Rat Thymocyte Apoptosis and Proliferation Variations in Chronic Exposure to Low-Dose Dichlorodiphenyltrichloroethane

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The aim of the investigation was to study apoptosis and proliferation of rat thymocytes when exposed to low-dose dichlorodiphenyltrichloroethane (DDT) provided for by its maximum allowable level in food products.

Materials and Methods. The experiment was carried out on 64 Wistar male rats administered DDT at doses of 1.890±0.086 and 7.77±0.17 μg/kg/day within 6 and 10 weeks. We performed a histological study of thymus preparations, immunohistochemistry of pro-apoptotic proteins Bax and p53, the assessment of the proliferative activity of thymocytes by a radionuclide method, and the corticosterone and interleukin-2 concentrations in rat blood serum using an enzyme immunoassay.

Results. The study of thymus of control and experimental rats 6 weeks after the experiment start showed the selected doses of DDT to activate the synthesis of pro-apoptotic proteins and initiate p53-dependent apoptosis of both low- and high-grade differentiated thymocytes resulting in focal depletion of thymic cortex. It causes a reactive increase of interleukin-2 production and thymocyte proliferation, however, results in no thymocyte population recovery. Subsequently, with DDT accumulation and exposure time increase, there is the enhanced death of both lymphocytes and reticular epithelial cells, and proliferative activity of thymocytes is suppressed despite the decreased secretion of glucocorticosteroids by adrenals, it being the basic acceleration mechanism of observable involutory changes of thymus.

Key words: thymus; dichlorodiphenyltrichloroethane; DDT; thymocyte apoptosis; thymocyte proliferation.

Thymocyte proliferation, differentiation and apoptosis are basic processes of thymus morphogenesis and regeneration. T cells die through apoptosis in thymus at the stage of antigen-dependent differentiation of T cells. Due to this fact, the study of the effect of ecological factors on the main morphogenetic processes (proliferation and apoptosis of cells in thymus) is one of the fundamental concepts of immunotoxicological researches. In recent decades, endocrinologists and immunologists have been actively studying the effect of low-dose endocrine disruptors on the body. The most wide-spread disruptor is dichlorodiphenyltrichloroethane (DDT), which can be found in all ecosystems of continents and oceans including Arctic and Antarctic regions, and can continue to persist in soil and water for a long time [1–3].

The aim of the investigation was to study apoptosis and proliferation of rat thymocytes when exposed to low-dose dichlorodiphenyltrichloroethane provided for by its maximum allowable level in food products.

Materials and Methods. The experiment was carried out on 64 Wistar male rats weighing 80–100 g. The animals were divided into control and test groups. The animals were kept in vivarium, animal care being in accordance with the handling standards and rules of laboratory animals as consistent with "International Guidelines for Biomedical Researches with Animals" (1985), laboratory routine standards in the Russian Federation (Order of Ministry of Healthcare of the Russian Federation dated 19.06.2003 No.267) and "Animal Cruelty Protection Act" dated 1.12.1999. The experiment was carried out in accordance with the regulations of experimental animal operation approved by Order of Ministry of Healthcare of USSR No.577 dated 12.08.1977.

The test animals were administered *o,p*-DDT (Sigma-Aldrich, USA) solutions with the concentration of 20 and 80 μg/L instead of water. The selection of doses was due to different background DDT content on various geographical territories, and different maximum permissible levels in food products (meat products: 0.1 mg/kg; milk products: 0.05 mg/kg; grain crops: 0.02 mg/kg) [4]. According to calculations, mean daily DDT doses were 1.890±0.086 and 7.77±0.17 μg/kg/day.

Control animals were given tap water. The access to water and food was free.

