13	1.00 ± 0.00
Segmented cells, %	23.00 ± 2.00	25.88 ± 2.13	23.75 ± 2.54	26.63 ± 1.68	28.63 ± 1.48	27.88 ± 2.33	27.67 ± 1.37	26.63 ± 1.89	28.75 ± 1.13
Monocytes, %	6.25 ± 0.49	5.75 ± 0.49	6.13 ± 0.40	5.75 ± 0.45	6.13 ± 0.48	5.75 ± 0.37	5.78 ± 0.22	6.38 ± 0.37	5.50 ± 0.19
Lymphocytes, %	67.50 ± 1.67	65.50 ± 2.01	67.75 ± 2.10	64.38 ± 1.35	59.75 ± 1.63*	63.00 ± 1.85	62.22 ± 0.94*	63.38 ± 2.05	63.00 ± 1.10*
Erythrocytes, 10^{12} cells/L	7.97 ± 0.62	6.49 ± 0.37	7.06 ± 0.55	8.48 ± 0.63	7.55 ± 0.32	7.71 ± 0.44	7.76 ± 0.43	6.86 ± 0.34	7.98 ± 0.54
Mean volume of erythrocyte, μm^3	56.02 ± 0.87	56.39 ± 1.29	57.73 ± 1.00	50.16 ± 0.80*	45.33 ± 1.17*	51.46 ± 1.21*	48.52 ± 0.94*	53.95 ± 0.63	47.19 ± 0.77*
Hemoglobin, g/L	167.00 ± 10.84	136.50 ± 6.99*	147.50 ± 7.81	154.75 ± 9.98	126.75 ± 4.79*	144.75 ± 5.49	132.22 ± 6.45*	136.00 ± 6.67*	136.25 ± 8.22
Hematocrit, %	22.23 ± 1.54	18.20 ± 0.89*	20.26 ± 1.27	21.18 ± 1.40	17.09 ± 0.75*	19.70 ± 0.76	18.72 ± 0.90	16.73 ± 1.40*	18.82 ± 1.29
Thrombocytes, 10^3/ μL	728.50 ± 58.08	812.00 ± 56.51	792.25 ± 41.97	875.25 ± 69.47	1181.5 ± 52.9*	925.7 ± 67.89*	978.6 ± 84.0*	841.75 ± 63.64	1037.7 ± 87.7*
Thrombocrit, %	0.22 ± 0.02	0.25 ± 0.01	0.24 ± 0.01	0.28 ± 0.02*	0.48 ± 0.03*	0.29 ± 0.03*	0.33 ± 0.03*	0.27 ± 0.02*	0.37 ± 0.04*
Total protein in blood serum, g/L	80.90 ± 3.75	80.33 ± 2.62	85.81 ± 2.27	78.14 ± 2.73	75.77 ± 3.25	81.60 ± 2.53	78.36 ± 3.93	79.29 ± 1.62	71.54 ± 1.22*
Albumin in blood serum, g/L	40.82 ± 1.20	40.48 ± 1.23	41.77 ± 1.82	37.71 ± 1.14	34.75 ± 1.47*	39.08 ± 0.43	35.36 ± 2.04*	38.91 ± 1.0	35.25 ± 0.82*
Globulins in blood serum, g/L	40.08 ± 2.83	39.85 ± 2.68	44.04 ± 1.68	40.43 ± 2.31	41.02 ± 1.98	42.52 ± 2.36	43.00 ± 2.62	40.38 ± 1.31	36.29 ± 0.88
A/G index	1.04 ± 0.06	1.05 ± 0.08	0.96 ± 0.06	0.95 ± 0.06	0.76 ± 0.1	0.94 ± 0.05	0.84 ± 0.07*	0.97 ± 0.04	0.98 ± 0.03
ALT activity in blood serum, U/L	78.95 ± 6.43	54.14 ± 2.31	66.85 ± 4.68	57.21 ± 3.46*	60.89 ± 4.36*	58.29 ± 2.67*	58.92 ± 5.89*	57.25 ± 5.44*	75.23 ± 22.41
AST activity in blood serum, U/L	292.75 ± 34.40	254.84 ± 10.95	315.13 ± 23.51	249.65 ± 28.10	288.75 ± 20.42	262.14 ± 29.08	365.20 ± 35.15	253.22 ± 36.11	376.39 ± 53.72
De Ritis coefficient	3.68 ± 0.24	4.74 ± 0.24*	4.78 ± 0.30*	4.34 ± 0.36	4.78 ± 0.22*	4.52 ± 0.48	6.40 ± 0.49*	4.38 ± 0.27	5.87 ± 0.54*
SDH (number of formazan granules per 50 lymphocytes)	646.55 ± 35.42	586.73 ± 30.89	643.17 ± 25.60	673.09 ± 33.88	638.17 ± 14.42	640.83 ± 10.86	663.45 ± 25.30	723.17 ± 13.71	687.80 ± 27.51
Homocysteine in blood serum, mol/L	4.29 ± 0.50	4.54 ± 0.46	3.88 ± 0.44	4.11 ± 0.44	3.92 ± 0.47	3.65 ± 0.78	4.20 ± 0.65	3.62 ± 0.54	4.05 ± 0.57
Glucose in blood serum, μmol/L	6.81 ± 0.44	6.29 ± 0.25	7.01 ± 0.29	7.33 ± 0.39	6.75 ± 0.34	7.06 ± 0.23	6.99 ± 0.70	7.24 ± 0.35	6.35 ± 0.43
Ca^{2+} in blood serum, mol/L	2.68 ± 0.06	2.84 ± 0.1	2.68 ± 0.03	2.61 ± 0.04	2.61 ± 0.04	2.61 ± 0.03	2.58 ± 0.11	2.68 ± 0.05	2.53 ± 0.04
Gamma-glutamyl transpep-tidase, U/L	5.56 ± 1.24	1.89 ± 0.48	4.55 ± 1.02	3.97 ± 0.56	6.22 ± 1.82	4.52 ± 1.58	6.62 ± 1.58	4.69 ± 0.96	6.21 ± 1.97
Amilase in blood serum, U/L	5732.38 ± 479.63	5146.89 ± 375.60	5397.50 ± 839.25	4842.38 ± 444.27	5039.75 ± 773.63	3917.63 ± 314.79	6401.00 ± 1491.1	5563.25 ± 829.28	6450.88 ± 293.2
LDH in blood serum, U/L	2600.00 ± 326.56	2519.13 ± 256.65	2716.88 ± 242.15	1853.50 ± 275.85	2230.63 ± 116.18	2073.25 ± 253.14	2937.89 ± 340.8	2276.75 ± 394.98	3052.88 ± 426.4

