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Evaluation of the combined effect of cadmium, benzo(a)pyrene and pyrene in general toxicity studies on Wistar rats

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Summary

Heavy metal cadmium (Cd) and polycyclic aromatic hydrocarbons benzo(a)pyrene (B(a)P) and pyrene (P) are ubiquitous and persistent environmental pollutants. Human beings are constantly exposed to mixtures of these substances. Exposure to Cd may cause changes in critical organs – kidneys and liver. B(a)P has an adverse effect on haemopoesis, digestive systems and on the liver. According to some data Cd, B(a)P and P may interact in all the metabolism phases of xenobiotics. The objective of our study was to investigate the combined effect of Cd, B(a)P and P on general toxicity of the organism of Wistar rats. Tests were conducted on the basis of the methodical recommendations: 407 and 408 of the OECD Guidelines for the Testing of Chemicals. In the experiment, 176 male Wistar-line rats were employed. Four different dose levels were used: 0.1, 0.5, 1.92, 4.0 mg/kg for Cd; 0.00015, 0.0015, 33.3, 10.0 mg/kg for B(a)P and 0.00075, 0.0075, 90.0, 20.0 mg/kg for P and their 4 combinations. The complex of substances studied induced changes in the biochemical blood, urinalysis, hematological parameters which indicated renal and liver function damage and evoked leukopenia symptoms. Evaluating the complex of the substances by these parameters it was noted that the combined action of substances had three types: antagonistic – 56.9%, additive – 27.4% and unknown origin – 15.7%.

Keywords: combined effect, rats

Heavy metals and polycyclic aromatic hydrocarbons are ubiquitous and persistent environmental pollutants. The human being is constantly exposed to the mixtures of these substances. Pollution sources of heavy metal cadmium (Cd) and polycyclic aromatic hydrocarbons – benzo(a)pyrene (B(a)P) and pyrene (P) are fairly identical – industry, transport, combustion systems. Through the ecological chain air–soil–plants, these xenobiotics get into food. Data of the World Health Organisation indicate that the most part of these substances (~90%) is taken by non-smokers with their food. Cadmium is extremely dangerous as it is easily absorbed and remains in tissues for a long time (11). Cd may result in renal diseases, hepatic dysfunction, osteoporosis, sterilisation, teratogenicity, various disorders of the nervous system, lower immunity. Benzo(a)pyrene is a carcinogen and has an adverse effect on reproductive, immunity, haemopoesis and digestive systems, on the liver. It is known that high doses of pyrene may cause renal lesions and hepatic dysfunction.

Mostly organism in natural environment is exposed not to isolated substances but to the whole complex of them. Not many data are available on the combined effect of Cd, B(a)P and P. The specific toxicity of the binary complex was most often studied by carcinogenic and genotoxic tests *in vivo* and *in vitro* systems. Some reports indicated that Cd and B(a)P or B(a)P and P may interact in all the metabolism phases of xenobiotics. Some findings (3, 6) show that Cd can be a bioactivator of this organic carcinogen, according to the other authors it can be an inhibitor (7, 13). The combined effect of Cd, B(a)P and P was not studied to observe non-specific toxic effect on the organism and on its main systems (kidneys, liver, blood system). In previous studies we have investigated only the combined action of binary complexes (Cd + P; Cd + B(a)P) on general toxicity or reproductive toxicity of laboratory animals (8, 9, 14). The toxic effect of the binary complex Cd + B(a)P on female rats reproductive function was higher than the effect of isolated substances – synergic and additive types have been manifested.

The aim of this study was to determine the combined effect of Cd, B(a)P and P at different doses and exposure duration on general toxicity on the organism of Wistar-line rats at the oral route of administration.

