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SUBACUTE TOXICITY OF ORALLY APPLIED ALPHA-CYPERMETHRIN IN SWISS MICE

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Abstract: The effect of a synthetic pyrethroid - alpha-cypermethrin administered per os for 28 days to Swiss mice was examined on phagocytic and bactericidal activity of neutrophils, and leukocytic image, IL-12 p70 level in blood plasma, as well as histologic and ultrastructural picture of the liver, heart, kidneys, lung and spleen. A synthetic pyrethroid alpha-cypermethrin, [(R,S)-alpha-cyano-3-phenoxybenzyl (R,S)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate], produced by the Chemical Plant in Jaworzno was used in the study. The preparation for the application per os was used in doses 1/2 LD_{50} (25 mg/kg body mass) and 1/5 LD_{50} (10 mg/kg body mass). The results were presented as mean ($\bar{x}$) ± standard error (SEM) and subjected to statistical analysis by the parametric t-Student test. Subacute poisoning of mice with alpha-cypermethrin in doses 1/2 LD_{50} and 1/5 LD_{50} resulted in decreased bactericidal activity of neutrophils. The dose 10 mg/kg body mass had a stronger stimulatory effect on phagocytic activity than 25 mg/kg body mass. Significantly higher numbers of monocytes and lymphocytes were observed in the blood of male mice poisoned with 1/5 LD_{50} alpha-cypermethrin. The administration of alpha-cypermethrin resulted for both doses in the decrease in IL-12 p70 serum secretion. The lowest IL-12 p70 level (pg/ml) was noted among female mice administered 1/2 LD_{50} of the preparation. The results of the study may indicate that the pyrethroid in the study had a suppressive effect on Il-12 p70 production. In mice administered 1/5 LD_{50} or 1/2 LD_{50} of the preparation examined, histopathologic and ultrastructural changes were observed in the liver and kidneys.

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Key words: alpha-cypermethrin, oral poisoning, Swiss mice, phagocytic test, NBT test, IL-12 p70, histologic and ultrastructural studies.

INTRODUCTION

Synthetic pyrethroids occupy an important position among commonly applied pesticides. These are modified derivatives of pyrethrins, i.e. natural substances obtained from the flowers of *Pyrrethrum cinerariaefolium* and *Pyrethrum carneum*. In the course of studies on modification of the chemical structure of pyrethrins a certain number of synthetic pyrethroids were obtained of improved physical and chemical properties and increased biological activity. With respect to chemical structure synthetic pyrethroids are esters of specific acids, e.g. 2-(4-chlorophenyl)-3-methylbutyric acid and alcohols, e.g. 3-phenoxy-benzyl alcohol. In 1977 one of the synthetic pyrethroids - cypermethrin (obtained for the first time in 1974) was allowed for turnover as a highly active synthetic pyrethrin insecticide, effective in the control of many pest species in agriculture, animal breeding and the household.

The absorption of cypermethrin from the digestive tract and its excretion takes a quick course. Cypermethrin, both -cis and -trans isomers are metabolized to phenoxybenzoic acid and cyclopropanecarboxylic acid.

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Table 1. Leukogram of Swiss mice in subacute poisoning with α-cypermethrin.

Experimental Group	Leukocytes		Neutrophils				Eosinophils		Monocytes		Lymphocytes	
			With lobate nucleus		With stick nucleus							
	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM	$\bar{x}$	SEM
1/5 LD_{50} α-cypermethrin Male mice N=10	7,740.0**	2,136.1	1,872.8	956.9	269.2	218.2	121.8	124.4	309.4*	118.4	5,166.8**	2,312.0
1/5 LD_{50} α-cypermethrin Female mice N=10	4,460.0	1,887.6	865.2	483.3	84.8	55.4	56.2	61.1	147.2	117.2	3,306.6	1,333.9
Control group Male mice N=10	3,680.0	327.1	1,711.2	652.0	89.6	63.9	57.8	48.9	70.8	36.6	1,750.6	860.3
Control group Female mice N=10	4,760.0	3,457.3	635.2	324.9	85.0	85.5	89.0	92.4	93.8	73.1	3,857.0	306.2

$\bar{x}$ - mean value; SEM - standard error; *-** Significantly higher compared to control group: * $p < 0.05$; ** $p < 0.005$.