(*continued on next page*)

Table 2 (*continued*)

Indices	Groups of rats by type of exposure [+]								
	Combi-ned cont-rol	B(a)P 0.5	B(a)P 1.0	Pb 0.5	Pb 1.0	B(a)P 0.5 + Pb 0.5	B(a)P 0.5 + Pb 1.0	B(a)P 1.0 + Pb 0.5	B(a)P 1.0 + Pb 1.0
Alkaline phosphatase, nmol/(s × L)	215.70 ± 14.77	173.35 ± 25.15	175.60 ± 20.79	242.99 ± 27.54	225.38 ± 27.20	160.85 ± 11.72*	194.20 ± 25.51	180.96 ± 13.76	113.85 ± 11.6*
Bilirubin in blood serum, mmol/L	1.05 ± 0.11	1.11 ± 0.12	0.97 ± 0.14	1.07 ± 0.17	1.23 ± 0.27	1.23 ± 0.20	1.30 ± 0.21	0.90 ± 0.06	1.24 ± 0.19
Reduced glutathione in whole blood, μmol/L	26.13 ± 1.15	28.48 ± 2.56	32.49 ± 2.03*	26.75 ± 0.89	22.71 ± 1.65	28.35 ± 1.75	26.06 ± 1.22	31.88 ± 4.08	26.00 ± 2.35
Catalse in blood serum, μmol/L	0.41 ± 0.06	0.44 ± 0.03	0.41 ± 0.04	0.45 ± 0.03	0.44 ± 0.03	0.45 ± 0.05	0.45 ± 0.05	0.46 ± 0.04	0.37 ± 0.05
SH-groups in blood serum, mmol/L	4.80 ± 0.69	3.45 ± 0.71	4.82 ± 0.60	4.80 ± 0.45	4.71 ± 0.92	4.28 ± 0.74	3.35 ± 1.11	5.08 ± 0.76	3.29 ± 0.66
Uric acid in blood serum, μmol/L	147.38 ± 10.93	141.00 ± 15.98	144.13 ± 15.32	121.75 ± 13.14	136.50 ± 10.57	134.38 ± 19.18	168.00 ± 16.90	150.38 ± 18.71	169.88 ± 18.10
Urea in blood serum, mmol/L	3.14 ± 0.51	2.63 ± 0.26	3.01 ± 0.68	3.26 ± 0.48	3.71 ± 0.23	2.59 ± 0.39	4.91 ± 1.11	3.08 ± 0.44	3.80 ± 0.38
Creatinine in blood serum, μmol/L	40.33 ± 2.87	40.93 ± 1.87	40.89 ± 3.00	35.36 ± 1.29	34.09 ± 1.81	36.39 ± 1.24	37.82 ± 3.01	40.30 ± 2.48	37.16 ± 2.23
Diuresis, mL	25.33 ± 5.40	21.93 ± 8.21	18.71 ± 3.30	17.90 ± 4.97	7.60 ± 3.06*	17.43 ± 2.54	17.57 ± 4.52	22.57 ± 3.98	29.00 ± 8.34
Urine pH	7.00 ± 0.47	6.07 ± 1.03	7.57 ± 0.38	7.60 ± 0.53	6.40 ± 0.24	7.00 ± 0.38	7.29 ± 0.29	7.29 ± 0.31	8.10 ± 0.46
Specific density of urine	1.018 ± 0.001	1.021 ± 0.004	1.021 ± 0.002	1.022 ± 0.002	1.023 ± 0.003	1.019 ± 0.001	1.019 ± 0.002	1.019 ± 0.002	1.017 ± 0.001
Coproporphyrine in urine, nmol/L	86.14 ± 14.99	224.92 ± 88.28	358.3 ± 155.01	504.7 ± 121.01*	429.3 ± 80.76*	796.9 ± 164.73*	585.3 ± 146.6*	716.4 ±196.9*	623.3 ± 83.7*