Material and methods

Tests on general toxicity were conducted on the basis of methodical recommendations 407 and 408 of the Organization For Economic Co-operation and Development (OECD) Guideline For Testing of chemicals: „Repeated Dose Oral Toxicity – Rodent: 28-day or 14-day Study" and „Subchronic Oral Toxicity – Rodent: 90-day Study". Blood and urine biochemical tests were performed by the clinical biochemical analyser Humalyzer 2000 (Germany). The amount of Cd in the standard feed was 0.025 mg/kg, no Cd was found in the drinking water or rape oil. In the experiments 176 Wistar-line male rats, aged 6-7 weeks, were employed. They were kept under standard laboratory conditions: at 22 ± 3°C regulated temperature, relative humidity 50 ± 20%, 12 h light/dark cycle. Throughout the study, animals were cared for in accordance with Republic of Lithuania law on the care, keeping and use of animals (1997; No. VIII-500) and the Guide for the Care and Use of Laboratory Animals (Washington, National Academy Press, 1996). Tested substances were administrated orally (1 ml/100 g body weight) by a gavage and one by one (respectively) for Cd + B(a)P + P complex group. Cadmium chloride and B(a)P, P (both are insoluble in water) were purchased from Fluka AG (Switzerland). Cd dissolved in water and B(a)P or P dissolved in rape oil were used. There were 20 groups (4 control groups) of laboratory animals, 7-10 animals per group. 2 controls groups (short-term and subchronic duration) received water, 2 others – rape oil. The following doses and exposure duration were investigated: 1) doses which corresponded to the 100 of average daily intake (ADI) per men: 0.1 mg/kg of Cd, 0.00015 mg/kg of B(a)P, 0.00075 mg/kg of P and their complex Cd + B(a)P + P 90 days; 2) doses which corresponded to 500 for Cd and 1000 for B(a)P and P of ADI per men: 0.5 mg/kg of Cd, 0.0015 mg/kg of B(a)P, 0.0075 mg/kg and their complex 90 days; 3) as cumulative doses (1/30 LD_{50}) of these substances: 1.92 mg/kg of Cd, 33.3 mg/kg of B(a)P, 90.0 mg/kg of P and their complex 14 days; 4) as an active doses in reproductive function studies: 4.0 mg/kg of Cd, 10.0 mg/kg of B(a)P, 20.0 mg/kg of P and their complex 28 days.

The animals were weighted weekly, and in subchronic study – from the fifth week – every 2 weeks. At the end of the experiments clinical urinalysis (overall daily quantity, relative weight, hippuric acid, urea, total protein), biochemistry (activity of the enzymes – alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), the blood serum level urea, creatinine, total protein, calcium (Ca)) and the hematology (haemoglobin concentration (Hb), the count of erythrocytes and leukocytes, deposition rate of erythrocytes, the formula of leukocytes) examinations were made. Relative weight calculation of various organs (liver, kidneys, lung, heart, pancreas, thymus, spleen, adrenals) and histopathological examination of some organs (liver, kidney, pancreas, spleen, stomach) were performed too. At the end of the experimental period, the rats were killed by decapitation.

Adverse effects of the Cd, B(a)P and P complex on general toxicity were characterised by four types of the combined action of substances (CAS): antagonistic, additive, synergic and unknown origin. An additive combined action was equal to the percentile sum of the effect of each individual agent given alone, meanwhile a synergic – greater than the sum and an antagonistic – lower than the sum (4), unknown origin – all others. The findings of tests were processed statistically (values are mean ± SE). The results were considered significant when $p < 0.05$.

Results and discussion

Integral parameters (appearance, consumption of food and body mass dynamics) of performed test showed, that various exposure duration and different doses of Cd + B(a)P + P complex (doses – 1.92 + 33.3 + 90.0 mg/kg – 14 days; 4.0 + 10.0 + 20.0 mg/kg – 28 days; 0.1 + 0.00015 + 0.00075 mg/kg – 90 days; 0.5 + 0.0015 + 0.0075 mg/kg – 90 days) haven't produced visible signs of animals' intoxication. There were not recorded any marked changes in the body mass among these animals groups and control or groups given only one substance. In several groups (Cd – 1.9; 4.0 and the complex 0.1 + 0.00015 + 0.00075 mg/kg) observed only a small decrease of body mass confirmed the data of other authors (10): only very high doses *per os* of these substances (11.6 mg/kg 30 days for Cd; 1.1 and 0.3 g/kg 100 days for B(a)P and P) evoked a marked loss of body mass. In different groups (Cd – 4.0 mg/kg – 1 animal; B(a)P – 10.0 mg/kg – 1 and Cd + B(a)P + P – 100 ADI – 1) several fatal cases registered might be due to the tumour of stomach, kidney and lung. In Cd + B(a)P + P group (1/30 LD_{50} 14 days) 3 animals with tumours of the lung, adrenals and macropathological changes of backbone were found.