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The first part of the animals of the control and test groups were sacrificed 6 weeks after the experiment had been started. And the second part of the animals was sacrificed 10 weeks after the experiment had begun. We took thymic and blood samples. After standard histological study using automated tissue processor Tissue-Tek VIP 5 Jr (Hygeco, France) we prepared thymic sections, which were hematoxylin and eosin stained. Histological preparations were studied by light microscopy using a microscope Leica DM2500, and computed morphometry using ImageScope program (Leica Microsystems Gmbh, Austria). In histological thymic preparations we determined cortex-medulla ratio. The expression of pro-apoptotic proteins p53 and Bax were immunohistochemically studied using primary rabbit polyclonal antibodies (Santa Cruz Biotechnology, USA). The reaction was imaged using UltraVision Detection System (Thermo Scientific, USA). Corticosterone (IBL, Germany) and IL-2 (Bender Medsystems, Austria) concentrations were determined by enzyme immunoassay. *Ex tempore* thymocyte proliferation [5] was determined using ^{3}H-thymidine.

The findings were statistically processed by Statistica program package (StatSoft Inc., USA) using parametric and nonparametric methods. The differences were considered significant if $p<0.05$.

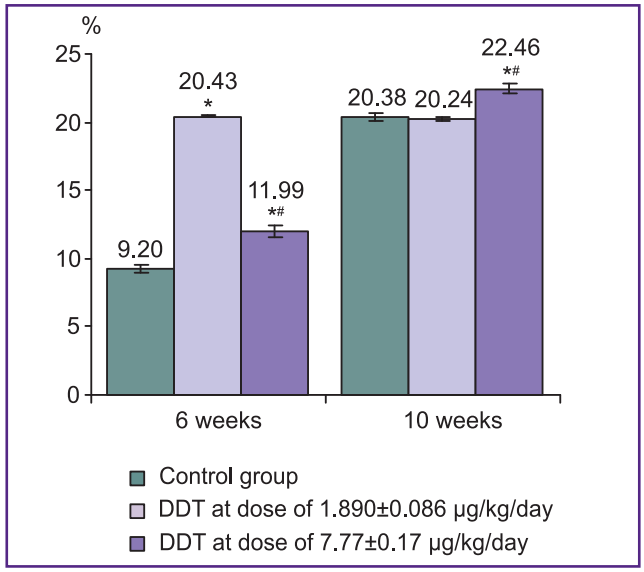

Figure 1. The content of thymocytes expressing protein p53, in the control and test groups of rats consumed DDT at doses of 1.890±0.086 μg/kg/day and 7.77±0.17 μg/kg/day within 6 and 10 weeks. * Significant difference of values from the control group; # rom a group with a lower DDT dose

Results. *Control rat thymus 6 weeks after the experiment start* had typical lobulation. The cortex averaged three-quarters of the thymic parenchyma. In cortex lymphocytes fitted tight to each other. In medulla there could be found single thymic bodies. Protein p53 expression was revealed approximately in 10% lymphocytes of the thymus (Figure 1). p53-positive cells were found in both a subcapsular layer, and also at the boundary of cortex and medulla of the thymus. Bax-positive cells were imaged only in the subcapsular layer (Figure 2 (a)).

After taking DDT at a dose of 1.890±0.086 μg/kg/day within 6 weeks the boundary between cortex and medulla in thymus became less clear. The percentage of cells expressing protein p53 was twice as high than that in the control group (See Figure 1). p53-positive cells arranged diffusely were found in cortex, as well as in medulla. The number of Bax-positive cells in a