Notes:
[+] The numbers 1.0 and 0.5 next to the designations Pb and B(a)P denote groups administered these toxicants in full dose as specified in "Materials and methods", and in corresponding half dose.
* Difference from the control group is statistically significant for $P < 0.05$ according to Student's *t*-test with Bonferroni correction.

preliminary sample decomposition, alkaline hydrolysis of fats and extraction of substances to be analyzed into 5 mL of methylene chloride were performed directly in 50cm^3 disposable Luer syringes with SPE 12-position Stopcocks (AHO-6048). («SF Medical Products Gmbh», Berlin, Germany). The resulting extract was used for analysis by high-performance liquid chromatography using the Agilent 1260 Infinity LC (Agilent Technologies, USA) chromatograph with fluorometric and diode-array detectors, gradient pump, and column thermostat set at 40 °C. The column 4.6 × 150 mm was filled with the Zorbax Eclipse XDB C 18 sorbent. The injection volume was 5 mm^3. The eluent (80 % acetonitrile + 20 % water) was fed in the isocratic mode with the flow rate of 1.0 mL/min. Fluorometric detection was performed at an excitation wavelength of 260 nm and emission wavelength of 415 nm, and diode-array detection at an analytical wavelength of 254 nm (the reference wavelength being 390 nm). Aliquots of samples to be analyzed were dosed using an auto-sampler. A standard sample of benzo(a)pyrene was prepared using benzo(a)pyrene solution in hexane (OOO «Ekroskhim», Saint-Petersburg, Russia). Samples of benzo(a)pyrene metabolites (3-hydroxibenzo(a)pyrene and benzo(a)pyrene-trans-7,8-dihydrodiol) were prepared using the standards of these substances supplied by TRC, Toronto, Canada.

The result of each chromatographic reading was quantified as the area under curve (AUC) in fluorescence units for the fluorimetric detector and in optical absorbance units for the diode-array detector. Respective calibration curves were linear in ranges 0.01–1.0 mcg/mL and 1.0–100 mcg/mL Respective Limits of Detection and of Quantitation were: (1) for the benzo(a)pyrene in blood 0.30–30.00 and 28.50–3000.0 mcg/100 mL, in urine 0.12–12.00 and 11.50–1200.0 mcg/100 mL, in feces 0.70–70.00 and 70.00–7000.0 mcg/100 g; (2) for (3-hydroxibenzo (a)pyrene in blood 0.40–40.00 and 40.00–4000.0 mcg/100 mL, in urine 0.15–15.00 and 15.00−15 000.0 mcg/100 mL, in feces 1.00–100.00 and 100.00–10000.0 mcg/100 g. (3) for benzo(a)pyrene-trans-7,8-dihydrodiol in blood 0.40–40.00 and 40.00–4000.0 mcg/100 mL, in urine 0.15–15.00 and 15.00−15 000.0 mcg/100 mL, in feces 1.00–100.00 and 100.00−10 000.0 mcg/100 g.

The statistical significance of differences between group-average results was estimated by Student's *t*-test with Bonferroni correction for multiple comparisons.

Mathematical modeling of responses to binary exposures was based on the Response Surface Method (RSM) [10]. In this methodology, the response surface $Y = Y(x_1, x_2)$ is described by the Eq. (1)

$$Y = f(x_1, x_2) \tag{1}$$

where Y is the quantitative effect (outcome) of a toxic exposure; x_1 and x_2 are the doses of the toxicants participating in the combination; $f(x_1, x_2)$ is a regression equation with some numeric parameters which can be found by fitting to experimental data. In the case of two-level exposures (even if one of the levels is equal to zero), the response surface may have one possible shape (hyperbolic paraboloid)

$$Y = b_0 + b_1x_1 + b_2b_2 + b_{12}x_1x_2 \tag{2}$$

It is inferred that two agents produce a unidirectional effect on response Y if both one-way response functions $Y(x_1, 0)$ and $Y(0, x_2)$ either increase or decrease with an increase in x_1 or x_2; on the contrary, two agents are assumed to be acting contra-directionally (oppositely) if one function increases while the other decreases. This mathematical model enables one to predict the magnitude of response Y for any combination of toxicant doses within the experimental range for each of them (rather than at two factual points only). The sectioning of the response surface on different levels corresponding to different meanings of the outcome **Y** or of the doses **x,** provides a family of Loewe isoboles that may have the same or a different form and/or different slopes and thus render the interpretation of binary combined toxicity types both easy and illustrative. In Section 3, we therefore will illustrate the results of the RSM modeling just in this way.