According to the blood biochemistry, urinalysis parameters and renal histopathological results we determined that various doses of different substances and their combinations induced different disturbances of renal function. According to the symptoms of impairment of renal nitrogen-excreting function, proteinuria, oliguria, hypostenuria and to renal histopathological data have been observed renal dysfunction. Due to high short-term doses of Cd (1.92; 4.0 mg/kg), P (90.0; 20.0 mg/kg) and Cd + B(a)P + P complexes, these symptoms were noted most frequently (tab. 1). The subchronic doses of these isolated substances or their complexes induced only in a few cases renal dysfunction (proteinuria, oliguria, hypostenuria). At least changes (oliguria) were found in the group given B(a)P. The short-term dose of complex (4.0 + 10.0 + 20.0 mg/kg) reduced the urine nitrogen level (urea by 47.3%, creatinine by 28.2%), increased the serum nitrogen level (urea by 29.6%, creatinine by 8.1%) and increased the level of total protein in urine (the antago-

Tab. 1. Short-term effect of Cd (1.92, 4.0 mg/kg), B(a)P (33.3, 10.0 mg/kg), P (20.0, 90.0 mg/kg) and their combination on biochemical blood, urine and hematological parameters of rats

Testing variants	Doses, mg/kg	UREA, mmol/l		Total protein, g/l		Calcium in serum, mmol/l	Leucocytes, 10^9/l
		in serum	in urine	in serum	in urine		
Control: – H_2O	–	3.8 ± 0.3	338.3 ± 33.4	60.5 ± 2.1	0.0	1.6 ± 0.1	5.1 ± 0.5
Control: – oil	–	4.4 ± 0.3	306.7 ± 39.6	55.8 ± 1.4	0.0	1.5 ± 0.1	5.3 ± 0.1
Cd	1.92	5.0 ± 0.2**	250.8 ± 16.6*	59.5 ± 1.0	3.4 ± 0.5***	1.7 ± 0.1	5.8 ± 0.2
	4.00	5.2 ± 0.5*	171.7 ± 30.2**	65.5 ± 1.5	2.3 ± 0.2***	1.8 ± 0.1	4.7 ± 0.4
B(a)P	33.3	3.6 ± 0.2*	360.6 ± 24.0	58.0 ± 1.0	0.9 ± 0.4	2.2 ± 0.1***	4.7 ± 0.2*
	10.0	2.7 ± 0.7*	329.4 ± 22.2	66.8 ± 4.1*	4.2 ± 0.4***	1.6 ± 0.2	4.4 ± 0.7
P	90.0	7.8 ± 0.3***	400.1 ± 27.7	57.3 ± 1.9	1.5 ± 0.8	2.1 ± 0.1***	4.3 ± 0.2***
	20.0	6.0 ± 0.4**	200.3 ± 17.8*	83.4 ± 2.1***	3.4 ± 0.8***	1.8 ± 0.1*	4.6 ± 0.2**
Cd + B(a)P + P	1.92 + 33.3 + 90.0	5.5 ± 0.3* BP, P	248.1 ± 38.2	66.0 ± 2.8** Cd, BP, P	1.8 ± 1.1	1.5 ± 0.1 BP, P	3.7 ± 0.1*** Cd, BP, P
	4.0 + 10.0 + 20.0	5.7 ± 0.3** BP	161.6 ± 27.4*** BP	78.4 ± 1.4*** Cd, BP	4.2 ± 1.0***	2.1 ± 0.2**	4.3 ± 0.4*