The half-life period of cis-cypermethrin in the fatty tissue in mice is 13 days, whereas in the case of trans-isomer - only one day [27].

Synthetic pyrethroids are neurotoxins. In mammals and insects they affect axons of the neurons of the peripheral and central nervous system and interact with the sodium ions transportation system through the cellular membrane [26, 41].

Pyrethroids may exert a toxic, suppressive or stimulatory effect on the immunological system [8, 9, 13, 22, 23, 25]. The defensive capability of neutrophils changes as a result of the dermal absorption of alpha-cypermethrin, which, when administered in the dose 1/10 LD_{50} stimulates phagocytic activity, and in the dose 1/2 LD_{50} causes its suppression [21].

Pesticides may cause the mobilization of the hemopoietic system manifested by statistically higher mean values of erythrocytes, hemoglobin, and hematocrit, as well as the general number of leukocytes and their subpopulations: neutrophils, lymphocytes and monocytes [37].

Pyrethroids may also affect the mechanisms of non-specific humoral immunity. After 28 days of application of alpha-cypermethrin per os its stimulatory effect on the synthesis of TNF-α (1724.3 pg/ml) was observed in Swiss mice. The synthesis of IL-2 was inhibited or stimulated according to the dose of the pyrethroid and the gender of the animals [19]. IL-12 induces IFN-γ production in peripheral blood lymphocytes (PBL), enhances the cytotoxicity of NK cells and stimulates the proliferation of PBL [15]. IL-12 synergizes with IL-2 to activate cytotoxic lymphocytes [35]. IL-12 is a pleiotropic cytokine that is thought to be a functional bridge between innate resistance and antigen-specific adaptive immunity [3]. There are many biological activities associated with IL-12, and many of them are associated with its role in Th1/Th2 responses. IL-12 is secreted by a variety of antigen-presenting cells, including B cells [15, 35], monocytes and macrophages [38], dendritic cells [24], neutrophils [2], Langerhans cells and keratinocytes [14]. Low levels of IL-12 production were detected in granulocytes stimulated with LPS (lipopolysaccharide) and IFN-γ [42]. IL-12 has also been detected in free nerve endings, dermal nerve fibres, peripheral nerves and in spinal cord tissues [40].

Three forms of IL-12 are known to be secreted from cells producing recombinant IL-12 - heterodimeric p70, homodimeric p40 ($p40_2$), and monomeric p40 [12]. The form of IL-12 for which a biological activity has been ascribed is p70 [15, 35]. In a murine system, secreted $p40_2$ has been shown to bind to the IL-12 receptor with the same affinity as p70 [12]. IL-12 is a heterodimer of molecular weight 70kDa, composed of 40kDa (p40) and 35kDa (p35) subunits that are covalently linked by a disulfide bond [35].

IL-12 is capable of stimulating the production of certain categories and subcategories of immunoglobulines and of inhibiting the secretion of others. Among the immunoglobulines, the secretion of which increases IL-12, are: IgG2a, IgG2b, and IgG3. The production of these imunoglobulines is associated with its participation in the response of Th1 cells. IL-12 inhibits the secretion of IgG1 and IgE - the immunoglobulines dependent on Th2. The effect of Il-12 is changeable according to doses and is not always accompanied by the inhibition of IgE secretion [11].

IL-12 has been considered as a cytokine which enhances the cytotoxicity of NK and LAK cells [15, 31], cytotoxicity of lymphocytes T and TIL [18, 28]. Antigen presenting cells (APC), such as macrophages and dendritic cells provide signals indispensable for the optimum activation of lymphocytes T [17].

The aim of the study was to evaluate subacute toxicity of the synthetic pyrethroid alpha-cypermethrin administered per os to Swiss mice, based on the level of phagocytic and bactericidal activity of neutrophils, the level of IL-12 p70 in blood plasma, as well as hematologic, histologic and ultrastructural studies.

Table 2. Results of phagocytic test and nitroblue-tetrazolium test (NBT) in mice after administration of α- cypermethrin.