3. Results and discussion

As can be seen from the data presented in Table 2, a number of dose-dependent indices of toxic effect (in particular, delayed body mass gain, increased relative mass of the liver, reduced mass of the testes, inhibition of exploratory behavior, and altered general motion activity) were

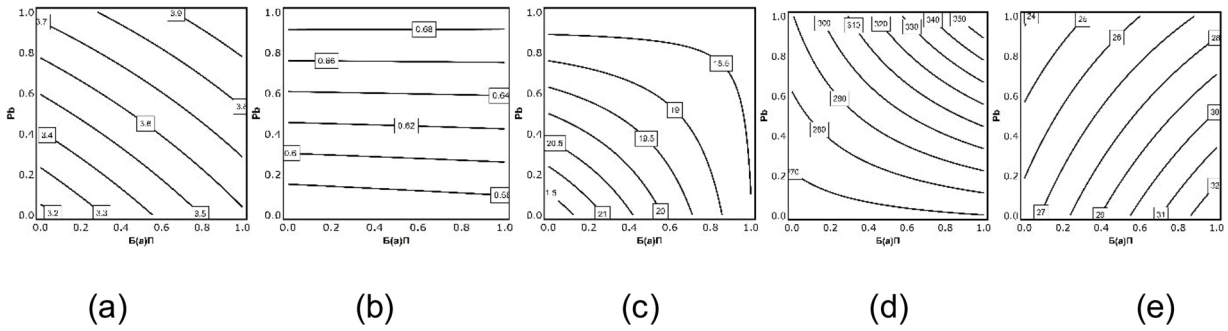

Fig. 1. Examples of isobolgrams characterizing the typological diversity of combined B(a)P + Pb subchronic toxicity depending on the effect by which it is estimated: (a) relative liver mass (additivity); (b) relative kidney mass (single-factor action of lead independent of the action of B(a)P; (c) hematocrit (subadditivity); (d) AST (superadditivity); (e) reduced glutathione (opposite actions). The doses on the axes are plotted as fractions of the actual ones. The numbers on the lines correspond to index magnitude.

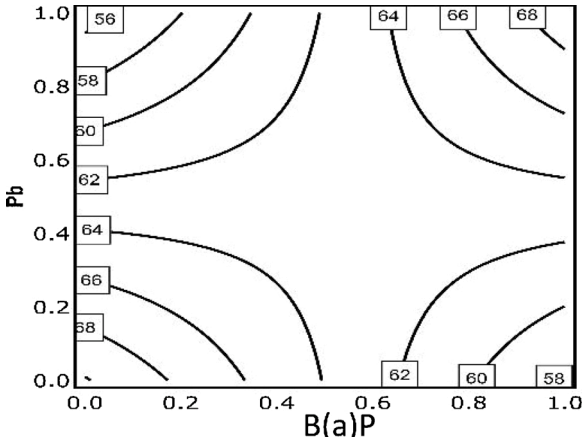

Fig. 2. An example of isobologram characterizing the typological diversity of combined B(a)P + Pb subchronic toxicity estimated by one and the same effect (reduced activity of alanine aminotranferase in blood serum) but depending on dose ratio: subadditivity for low doses and superadditivity for high doses of both toxicants; different forms of opposite action where high doses of one agent are combined with low doses of the other. The doses on the axes are plotted as fractions of the actual ones. The numbers on the lines correspond to index magnitude.

revealed in response to the action of both toxic agents. However, some indices of this kind were observed for the impact of lead only, for instance: leukocytosis and thrombocytosis[3], increased kidney mass accompanied with reduced diuresis, and inhibition of the protein-producing function of the liver judging by the total protein and albumin contents of the blood. Moreover, the indices of toxic impact on the red blood, such as reduced hemoglobin content, hematocrit and mean corpuscular volume of erythrocytes, were dose-dependent only under the effect of lead. The concentration of coproporphyrin in urine was increased in response to the impact of both substances; however, it was more marked under the action of lead and statistically significant for this action only. Thus, what we see is not only integral but also commonly known specific manifestations of lead toxicodynamics [40–42].

At the same time, considering the important role of the liver in the biotransformation of organic toxicants mentioned in the Introduction, special attention should be given to such functional indices of damage to this organ caused by lead alone and, especially, its combination with B(a)P as increase in its relative mass and reduced concentration of total protein and albumin in the blood serum.

As for many other previously studied binary intoxications [10,11,13,15,29,30,43], the type of combined action displayed by benzo(a)pyrene and lead together varies from synergism to independent single-factor action or even opposite action depending:

- on the effect by which the type of combined action is determined (see examples in Fig. 1),
- for some effects, on their level and dose ratio as well (see the example in Fig. 2).