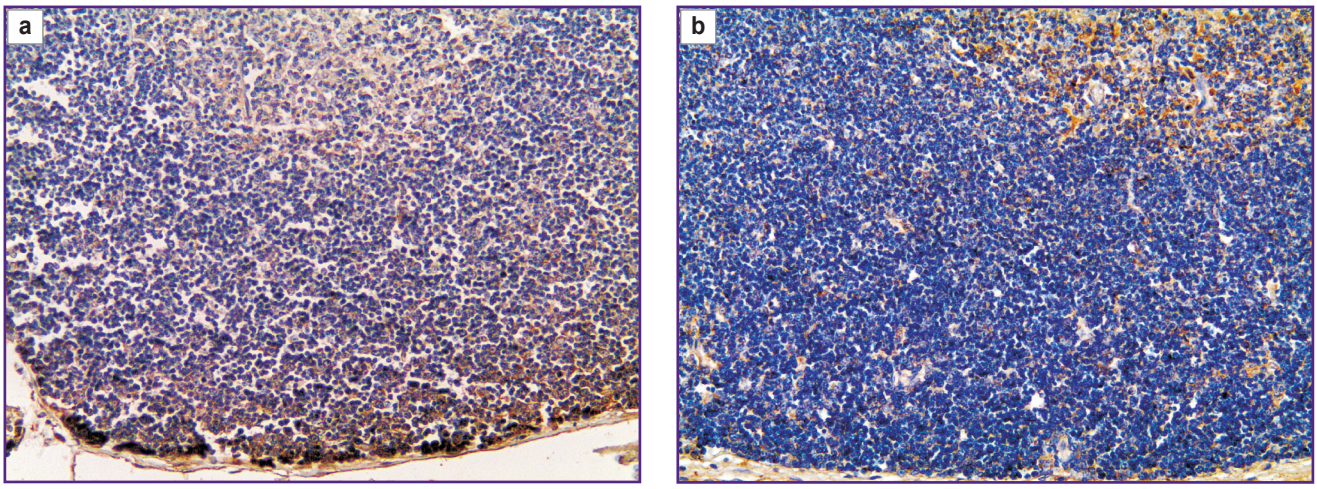

Figure 2. Bax protein expression by thymocytes: (a) in control rats 6 weeks after the experiment start; Bax-positive cells are seen in a subcapsular layer only; hematoxylin counterstaining; ×200; (b) in rats of a test group consuming DDT at a dose of 7.77±0.17 μg/kg/day for 6 weeks; Bax-positive cells are found more frequently in medulla, and scarcely found in a subcapsular layer of the cortex; hematoxylin counterstaining; ×200

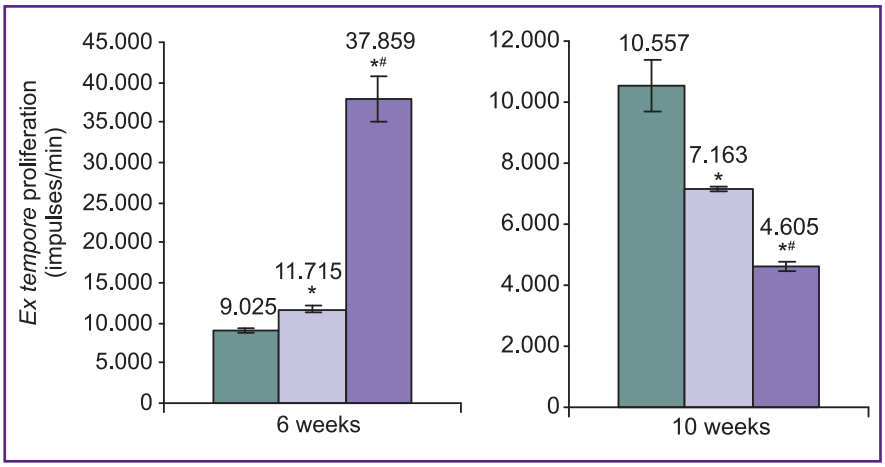

Figure 3. *Ex tempore* prolifarative activity of thymocytes of the control and test groups 6 and 10 weeks after the experiment start. * Significant difference from the control group; # from a group with a lower DDT dose

subcapsular layer increased, they were also revealed in deeper cortical layers (Figure 2 (b)). Single Bax-positive cells were found in medulla. The study of rat thymocyte proliferation activity showed a significant increase of *ex tempore* thymocyte proliferation compared to control animals (Figure 3). There was a significant decrease of corticosterone concentration in rat blood serum compared to the control animals of the similar experimental period (See the Table). IL-2 concentration increased as well, though insignificantly.