Experimental Group	Phagocytic index		NBT index	
	$\bar{x}$	SEM	$\bar{x}$	SEM
1/5 LD_{50} α-cypermethrin Male mice N=10	71.2**	3.8	6.4*	3.0
1/5 LD_{50} α-cypermethrin Female mice N=10	74.2**	3.2	6.8*	3.0
1/2 LD_{50} α-cypermethrin Male mice N=10	69.6*	2.1	7.5*	3.0
1/2LD_{50} α-cypermethrin Female mice N=10	69.7*	2.6	5.4**	3.1
Control group Male mice N=10	62.7	7.4	10.6	3.0
Control group Female mice N=10	62.7	3.2	10.5	3.0

$\bar{x}$ - mean value; SEM - standard error; *-** Significant difference compared to control group: * $p < 0.05$; ** $p < 0.005$.

Table 3. Level of IL –12 p70 in blood plasma of Swiss mice in subacute poisoning with α-cypermethrin.

Experimental group	IL-12 p70 level (pg/ml)	
	$\bar{x}$	SEM
1/5 LD_{50} α-cypermethrin Male mice N=10	2.2	1.5
1/5 LD_{50} α-cypermethrin Female mice N=10	7.0	11.4
1/2 LD_{50} α-cypermethrin Male mice N=10	1.3	2.6
1/2 LD_{50} α-cypermethrin Female mice N=10	0.2*	0.6
Control group Male mice N=10	3.3	3.5
Control group Female mice N=10	8.3	9.8

$\bar{x}$ - mean value; SEM - standard deviation; * Significantly lower compared to control group: $p < 0.05$.

MATERIALS AND METHODS

A synthetic pyrethroid - alpha-cypermethrin, [(R,S)-alpha-cyano-phenoxybenzyl (R,S)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate], produced by the Chemical Plant in Jaworzno was used.

The application liquid was in the form of emulsion consisting of arabic gum, olive oil and water in proportion 1 : 2 : 1.5. The preparation for the application per os was used in doses 1/2 LD_{50} (25 mg/kg b.m.) and 1/5 LD_{50} (10 mg/kg b.m).

The studies were conducted on male and female Swiss mice. The animals were fed with standard fodder LSM [16] and watered *ad libitum*. At the beginning of the experiment the body mass of mice ranged from 21-24 g.

The study covered three groups of animals, two experimental groups and one control group. Each group consisted of 20 mice (10 males and 10 females). The experimental groups were administered per os 1/5 or 1/2 LD_{50} of the preparation for 28 days, except for Saturdays and Sundays. The animals of the control group were administered per os the sole emulsion during this time and in conditions corresponding to the experimental group. After 28 days of the experiment, the animals were anaesthetized and blood taken from the heart in order to evaluate the activity of granulocytic system and to determine the IL-12 p70 level in blood plasma. For evaluation of phagocytic properties of neutrophils, phagocytic reaction with Bacto-Latex (Difco, USA) was conducted [36]. The bactericidal activity of neutrophils was investigated by nitroblue-tetrazolium test (NBT) [30]. In both tests full peripheral blood was used. In each test, 100 neutrophils were counted. In phagocytic test, the cells which contained at least 3 latex grains were considered as positive. In NBT test, cells containing formazan deposits of at least the size of one lobe of nucleus were recorded as positive. The numbers of positive cells per 100 neutrophils analysed were determined as indices of the tests applied.

The following organs were taken in order to evaluate histologic and ultrastructural changes: the liver, heart, kidneys, lungs and spleen. The material for histological study was fixed in neutralized formalin diluted in proportion 1 : 9. The preparations were dyed with H+E. Ultrastructural and histologic studies were conducted in parallel. Material for the study by electron microscope was fixed in 5% glutaraldehyde solution, in 0.1 M. cacodylate buffer of pH 7.2-7.4 for a period of 5 hours. The material was washed in buffer with the addition of 7% saccharose, and then fixed for 1 hour in 1% solution of OsO_4 in Michaelis buffer of pH 7.2-7.4. The material was dehydrated in ethyl alcohol, the concentrations increasing from 50% to absolute and embedded in Epon 812. Polymerization was carried out at temperature 60°C. Ultrathin specimens were cut with the use of Tesla BS 490 ultramicrotome, then observed, and photographed using BS 613 Tesla electron microscope.