In general, out of the 56 effects for which we constructed the isoboles, additivity was revealed in 10 cases; predominantly superadditivity, in 3 cases; subadditivity of unidirectional action or even explicit antagonism (i.e. opposite actions), in 21 cases; a complex type depending on dose ratio, in 8 cases (see Fig. 2). Moreover, a combination of high doses of both toxicants gave superadditivity; in 10 cases, there was a single-factor action of lead; and in 4 cases, a single-factor action of benzo(a)pyrine.

Thus, overall 21 effects (37.5 %) were found to produce the most adverse additive or even superadditive combined action. It is particularly noteworthy that, as shown in Fig. 1, additivity was revealed (over the entire range of doses used) for such an important effect as increased liver mass coefficient, keeping in mind again that the liver is a key organ for biotransformation of organic xenobiotics.

The data on a comparative cytological analysis of tissue touching imprints presented in Table 3 provide evidence that both toxic agents, lead in particular, and their various combinations have caused degenerative and inflammatory changes in organs with signs of hyperergy (eosinophylic reaction). We should draw attention again to cytological indices of liver injury under isolated and, particularly, combined action of lead: increased percentage of degenerated hepatocytes and Kupffer cells[4] and neutrophils.

As follows from Table 4, where exposure to B(a)P went along with lead intoxication, the concentrations of non-biotransformed B(a)P in all the media analyzed by us were higher than where B(a)P acted alone, even though in each individual object of chemical analysis this difference was not statistically significant. The probability of an accidental 4-fold repetition of its sign is equal to only 0.0625, meaning that with such reproducibility the inter-group difference observed could be considered as statistically significant at $P < 0.1$.

Along with non-transformed B(a)P, we also determined two of its metabolites which are products of the 1 st (oxidative) phase of B(a)P biotransformation [20], namely: 3-hydroxi-benzo(a)pyrene, capable of

[3] These hematological shifts are rarely used in toxicological characterization of lead, but they are described for lead intoxication in a number of papers [35–37], including ours [10,38,39].

[4] Indirect index of enhanced hepatocyte apoptosis, since Kupffer cells play an active role in the elimination of cell fragments resulting from apoptosis (e.g., [44]).

Table 3
Cytological indices (%) of tissue touching imprints under isolated and combined subchronic intoxication of rats with lead and benzo(a)pyrene.

