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Systemic Biomarkers and Liver Morphology in Rats during Chronic Low-Dose Toxicant Administration against the Background of Vitamin Deficiency

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Liver morphology, intensity of apoptosis, and activity of xenobiotic metabolism enzymes were studied in a chronic model experiment in rats receiving a mixture of 6 pesticides against the background of life-long diets with adequate and insufficient supply of water-soluble vitamins. The dose of each pesticide in the mixture did not exceed the acceptable daily intake (1 ADI). It was found that chronic exposure to low doses of anthropogenic toxicants in combination with permanent vitamin deficiency provokes a number of liver changes, such as increased apoptosis activity, cytochrome P450 system depletion, steatosis, and inflammatory infiltration, which is a potential health risk factor.

Key Words: *toxicological studies; liver morphology; apoptosis; biomarkers; in vivo model experiments*

Over the past years there are appearing more and more publications devoted the negative impact of anthropogenic contaminants in doses considered safe for consumers, combined with a number of physical, chemical, and social stress factors accompanying technological and sensory overload of modern human life [1,2]. Long-term population studies conducted in different countries indicate a suboptimal dietary structure of the population characterized by a shift in the balance of proteins, fats, and carbohydrates, excessive caloric intake, insufficient consumption of macro- and micro-elements, as well as of the most vitamins and minor substances [3,4]. Of the above disorders, the effect of chronic vitamin deficiency on the adaptive potential of the body has the greatest evidence base [5,6]; therefore, alimentary models with vitamin deficiencies are used for toxicological studies of low-toxicity objects.

Within the framework of the assessment of the risk of natural and anthropogenic toxicants, the maximum non-acting doses (no observed adverse effect level, NOAEL) and (ADI, acceptable daily intake) of individual substances are usually determined without taking into consideration their possible combined effect against the background of factors reducing the adaptive potential. Concerning modern requirements for life quality and longevity, there is an increasing demand to revise ADI calculation approaches for toxicants whose complex chronic effects represent a potential risk factor for human health [7,8].

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Our aim was evaluation of the combined effect of chronic exposure to low doses of anthropogenic toxicants and permanent vitamin deficiency on the liver.

MATERIALS AND METHODS

A chronic (777 days) toxicological experiment was performed on male Wistar rats (n=200) obtained from Stolbovaya Branch (Scientific Center of Biomedical Technologies of the Federal Medical-Biological Agency of Russia). The initial age of the animals was 30 days and body weight was 90.9±0.9 g. The rats were divided into 4 groups (50 animals per group): two control and two test groups. Animals of the first control group (C-100) received a basic semi-synthetic casein diet [9] with normal (taken as 100%) content of water-soluble vitamins (B1, B2, B3, B5, B6, B7, B12, K3, and folic acid). The second control group (C-25) received a diet where the content of water-soluble vitamins was reduced to 25% of normal [8]. Animals of the experimental groups received ration with a mixture of pesticides (active substances: diquat dibromide, imazamox, acifluorfen, bentazone, imazethapyr, and tepraloxydim) and 100 and 25% daily norm of vitamins: groups E-100 and E-25, respectively. The daily intake of each pesticide did not exceed 1 ADI. The rats received the above experimental diets with normal or modified vitamin composition and with pesticides throughout the study period [8].

Animal experiments were conducted in accordance with GOST 33215-2014 and GOST 33216-2014. The rats were kept in plastic cages with sawdust bedding (2 rats per cage) in a warm (21-23°C) and ventilated room with natural light. They received water and feed *ad libitum*. Feed consumption, body weight dynamic, and general condition of the animals were controlled during the experiment.

The experiment included complex gravimetric, hematological, biochemical, and morphological studies; intensity of apoptosis and activities of some xenobiotic metabolism enzymes in the liver were also determined [10]. Apoptosis activity was assessed by alkaline gel electrophoresis of isolated cells (DNA comet assay) [11]. The degree of morphological changes in the liver was assessed according to the SAF (steatosis, activity, fibrosis) score that includes semiquantitative assessment of steatosis, ballooning and lobular inflammation grading, as well as the stage of liver fibrosis [12,13].