Explanations: significant difference compared to the control: *p < 0.05; **p < 0.01; ***p < 0.001; significant (p < 0.05): Cd – compared to cadmium group; BP – compared to B(a)P group; P – compared to P group

Tab. 2. Subchronic effect of Cd (0.1, 0.5 mg/kg), B(a)P (0.00015, 0.0015 mg/kg), P (0.00075, 0.0075 mg/kg) and their combination on biochemical blood, urine and hematological parameters of rats

Testing variants	Doses, mg/kg	UREA, mmol/l		Total protein, g/l		Hippuric acid in urine, mg/ml	Leucocytes, 10^9/l
		in serum	in urine	in serum	in urine		
Control: H_2O	–	6.8 ± 0.3	533.2 ± 48.9	82.5 ± 2.7	1.4 ± 0.6	17.6 ± 2.8	5.5 ± 0.2
Control: oil	–	7.3 ± 0.4	240.7 ± 14.6	82.1 ± 2.3	1.4 ± 0.2	23.1 ± 1.8	5.8 ± 0.2
Cd	0.1	6.7 ± 0.1	811.2 ± 36.4**	76.5 ± 2.5	2.2 ± 0.2	17.1 ± 3.3	3.8 ± 0.4**
	0.5	5.5 ± 0.2**	277.0 ± 12.0***	75.6 ± 1.4*	3.1 ± 0.3*	22.7 ± 2.5*	4.8 ± 0.3
B(a)P	0.00015	7.1 ± 0.6	329.9 ± 27.2*	71.2 ± 2.3	5.0 ± 0.5***	16.3 ± 1.4**	5.0 ± 0.2*
	0.00150	5.0 ± 0.2***	249.1 ± 19.6	75.6 ± 1.8*	2.7 ± 0.3**	25.3 ± 3.7	3.6 ± 0.2***
P	0.00075	5.0 ± 0.3***	306.7 ± 18.6**	79.8 ± 1.6	3.3 ± 0.2***	14.1 ± 1.5***	4.4 ± 0.3**
	0.00750	5.1 ± 0.1***	613.9 ± 45.9***	80.1 ± 2.2	5.5 ± 0.8***	43.1 ± 7.3*	3.9 ± 0.3***
Cd + B(a)P + P	0.1 + 0.00015 + 0.00075	5.8 ± 0.2*** Cd, BP	304.0 ± 43.8 P	85.7 ± 1.7 Cd, BP, P	5.0 ± 0.5*** Cd, P	29.1 ± 5.4 BP, P	3.7 ± 0.3*** BP
	0.5 + 0.0015 + 0.0075	4.6 ± 0.2*** Cd	419.1 ± 49.2** BP, P	75.4 ± 1.8*	4.5 ± 0.5*** Cd, BP	15.9 ± 1.0** Cd, BP, P	4.5 ± 0.5*

Explanations: as in tab. 1.

nistic, additive and unknown origin types of combined action of substances (CAS) were manifested). The short-term complex dose (1.92 + 33.3 + 90.0 mg/kg) resulted in 25.0% increase of serum urea level, in 23.1% reduction of creatinine in urine and in 37.6% reduction of daily diuresis (from 10.1 ± 0.8 to 6.3 ± 1.1 ml, $p < 0.05$). Proteinuria of different origin was caused by Cd (0.5; 1.92; 4.0 mg/kg), B(a)P (0.00015; 0.0015; 10.0 mg/kg), P (0.00075; 0.0075; 20.0 mg/kg) and by the complexes (100 ADI; 0.5 + 0.0015 + 0.0075; 4.0 + 10.0 + 20.0 mg/kg; antagonistic types of CAS) (tab. 1, 2). We determined three eventual reasons concerning the increase of total protein level in urine. The first reason – renal dysfunction – was manifested in groups exposed to Cd (0.5; 1.92; 4.0 mg/kg) and P (0.0075 mg/kg). The lesions of renal proximal tubules were confirmed histologically. The second reason – the impaired pancreatic secretary function – was manifested in groups given B(a)P (100, 1000 ADI) and P (100 ADI). It was confirmed by histological examination of the pancreas as well as some changes of biochemical blood parameters – reduced Ca (from 2.9 ± 0.3 to 2.1 ± 0.2 mmol/l, $p < 0.05$) and enhanced creatinine (from 54.2 ± 1.6 to 65.6 ± 4.2 µmol/l, $p < 0.05$) levels in blood serum under the effect of B(a)P (100 ADI). The third reason – increased total protein level in blood serum – was manifested in the group given B(a)P (10.0 mg/kg). The increase of total protein level in urine could be determined by several above mentioned reasons. For example both subchronic doses of