Index	Groups of rats by type of exposure [+]								
	Combined control	B(a)P 0.5	B(a)P 1.0	Pb 0.5	Pb 1.0	B(a)P 0.5 + Pb 0.5	B(a)P 0.5 + Pb 1.0	B(a)P 1.0 + Pb 0.5	B(a)P 1.0 + Pb 1.0
Liver									
Epithelial cells of ducts	10.83 ±0.95	9.83 ±0.60	9.17 ±0.60	10.83 ±0.70	11.50 ±0.76	10.67 ±0.67	10.29 ±0.42	10.27 ± 0.58	10.00±0.58
Hepatocytes	73.00±5.23	57.50±4.00 *	62.67±4.55	41.67±2.01 *	58.67±3.67 *	68.17±1.45	66.00±1.50	65.80±1.35	46.33±1.28 *+
Degeneratively altered hepatocytes	3.83±0.48	10.33±1.15 *	8.83±1.45 *	15.33±1.12 *	8.50±0.76 *	8.33±0.49 *	9.29±0.75 *	9.93±0.87 *	17.83±1.62 *+
Neutrophils	4.17±0.48	10.67±1.28 *	8.50±1.43 *	15.50±1.18 *	12.83±1.35 *	5.17±0.31	7.00±0.62 *	7.13±0.73 *	16.33±1.43 *
Eosinophils	1.67±0.33	6.17±1.30 *	5.17±1.45 *	9.17±1.30 *	5.00±1.00 *	3.00±0.37 *	3.14±0.63	2.27±0.17	3.67±0.76 *
Binuclear cells	0.83±0.17	1.17±0.17	1.17±0.17	1.33±0.21	1.33±0.21	1.33±0.21	1.29±0.18	1.27±0.21	1.00±0.00
Kupffer macrophages	2.67±0.33	3.83±0.91	3.33±0.61	5.00±0.58 *	3.17±0.40	2.83±0.31	2.43±0.20	2.67±0.21	4.33±0.56 *
Fibroblasts	0.67±0.21	0.50±0.22	0.67±0.21	1.17±0.40	0.50±0.22	0.50±0.22	0.57±0.20	0.67±0.31	0.50±0.22
Kidneys									
Proximal tubule cells	69.50±1.34	59.17±0.98*	61.50±1.80*	46.33±2.35*	49.33±1.69*	67.00±2.19	60.14±1.37*	61.60±2.47*	56.50±2.33*
Degenerative cells of proximal tubules	10.00±0.52	15.50±0.99*	12.67±1.02 *	21.17±1.51 *	20.83±0.95*	9.67±0.56	11.71±0.57*	12.20±0.73*	13.83±0.95*
Distal tubule cells	7.67±0.56	8.67±0.67	9.67±1.05	8.33±0.71	7.00±0.58	8.33±0.71	8.86±0.51	7.07±0.91	8.50±0.76
Degenerative cells of distal tubules	4.67±0.33	9.83±0.60*	8.67±0.71*	11.83±0.95 *	11.83±1.01*	6.83 ± 0.60*	8.57±0.37*	9.47±1.03*	10.67±0.61*
Neutrophils	4.50±0.62	2.50±0.43*	3.00±0.58	5.83±0.60	5.50±0.43	2.50±0.43*	4.14±0.40	2.80±0.43*	2.83±0.60+
Monocytes	2.00±0.45	2.00±0.26	2.50±0.43	3.33±0.49	2.83±0.31	2.50±0.50	3.86±0.51*	3.93±0.42*	5.50±0.43*
Eosinophils	0.67±0.33	1.50±0.22	1.33±0.21	2.00±0.37*	1.83±0.31*	2.17±0.31*	1.29±0.18	1.87±0.31*	2.00±0.37*
Fibroblasts	1.00±0.26	0.83±0.31	1.17±0.17	1.17±0.31	0.83±0.17	1.00±0.26	1.43±0.37	1.07±0.21	0.67±0.21
Spleen									
Mature lymphocytes, prolymphocytes	84.80±0.80	80.33±2.50	83.33±1.89	83.50±0.85	81.83±1.62	77.40±1.81*	82.33±3.02	79.50±2.89	81.00±2.58
Lymphoblasts	0.80±0.20	1.00±0.00	1.33±0.21	1.00±0.26	0.83±0.17	0.80±0.20	1.17±0.31	1.00±0.00	1.33±0.21
Reticular cells	0.40±0.24	0.17±0.17	0.33±0.21	0.33±0.21	0.50±0.22	0.40±0.24	0.33±0.21	0.36±0.22	0.33±0.21
Plasma cells	1.40±0.24	1.67±0.49	2.00±0.52	1.17±0.17	1.50±0.34	2.40±0.75	1.67±0.33	2.64±0.72	3.33±1.17
Macrophages	1.00±0.00	1.17±0.17	1.17±0.17	1.50±0.34	1.33±0.21	1.60±0.24*	1.67±0.21	1.43±0.17	2.00±0.37*
Neutrophils	6.80±0.86	12.67±1.84 *	8.83±1.85	8.50±1.26	9.17±1.76	14.60±1.33 *	10.00±2.58	12.57±1.74	9.67±2.40
Eosinophils	4.40±0.75	2.83±0.65	3.00±0.93	4.00±0.82	4.83±1.14	2.80±0.80	2.67±0.67	2.29±0.42	2.33±0.49*
Mesenteric lymph nodes									
Mature lymphocytes, prolymphocytes	88.83±1.38	88.50±2.16	88.83±1.40	88.67±1.67	86.83±1.72	85.33±0.49*	83.60±1.81*	87.50±1.82	88.20±1.36
Lymphoblasts	1.83±0.31	1.33±0.21	1.33±0.42	1.33±0.21	1.00±0.26	1.33±0.33	1.00±0.00*	1.29±0.17	1.40±0.24
Reticular cells	0.17±0.17	0.17±0.17	0.50±0.22	0.50±0.22	0.50±0.22	0.17±0.17	0.60±0.24	0.29±0.21	0.60±0.24
Plasma cells	5.17±0.91	7.00±2.18	5.33±1.23	4.83±1.47	5.67±1.58	6.50±1.06	7.80±1.83	4.86±0.48	5.60±1.47
Macrophages	1.50±0.22	1.33±0.21	1.67±0.33	1.33±0.21	1.67±0.33	1.67±0.33	1.80±0.37	1.79±0.37	1.60±0.24
Neutrophils	1.83±0.54	1.33±0.21	1.50±0.50	2.33±0.99	2.83±0.75	3.67±0.99	3.60±1.21	3.00±1.09	1.60±0.24+
Eosinophils	0.67 ± 0.21	0.67±0.21	0.83±0.17	1.00±0.26	1.50±0.22 *	1.33±0.21 *	1.60±0.40	1.36±0.33	1.00±0.32

Notes:
[+] Numbers 1.0 and 0.5 next to the designations Pb and B(a)P denote groups receiving these toxicants in full dose specified in "Materials and methods". or in corresponding half dose.
* Difference from the control is statistically significant for $P < 0.05$ according to Student's *t*-test with Bonferroni correction.

Table 4
Concentration of non-transformed benzo(a)pyrene in the blood, urine, feces and liver tissues of rats upon the end of subchronic intraperitoneal exposure to benzo (a)pyrene alone or in combination with lead ($x \pm s_x$) in full doses [+].