In the rats administered DDT at a dose of 7.77± 0.17 μg/kg/day within 6 weeks the thymic structure had no marked differences. A part of cells expressing protein p53 was higher than that of the control group, and was lower than in rats given DDT at a dose of 1.890± 0.086 μg/kg/day within the similar period (See Figure 1). p53-positive cells were arranged mainly at the boundary of cortex and medulla. Bax-positive cells were found more frequently in medulla, while in the subcapsular layer of cortex there were few of them (See Figure 2 (b)). There was a significant enhancement of *ex tempore* thymocyte proliferation: three times as much compared to both control and the previous test group (See Figure 3). Corticosterone concentration in blood serum appeared to decrease compared to the control animals and the test animals administered a lower DDT dose, IL-2 concentration reduced (See the Table).

Thymus structure in control rats 10 weeks after the experiment start had no significant differences compared to the previous study period. The cortex-medulla ratio did not change. Moreover, the number of cells expressing protein p53 grew more than twofold (See Figure 1) due to the development of age involution. p53-positive cells located mainly in the subcapsular layer. The number of Bax-positive cells increased significantly, they arranging diffusely both in cortex, and medulla. Bax-positive cells were also found among reticular epithelium cells. Thymocyte proliferation indices in the control group 10 weeks later did not differ from those of the previous study period. Age dynamics also consisted in IL-2 content decrease in blood serum (See the Table).

After 10 weeks of DDT consumption at a dose of 1.890±0.086 μg/kg/day, a part of rat thymic cortex did not differ from the control animals, as well as from the animals administered DDT at the same dose within 6 weeks. Focal depletion of thymic cortex due to the death of lymphocytes was more marked compared to controls and the test group given the same DDT dose within a less time period. The percentage of cells expressing protein p53 underwent no changes compared to the control group. p53-positive cells were located diffusely in both cortex, and were arranged by groups in medullar. The number and localization of Bax-positive cells did not change either compared to controls. Thymocyte proliferation *ex tempore* decreased compared to the control values. There was also observed a significant decrease of serum corticosterone concentration compared to the control animals, and a significant increase of IL-2 concentration.

After DDT intake at a dose of 7.77±0.17 μg/kg/day within 10 weeks the rats were found to have the reduction in thymus lobule size. A cortical layer appeared to

Altered concentrations of IL-2 and corticosterone in blood serum of rats administered DDT at different doses within 6 and 10 weeks (M±m)

Indices	DDT consumption 6 weeks			DDT consumption 10 weeks		
	Control group	1.890±0.086 μg/kg/day	7.77±0.17 μg/kg/day	Control group	1.890±0.086 μg/kg/day	7.77±0.17 μg/kg/day
IL-2 (pg/ml)	118.39±16.14	129.17±33.15	76.07±9.25*	69.07± 9.58^	249.24±24.48*^	121.94±12.68*^#
Corticosterone (ng/ml)	139.09±10.99	108.41±1.94*	103.25±6.61*	127.12±8.99	108.09±2.75*	97.36±5.61*

N o t e. * Significant difference from a control group; # from a group administered a lower DDT dose; ^ from a group with a shorter research period.

have the areas with lymphocyte death and cells with hyperchromatic pyknotic nuclei. p53 protein expression by thymocytes grew compared to the control and test groups of the similar experimental period. p53-positive cells were found in a cortex and medulla, their number in a subcapsular layer increasing. The number of Bax-positive cells, both lymphocytes and reticular epithelial cells in medulla grew. However, in a cortical layer of these animals Bax-positive lymphocytes were less common than in other animal groups. Proliferative activity of thymocytes decreased compared to both control and test animals which were given the lower dose within 10 weeks (See Figure 3). Blood serum corticosterone concentration in rats of this group was no different from the previous period of research, and was significantly lower than in the control group. Serum IL-2 level increased compared to that of the controls, though was significantly lower than in the control group.

Discussion. Currently, at least two forms of thymocyte apoptosis are known, one of which is due to the glucocorticoid action [6]. The second thymocyte apoptotic form proceeds with the participation of protein p53 expressed by thymocytes [7, 8].