The material for the study was sampled on days 184, 356, and 549 of the experiment, 10 rats of each group were used for each point. Here we present the results of morphological studies of the liver, as well as the intensity of apoptosis and activity of xenobiotic metabolism enzymes in the liver on day 549.

The data were processed by methods of parametric statistics. The normality of distribution of quantitative signs was determined using the Kolmogorov–Smirnov test; variance equality was determined using the Cochran's test. Significance of differences between the mean values satisfying the conditions of normal distribution and equality of variance was assessed using one-way ANOVA, the critical level of significance (p) was taken to be 0.05 [14]. In accordance with the study design, quantitative characteristics of the test groups were compared with the corresponding control groups.

RESULTS

The general condition of all rats was satisfactory; no differences in the general appearance, behavior, and fur quality were found between the groups. Feed intake by rats of the control and test groups was about 15-19 g/rat/day at the beginning of the experiment and about 20-22 and 18-20 g/rat/day in the period from 100 to 200 days and from 250 to 777 days of the experiment, respectively. Analysis of body weight dynamics (Fig. 1) revealed some decrease in body weight

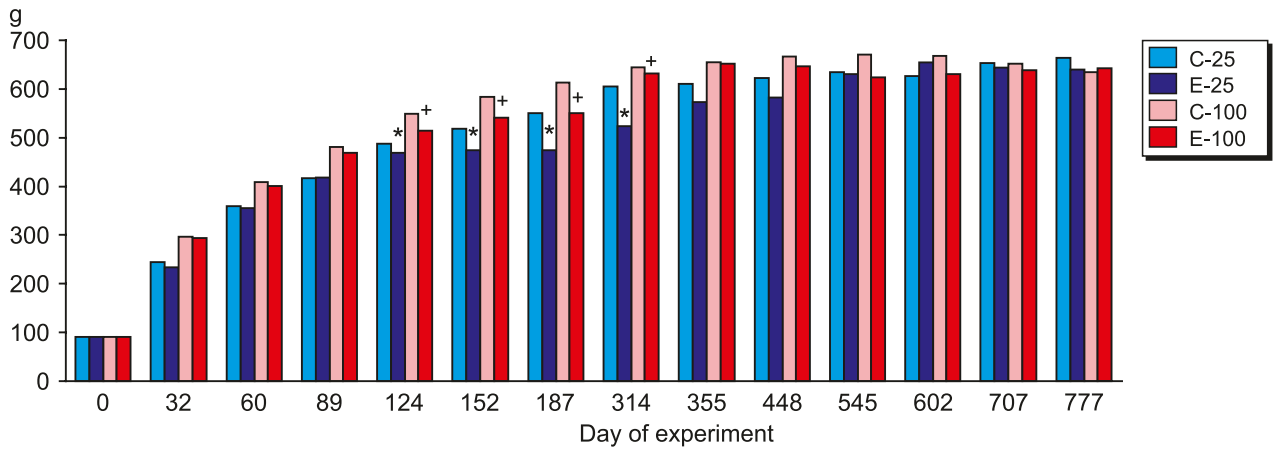

Fig. 1. Body weight dynamics in rats. p<0.05 in comparison with *C-25, $^{+}$C-100.

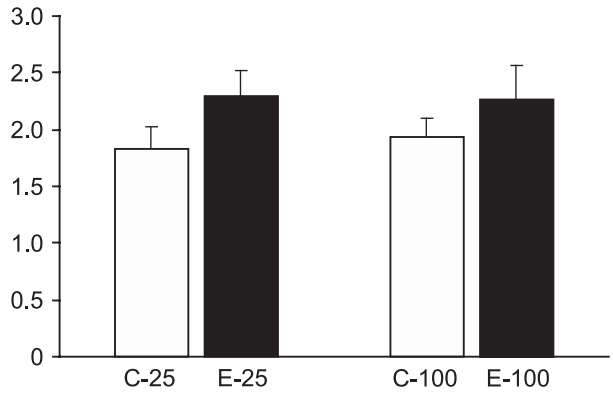

Fig. 2. Apoptosis index in the liver of rats on day 549 of the experiment.

gain in rats receiving diets containing 25% vitamins (C-25 and E-25) in comparison with rats receiving complete diets (C-100 and E-100): differences in the range 12-18% were observed during the first year, *i.e.* from 7 to 356 days of the experiment. Significant ($p<0.05$) differences between the control and corresponding test groups were observed only during the period of 120-330 days of the experiment: the body weight of E-100 group rats was lower by 6-9% than in the C-100 group, and body weight of E-25 group rats was lower by 5-16% than that of C-25 rats. During the second year, as the number of animals in the groups decreased, the above mentioned differences disappeared (Fig. 1).