Blood for the determination of IL-12 p70 was centrifuged and plasma separated from the blood cell mass. Samples of mice plasma were stored at -70°C. Before running the ELISA they were kept at room temperature. ELISA - enzyme-linked immunosorbent assay with IL-12 (p70) - endogen mouse interleukin-12 (p70) ELISA, produced by Endogen Inc. (USA) and

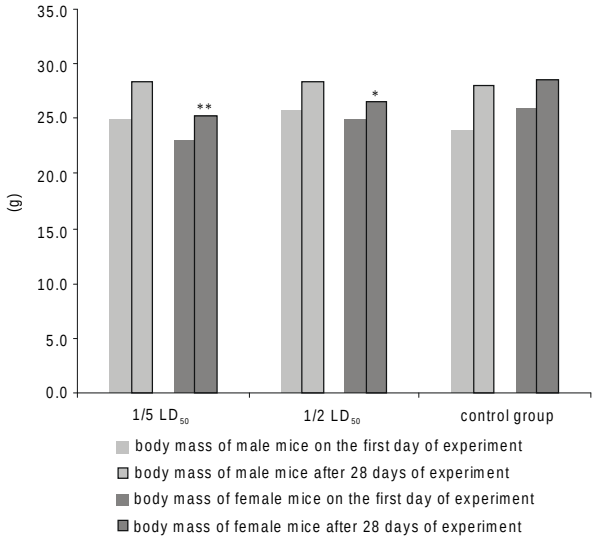

Figure 1. Body mass of Swiss mice poisoned with alpha-cypermethrin per os; *-** Significantly lower compared to control group: * $p < 0.005$, ** $p < 0.0005$.

bought in Warsaw (Biomedica) was used. Microtiter plates (Nunc-Immuno-Modules) were bought at Biocom Systems. In this assay 50µl of each plasma in duplicate was used. Reagent preparations consisted of Biotinylated Antibody Reagent, Streptavidin-HRP Solution, Premixed TMB Substrate Solution and Stop Solution. Absorbance was measered on an ELISA reader set (Metertech Σ 960) at 450 nm within 30 minutes of stopping the reaction. A standard curve was used to determine the amount of mouse IL-12 (p70) in the examined samples [10].

The results were presented as mean ($\bar{x}$) ± standard error (SEM) and subjected to statistical analysis by the parametric t-Student test.

RESULTS

Body mass. On the first day of the experiment the body mass of animals ranged from 23.0-26.0 g, and during the first week of poisoning with alpha-cypermethin it decreased by 0.2-1.3g, irrespective of the dose of the preparation administered. During the following days of the experiment a slow increase in the body mass was observed up to the initial level. On the 28th day, the body mass of mice of the experimental group ranged from 25.2-28.6 g, while the increase in body mass was 1.7-4.0 g. Statistically lower mean values of the body mass compared to the control group (Fig. 1), were noted only in female mice exposed to 1/5 LD_{50} alpha-cypermethrin ($p < 0.005$) and 1/2 LD_{50} alpha-cypermethrin ($p < 0.05$) after 28 days administration of the preparation per os.

Number of blood cells. Subacute poisoning per os with alpha-cypermethrin resulted in a statistically significant increase in the number of leukocytes ($p < 0.005$) only in male mice administered the preparation in the dose 1/5 LD_{50}, compared to the control group. In addition, in this group a statistically significant increase was noted in the number of monocytes ($p < 0.05$) and lymphocytes ($p < 0.005$) (Tab. 1). The preparation administered had no effect on the change in the parameters of the erythrocytic system (number of erythrocytes, level of hemoglobin and hematocrit - data not shown).