Where determined, and units of concentration	Exposure to:	
	B(a)P	B(a)P + Pb
Blood, ng/mL	84.87 ± 37.05	124.07 ± 51.70
Urine, ng/mL	5.23 ± 4.90	9.25 ± 5.68
Feces, ng/g	165.69 ± 101.48	297.99 ± 75.05
Liver homogenate, ng/g	616.22 ± 177.84	869.59 ± 469.42

+ B(a)P contents in the organism of the majority of control rats was found to be below the sensitivity limit of the method of determination.

conjugation reactions (a typical metabolite of this kind) and benzo(a) pyrene-7,8-dihydrofiol, capable of covalent binding with the DNA (a typical «harmful» metabolite). Although in the majority of the analyzed substrates the

concentration of the first of these metabolites was below the sensitivity of the method, we still detected it in the blood of rats administered only B(a)P (0.26 ± 0.26 ng/mL) but failed to detect it in the ones which received B(a)P + Pb. Respective concentrations in the liver of rats from the same two groups were 55.70 ± 42.33 and 17.27 ± 7.64 ng/g. This suggests a tendency in this inhibition of B(a)P oxidative metabolism towards this product. The second of the above-mentioned metabolites resulting from the oxidative phase of B(a)P biotransformation was detectable in a considerably greater number of substrates, but also with some tendency towards reduction under the concomitant effect of lead: 4.13 ± 2.14 and 3.04 ± 2.79 ng/mL in the blood and 21.23 ± 6.96 and 19.14 ± 7.13 ng/g in the liver, respectively.

In spite of the insufficiently high statistical significance of the intergroup differences in the B(a)P toxicokinetic indices considered above, concordance between them suggests that these differences are hardly accidental. In other words, the results of determination of B(a)P and its two metabolites in the organism, at any rate, do not contradict the assumption made in the Introduction (and illustrated by the scheme in Fig. 3) that lead may inhibit the oxidative phase of B(a)P biotransformation.

An answer to the most important question concerning what prevails as an expected outcome of this inhibition (either a favorable reduction of the probability of DNA damage related to unit mass of B(a)P accumulated in the organism, or adverse increase in this mass due to a reduced probability of conjugation and thus elimination of B(a)P from the organism) is provided by the results of a RAPD-test. An increase in the DNA fragmentation coefficient (Cfr) compared with its control value (0.4245 ± 0.0098) was statistically significant ($P < 0.05$) under the action of B(a)P (0.5465±0.01638) and was not significant under the action of lead (0.4523 ± 0.0088). Nevertheless, in the group administered the B(a)P + Pb combination, Cfr proved to be maximal (0.5857 ± 0.0197, $P < 0.05$). Moreover, the isobolgrams (Fig. 4) point to an

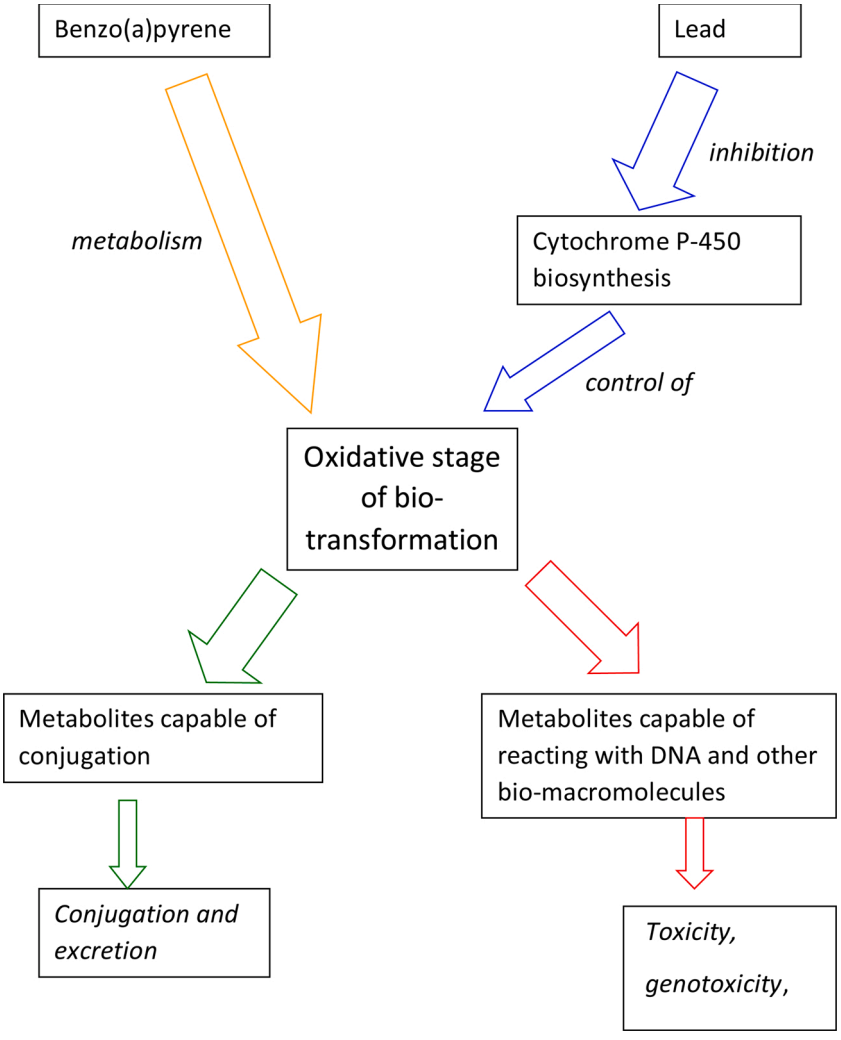

Fig. 3. A hypothetical mechanism underlying the impact of lead on the toxicokinetics and toxicodynamics of benzo(a)pyrene.