complex induced the pancreatic and renal dysfunction, which was confirmed histologically. The diameter of proximal convoluted tubules located within the renal cortex was very uneven, the lysis of cells and changes in secretary cells of pancreas were observed. Due to effect of P (20.0 mg/kg) and Cd + B(a)P + P complex (4.0 + 10.0 + 20.0 mg/kg), renal dysfunction and increase of total protein level were noticed. The fact that proteinuria was induced by Cd doses above 1.92 mg/kg has been found by other authors (2) too and has been confirmed by our histological examinations: renal proximal tubules were decomposed. However, our data showed that proteinuria has not been induced by Cd dose 1.92 mg/kg in complex with B(a)P + P, what showed antagonistic type of CAS.

Results obtained showed that the complexes of three substances (doses – 1/30 LD_{50}, 4.0 + 10.0 + 20.0 mg/kg) resulted in 18.3-40.5% increase of total protein level in the blood serum, whereas the last dose resulted in 40.0% increase of Ca level in the blood serum too (accordingly unknown origin, antagonistic and additive types) (tab. 1). Due to exposure to isolated doses of B(a)P (10.0 mg/kg) and P (20.0 mg/kg), the level of total protein in serum increased by 19.7-49.5% and further P (20.0; 90.0 mg/kg) and B(a)P (33.3 mg/kg) increased Ca levels in serum by 20.0-46.7%. We have found that enhanced Ca levels in blood serum were related either to alkaline phosphatase activation (B(a)P and P – 1/30 DL_{50}; from 643.7 ± 57.5 to 968.8 ± 90.1 and 805.5 ± 70.4 U/L, $p < 0.05$) or to increase of total protein in blood serum (Cd + B(a)P + P – 4.0 + 10.0 + 20.0 and P – 20 mg/kg). At some oncological diseases increase the levels of Ca, pathological proteins and activity of alkaline phosphatase in blood serum are obtained. All these symptoms are indicative of bone cancer, myelomas. B(a)P is known for its negative effect on myelocytes (5); its 6-39 mg/kg doses *per os* induce cancer and leuk(a)emia (1). The subchronic doses of combinations of these substances had no effect on the level of calcium in serum.

The damaged hepatic function was characterized by activation of the specific hepatic enzymes ALT and AST in blood serum, hepatic urea and protein synthesis, hepatic detoxification and histopathological data. Our short-term investigations have shown that only isolated substances induced hepatic damage signs, whereas short-term doses of complex didn't induce them and in most cases antagonistic types were manifested. A 40.3-44.8% elevation in the activity of AST (from 226.3 ± 8.3 to 327.7 ± 34.3 and 317.6 ± 35.3 U/L, $p < 0.05$) was observed in rats given Cd (1.92; 4.0 mg/kg) and the 18.2-38.6% decrease serum urea level was observed in rats given B(a)P (10.0; 33.3 mg/kg) (tab. 1). Our subchronic tests have shown that only complexes of substances induced hepatic dysfunction. Due to the both subchronic doses of the complex serum urea level decreased by 20,6-37% and additive as well as antagonistic types of CAS were manifested (tab. 2). The exposure to highest subchronic dose disturbed the protein synthesis of the liver – it decreased by 8.2% (antagonistic type) and reduced the hepatic detoxification function – hypuric acid in urine reduced by 31.2% (unknown type). Due to the exposure to these doses pathomorphological hepatic changes occurred: blind lobules, dark nucleus, lysis of hepatocytes, somewhere there were no nucleus and only splits of cytoplasm were observed in the sinuses of capillaries.