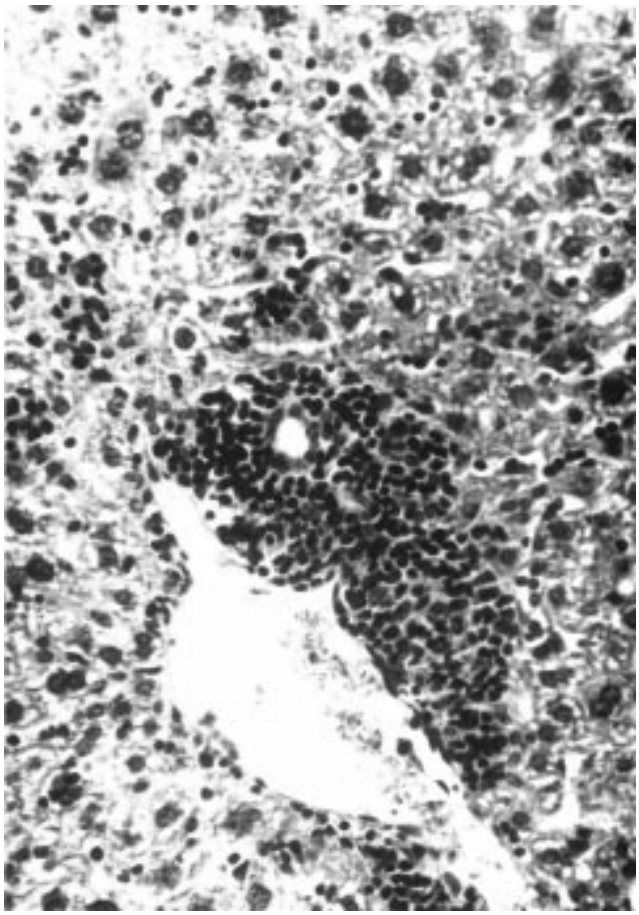

Figure 2. Liver of a mouse after per os application alpha-cypermethrin (1/5 LD_{50}). The infiltrate of inflammatory cells (lymphocytes and plasmocytes) concentrated in the portal tract. H-E, × 80.

Granulocyte activity. The poisoning of animals with alpha-cypermethrin resulted in a statistically significant increase in phagocytic activity, compared to the control group, both in the case of application of 1/2 LD_{50} ($p < 0.05$), and 1/5 LD_{50} ($p < 0.005$) (Tab. 2).

The bactericidal activity of neutrophils, manifested by the presence of dark-blue water insoluble formazan granules produced as a result of nitroblue-tetrazolium conversion, was statistically significantly lower in all experimental groups, compared to the control group (Tab. 2).

Alpha-cypermethrin administered per os in the dose 1/5 LD_{50} caused a decrease in the NBT index, compared to the control group (10.6 for male mice and 10.5 for female mice), to the values 6.4 and 6.8 respectively ($p < 0.05$). The administration of 1/2 LD_{50} of the preparation resulted in a decrease in the NBT index to 7.5 in male mice ($p < 0.05$) and 5.4 in female mice ($p < 0.005$).

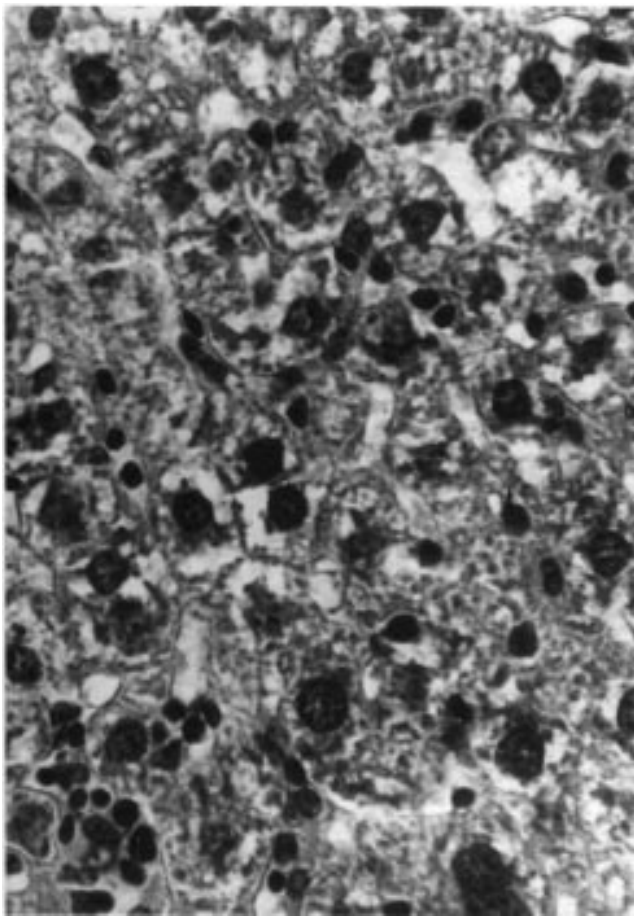

Figure 3. Liver of a mouse after per os application alpha-cypermethrin (1/2 LD_{50}). The infiltrate of individual mononuclear cells, and parenchyma degeneration of hepatocytes were noted. H-E, × 160.