The study of apoptosis in the liver revealed an increase in the activity of cell death in the test groups of rats in comparison with the control groups (Fig. 2). Thus, this parameter in E-25 group was higher by 25% ($p>0.05$) than in C-25 group and in E-100 group it was higher than in the C-100 group by 17% ($p>0.05$). It should be noted that a similar tendency to an increase in apoptosis activity against the background of toxic exposure, as well as more pronounced increase of apoptosis activity in E-25 group were observed at the earlier stages of the experiment (days 184 and 356).

The activities of the xenobiotic metabolism enzymes (cytochrome P450 isoforms: ethoxy-, methoxy- and pentoxyresorufin dealkylases) in rats of E-25

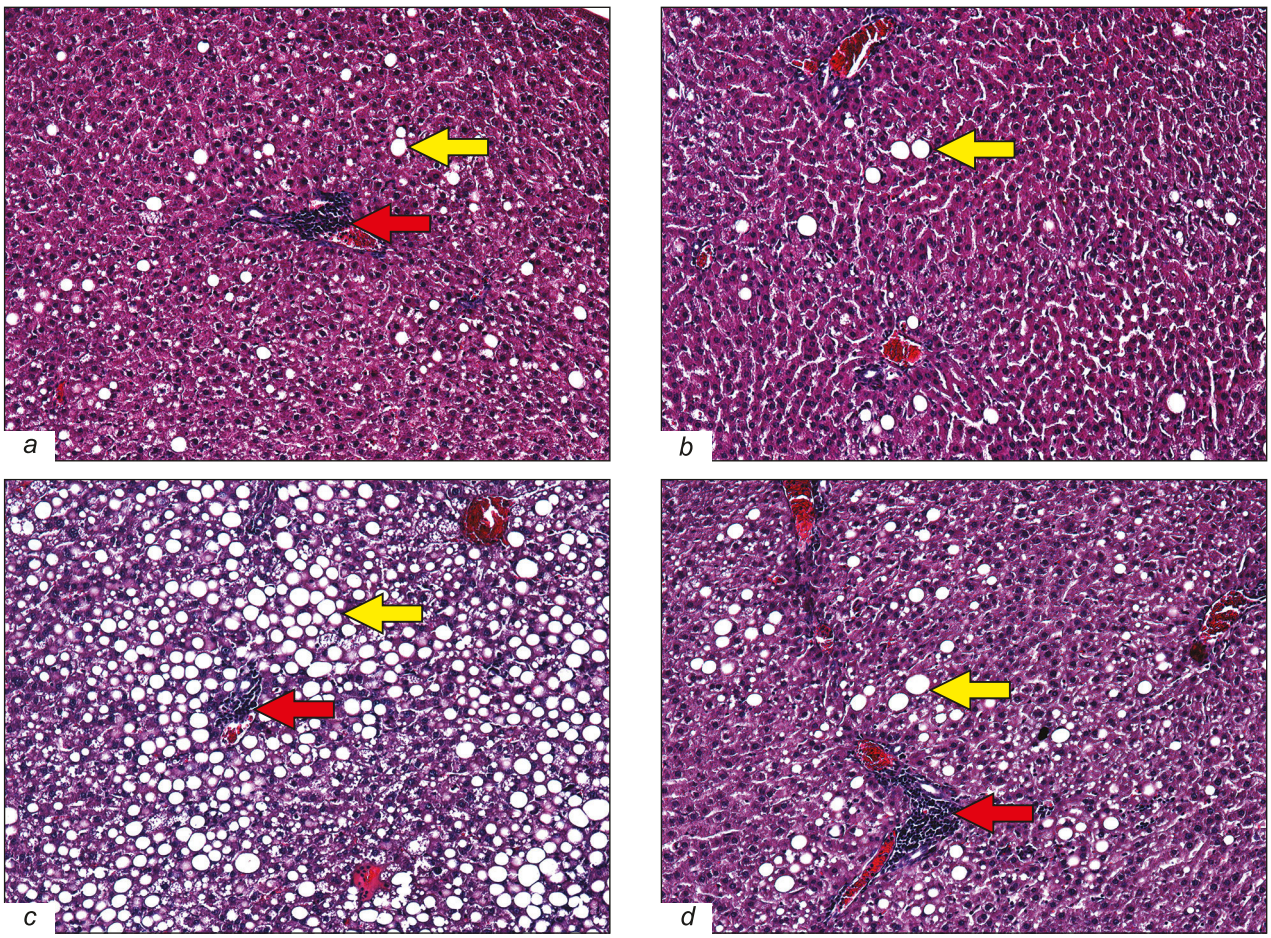

Fig. 3. Microphotographs of the liver from mice of groups C-25 (*a*), C-100 (*b*), E-25 (*c*) and E-100 (*d*). Hematoxylin and eosin staining, ×100. Red arrows show accumulation of lipid, yellow arrows show inflammatory infiltration.

group were lower by on average 40% ($p<0.05$) than in C-25 group, and in animals of the E-100 group, these activities were lower than in C-100 group by 16% ($p>0.05$). This decrease was probably a consequence of cytochrome P450 system depletion caused by chronic exposure to toxicants: on days 184 and 356 of the experiment, activity of these enzymes in E-25 group was higher by 64 and 80% ($p<0.05$) and in E-100 group it was higher by 12 and 28%, respectively, than in the corresponding control groups.

Microscopic examination of livers in all groups revealed intact structure of the hepatic lobules and hepatocyte cords, no periportal and centrilobular fibrosis were detected (Fig. 3). However, increased accumulation of lipids in the cytoplasm of the hepatocytes and signs of lobular inflammation were noted in a significant number of animals: the mean steatosis score in C-25 group corresponded to 0.5 points (up to 30% of rats in the group had lipid accumulation), the mean lobular inflammation score was 0.2 points; in E-25 group the mean steatosis