The study of rat thymic histological preparations of all the groups showed primarily the death of thymocytes manifested by the presence of the cortical depletion areas. To establish the mechanisms of thymocyte apoptosis initiation when exposed to low-dose DDT we determined blood serum corticosteroid concentration, which is the main glucocorticoid in rats. All the groups in our study were found to have decreased corticosterone level compared to the control values. The fact suggests that in rat thymus not glucocorticoid-induced apoptotic way is involved under DDT action.

DDT at a dose of 1.890±0.086 μg/kg/day given to rats within 6 weeks resulted in thymocyte apoptosis enhancement consisting in two-fold increase of the percentage of Bax-positive cells, and cells expressing protein p53 compared to the controls. Moreover, differentiated lymphocytes located in medulla appeared to be more sensitive to DDT action, and it was proved by the shift of Bax expression from a subcapsular layer to the deeper cortical and medullary layers.

In the rats taken DDT at a dose of 7.77±0.17 μg/kg/day for 6 weeks, as in a group of rats with DDT dose of 1.890±0.086 μg/kg/day lymphocyte death was noted in the cortex substance.

Significant enhancement of cell apoptosis revealed in a group of animals given a lower DDT dose within the same period suggests that in higher DDT dose cell death could be observed earlier and resulting in the cortical depletion areas. Cell death caused IL-2 concentration increase, IL-2 being a factor of lymphocyte proliferation [9, 10] followed by a reactive enhancement of thymocyte proliferation. Increased proliferation activates p53-dependent apoptotic pathway, however, when exposed to DDT, no relation between the parameters was found that proves the role of low insecticide doses in thymocyte death. Thus, even a low-dose DDT can enhance cell death in thymus, primarily, by p53-dependent apoptotic pathway.

10 weeks after the experiment start, rat thymus showed the differences due to both DDT action and age. Control animals showed lymphocyte death enhancement as evidenced by a twofold increase of p53-positive cells and the appearance of lymphocyte death areas in cortex. These facts suggest the start of thymolysis in rats, which develops after the entry into puberty [11].

In contrast to the control group, where lymphocytes of a subcapsular layer were primarily exposed to apoptosis, in a group of rats given DDT at a dose of 1.890± 0.086 μg/kg/day within 10 weeks, both differentiating and high-differentiated lymphocytes went through apoptosis leading to the increase of cortical depletion areas. The fact that the percentage of p53-positive cells was no different from that of the controls can be explained by a significant thymocyte death rate at an earlier stage. Combined with their decreased proliferative activity, it restricts the recoverability of the cell population despite an increased growth factor synthesis of IL-2 lymphocytes.

Thymus of rats administered DDT at a dose of 7.77±0.17 μg/kg/day for 10 weeks showed increased protein p53 expression by thymocytes compared to the control and test groups given a lower DDT dose within the same period. Bax-positive cells were found more frequently, among reticular epithelial cells as well. In our previous studies [12, 13] we found the increase in the number of thymic bodies at a degradation stage. This fact also proves the enhancement of reticular epithelium death, which in its turn is a factor contributing a decreased thymocyte proliferation and differentiation. The comparison of morphological and functional changes of the thymus 10 weeks after the experiment started showed that their nature is similar in the control and test groups, though thymocyte death rate in DDT consumption was accelerated. Since in the control group of a longer experimental period there were found age-related thymus alterations, the similarity of the indices can suggest the faster involutional processes in rats given low-dose DDT despite the reduced secretion of corticosteroids and increased IL-2 synthesis.

Conclusion. Chronic exposure to low-dose dichlorodiphenyltrichloroethane within maximum allowable levels of its content in food products causes apoptotic death of thymocytes, mainly in the participation of p53-dependent apoptotic pathway. Thymocyte apoptotic activation results in the reactive enhancement of their proliferative activity, though a longer exposure to the insecticide inhibits cell proliferative potential. The increase in dose and the exposure time of dichlorodiphenyltrichloroethane enhances death of both: lymphocytes and reticular epithelial cells leading to the growth of involutional changes of thymus despite a reduced corticosterone synthesis.

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Conflicts of Interest. The authors have no conflicts of interest related to the present study.

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