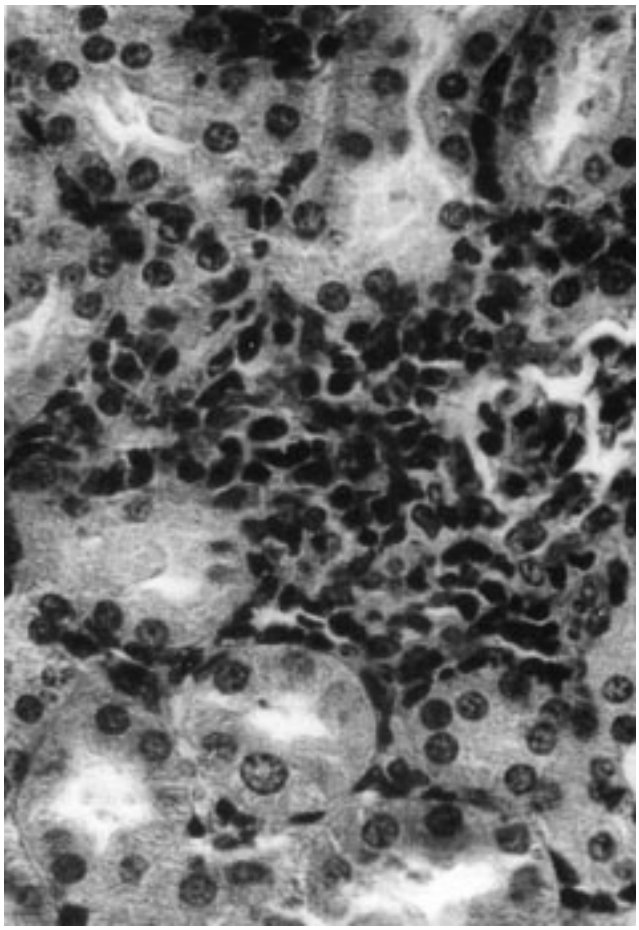

Figure 4. Kidney of a mouse after per os application alpha-cypermethrin (1/2 LD_{50}). A few infiltrations of mononuclear inflammatory cells between the proximal tubules were noted. H-E, × 160.

IL-12 p70 level. A significantly lower level of IL-12 p70 in plasma ($p < 0.05$), compared to the control group, was observed in female mice poisoned with 1/2 LD_{50} alpha-cypermethrin. The level of interleukin examined proved to be lower than in the control group, also in male mice administered the preparation in doses 1/2 LD_{50} and 1/5 LD_{50}. These results, however, were not statistically significant (Tab. 3).

Histological and ultrastructural changes. The analysis of histologic specimens from animals poisoned with alpha-cypermethrin in the doses 1/2 and 1/5 LD_{50} indicated the presence of numerous fine lymphoid infiltrations in the livers of the male and female mice. In the livers of male mice numerous fine infiltrations were noted in the area of the central veins of the lobules in the portal tract and between hepatocytes (Fig. 2). In individual mice which were administered 1/2 LD_{50} of the preparation, infiltrations of individual mononuclear cells and parenchymatous degenerations of hepatocytes were observed (Fig. 3). Subcapsular clear stimulation of reticular-endothelial system was noted.

The submicroscopic structure of hepatocytes showed a considerable increase in the lipid-like bodies (L) of various sizes (Fig. 5). These changes were usually accompanied by an increased number of fine peroxysomes (P) (Fig. 6).

In the kidneys, after administration of higher dose of preparation a few infiltrations of mononuclear cells between the proximal tubules were noted (Fig. 4). In the cells of proximal tubules in the kidney an increase in the number and size of autophagous vacuoles (AV) was observed (Fig. 7), as well as a considerable accumulation of electron dense bodies (DB). In addition, a clear widening of the Golgi structures (G) was noted (Fig. 8).

DISCUSSION

Studies by Tulińska *et al.*, showed that supercypermethrin administered intragastrically to Wistar rats through 28 days in the dose 1/40 LD_{50} caused an increase in the humoral and cellular resistance, while the doses 1/20 and 1/14 LD_{50} had a suppressive effect [39]. According to the studies by Madsen, alpha-cypermethrin may cause an increase in the activity of the spleen cells NK [25]. Desi *et al.* [8], describes a suppressive effect of alpha-cypermethrin on the humoral and cellular response.