score was 1 point (more than $^{2}/_{3}$ of rats had 30 to 50% lipid accumulation), lobular inflammation was 0.5 points; in groups C-100 and E-100, the mean steatosis score was 0.7 and 0.9 (in more than $^{2}/_{3}$ and $^{4}/_{5}$ rats, respectively, up to 30% of hepatocytes contained lipid accumulation) and lobular inflammation was 0.1 and 0.4 points. According to the SAF score, the degree of morphological changes in the liver was S1A0F0 in C-25 group, S1A1F0 in E-25 group, and S1A0F0 in C-100 and E-100 groups.

Evaluation of serum biochemical parameters in animals of E-25 and E-100 groups indicate the absence of pronounced differences from the corresponding control groups. No changes typical of liver damage were found. Liver weight in the control and test groups also did not differ, therefore, degenerative processes in the liver revealed by morphological study can be attributed to asymptomatic (compensated) stage of hepatosis [15]. Rat mortality throughout the study period did not exceed the values typical for animals of this species and age and was 32 and 38% in groups C-25 and C-100, and 36 and 36% in groups E-25 and E-100, respectively. The main cause of death at all experiment stages was pneumonia.

Therefore, the combined effects of chronic exposure to low doses of anthropogenic toxicants and permanent vitamin deficiency provoke a number of liver changes, such as increased apoptosis activity, cytochrome P450 system depletion, steatosis, and inflammatory infiltration. However, none of them was the cause of reduced lifespan or decreased activity under the experimental conditions. At the same time, it should be emphasized that these disorders can transform into a potential health risk factor under the influence of extended range of stressors affecting the organism throughout the life. The obtained data demonstrate the negative complex effect of toxicants entering the organism in conditionally no-effect doses, which indicates the necessity to adjust the approaches to ADI calculation.

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