Cd + B(a)P + P combination caused elevation of relative weight of the internal organs: pancreas (doses – 1/30 LD_{50}; 100 ADI; 0.5 + 0.0015 + 0.0075 mg/kg), liver (1/30 LD_{50}), thymus gland (doses – 1/30 LD_{50}; 4.0 + 10.0 + 20.0; 0.5 + 0.0015 + 0.0075 mg/kg) and spleen (doses – 1/30 LD_{50}; 4.0 + 10.0 + 20.0 mg/kg).

Under exposure to the tested complex of substances the indices reflecting hepatic and renal functions were changing. According to the tested indices of blood biochemistry and urinalysis, in the Cd + B(a)P + P group the following CAS types were identified: antagonistic – 62.5% of cases, additive – 15.6% and unknown origin – 21.9%. With only one active substance in the complex (e.g. 3 + 0 + 0 = 3), 40.0% of CAS types were additive. The effect of a complex of substances in 9.4% of cases differed from separate effects of the constituent parts alone.

Combined effect of Cd, B(a)P and P on hematological indices was manifested by the reduced blood leukocytes count, slight, phasic changes in the leukocytes formula and sometimes by slight anaemia. Morphological blood tests showed that combined effect of three substances induced leukopenia symptoms in all cases – decreased blood leukocytes count by 18.7--36.2% which exceeded 2σ control limit and in two cases (doses – 1/30 LD_{50}; 100 ADI) these values were lower than those indicated by Townsend (12) as value limits for leukocytes of Wistar rats (tab. 1, 2). It should be noted that in short-term investigations concerning leukocytes count were manifested additive types of CAS, whereas in subchronic investigations were manifested antagonistic types. Due to the short-term dose of complex (1/30 LD_{50}) the count of erythrocytes reduced by 12.5% (from 4.0 ± 0.1 to 3.5 ± 0.1 10^{12}/l, $p < 0.01$) and blood Hb level reduced by 11.1% (from 133.1 ± 5.5 to 118.3 ± 2.4 g/l, $p < 0.05$). Three doses of the complexes caused slight alterations in the leukocytes formula by increasing eosinophiles (doses – 100 ADI), lymphocytes (1/30 LD_{50}; 100 ADI) and neutrophiles (1/30 LD_{50}; 4.0 + 10.0 + 20.0 mg/kg) percentage. As regards the hematological indices, three CAS types could be distinguished: antagonistic (47.4%), additive (47.4%) and unknown origin (5.2%). With only one active substance in a complex (e.g., 5 + 0 + 0 = 5), 44.4% of all types were additive. The effect of complex substances on blood composition in 5.6% of cases differed from the isolated effects of the constituent parts.

Conclusions

1. Cadmium, benzo(a)pyrene, pyrene and their combination by the symptoms of impairment of renal nitrogen-excreting function, proteinuria, oliguria, hypostenuria and by renal histopathological data might be graded in descending order as follow: Cd ≥ Cd + B(a)P + P > P > B(a)P.

2. All tested substances and their combination by their hepatotoxicological effect might be graded in descending order as follow: Cd + B(a)P + P ≥ Cd > P > B(a)P.

3. According to the blood morphological parameters all tested substances by their hematotoxicological effect might be graded in descending order as follow: P ≥ Cd + B(a)P + P > B(a)P > Cd.

4. In the groups of Cd + B(a)P + P complex by blood biochemistry, urinalysis and hematological parameters the antagonistic type of CAS was dominated – it was manifested in 56.9% of cases, whereas additive or unknown origin type was manifested rarer – accordingly in 27.4% and 21.9% of cases.

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