The present study shows that alpha-cypermethrin administered per os to Swiss mice in the doses 1/5 and 1/2

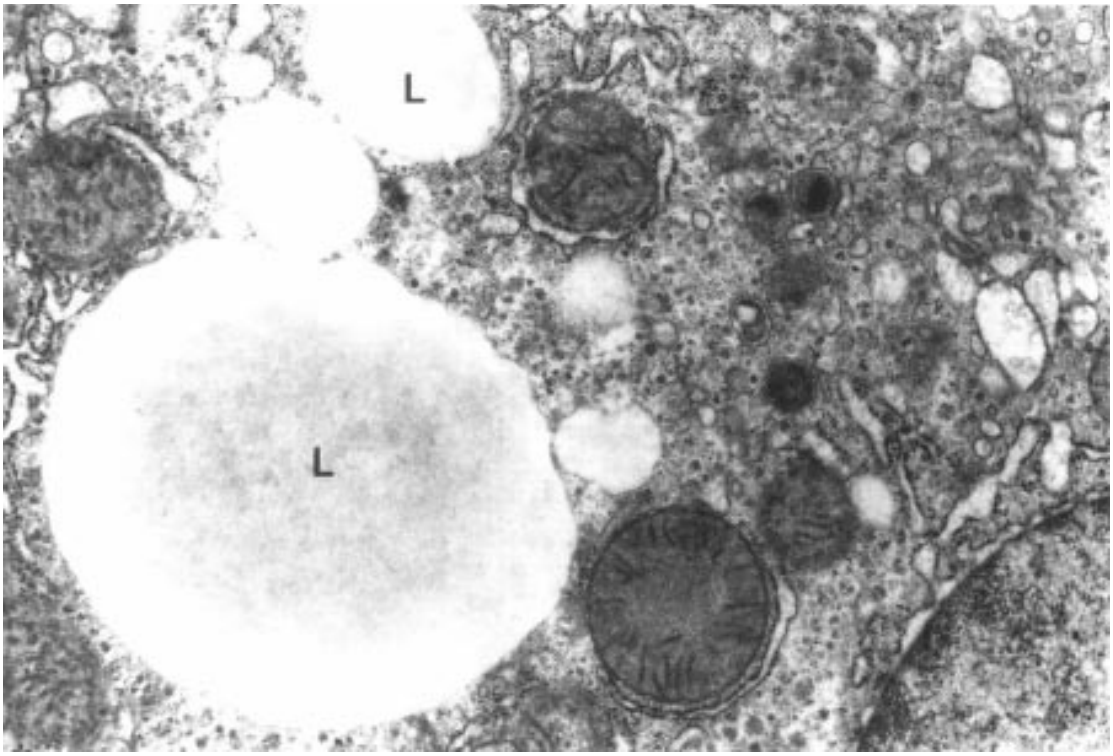

Figure 5. Liver of a mouse after per os application alpha-cypermethrin (1/2 LD_{50}). Considerable increase in the lipide-like bodies (L) of various sizes were noted. EM, × 18 000.