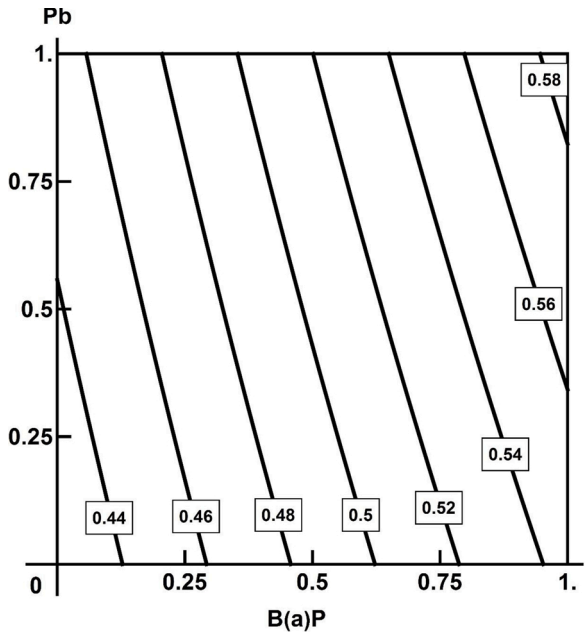

Fig. 4. Isobolograms characterizing the additive type of action produced by B(a)P + Pb combined subchronic toxicity on the fragmentation coefficient (Cfr) of genomic DNA in the nucleated blood cells. The doses on the axes are plotted in fractions of the actual ones. The numbers on the lines correspond to Cfr value.

explicit additivity of the B(a)P + Pb combined action on this index, although with the predominance of the effect of B(a)P.

Indirect evidence supporting our assumption that this enhancement of B(a)P genotoxicity is really due to developing intoxication is a complete absence of the genotoxic effect (0.4411 ± 0.0135) in rats which received the same toxic combination against the background administration of the bioprotective complex (BPC) while the BPC by itself, without toxic exposure, affected the Cfr but negligibly (0.4220 ± 0.0077). Meantime, this BPC reduced noticeably the toxic injury of the liver. Thus, the percentage of degeneratively altered hepatocytes in the tissue touching imprints of this organ, equal to 3.83 ± 0.48 in the control group, increased statistically significantly under B(a)P + Pb combined action in full dose to 17.83 ± 1.62, while exposure to the same combination along with BPC administration raised this percentage over the control value only to 4.33 ± 0.56 (the difference between the latter two values is statistically significant at $P < 0.05$), although the BPC itself did not have any effect on the control value.

4. Conclusion

1 The subchronic action of benzo(a)pyrene (B(a)P) and lead (Pb) in combination causes a number of toxic effects induced by both substances alongside some effects specific to lead intoxication.
2 An analysis of combined toxicity typology carried out with the help of the Response Surface Method has revealed a diversity of types (ranging from synergism to independent single-factor or even opposite action) depending on which effect the type of combined action is estimated by and, for a number of effects, on the dose ratio.
3 The important role of the liver as an organ responsible for benzo(a)pyrene biotransformation attaches special importance to the fact that many of the B(a)P to Pb dose ratios caused a markedly greater injury of this organ than the impacts of corresponding doses separately.
4 Data on the concentrations of non-transformed B(a)P and its two metabolites in the organism favor the hypothesis that Pb inhibits the oxidative biotransformation of B(a)P, assumingly due to impaired synthesis of cytochrome P450.
5 The above can explain the particularly pronounced genotoxic effect of combined intoxication, which is practically absent if it developed against background administration of a complex of bioprotectors.

CRediT authorship contribution statement

Ilzira A. Minigalieva: Methodology, Project administration, Writing - original draft. **Tatiana N. Shtin:** Investigation, Data curation. **Oleg H. Makeyev:** Investigation. **Vladimir G. Panov:** Investigation, Data curation. **Larisa I. Privalova:** Investigation. **Vladimir B. Gurvic:** Supervision. **Marina P. Sutunkova:** Writing - review & editing. **Tatiana V. Bushueva:** Investigation. **Renata R. Sakhautdinova:** Investigation. **Svetlana V. Klinova:** Investigation. **Svetlana N. Solovyeva:** Investigation. **Ivan N. Chernyshov:** Investigation. **Eugene A. Shuman:** Investigation. **Artem A. Korotkov:** Investigation. **Boris A. Katsnelson:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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