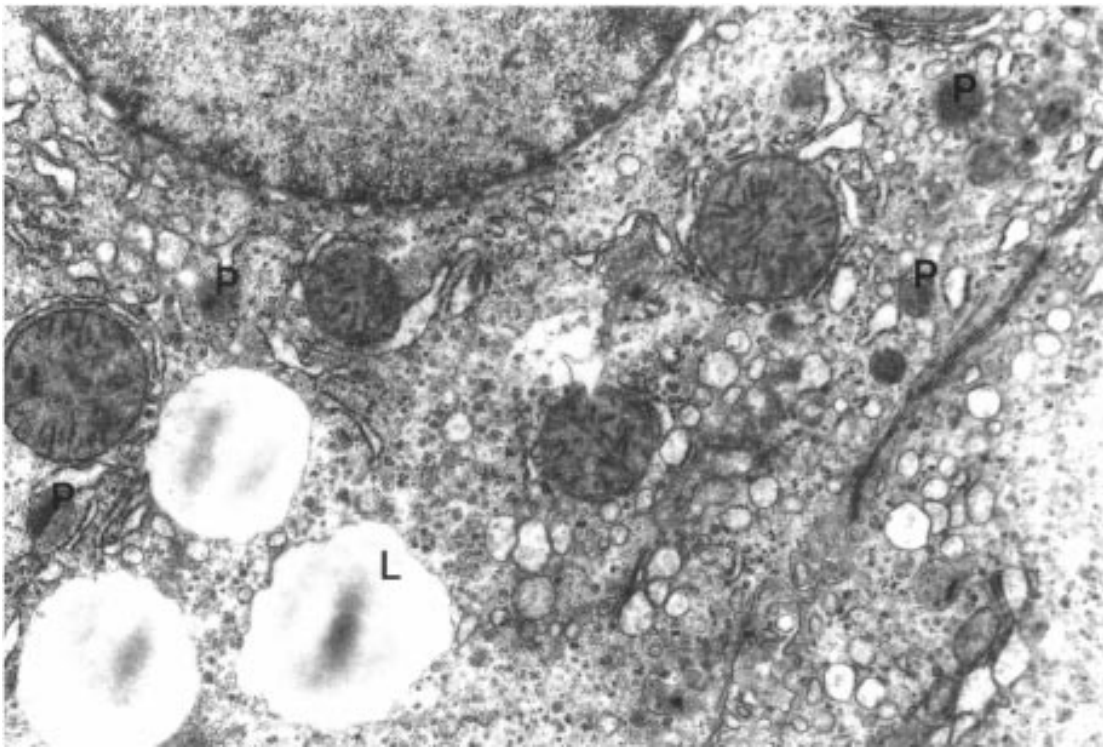

Figure 6. Liver of a mouse after per os application alpha-cypermethrin (1/2 LD_{50}). Increased number of peroxysomes (P) in some hepatocytes. EM, × 13 000.

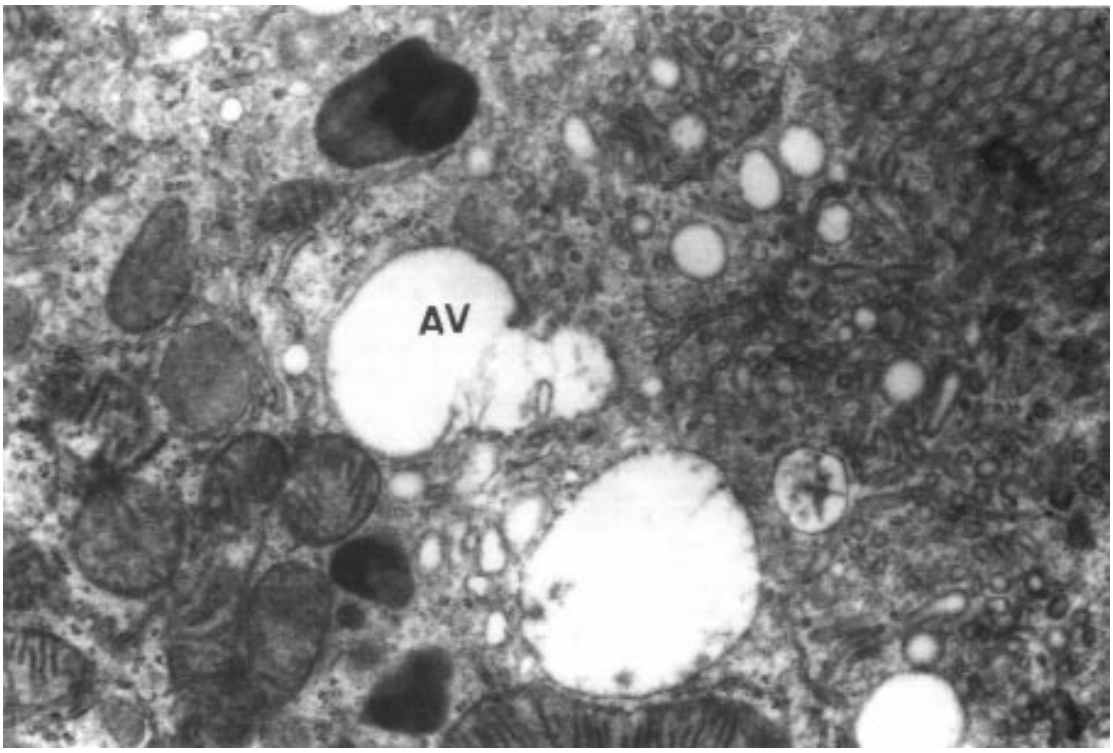

Figure 7. Kidney of a mouse after per os application alpha-cypermethrin (1/2 LD_{50}). In the cells of proximal tubules in the kidney an increase in the size of autophagous vacuoles (AV) was observed. EM, × 18 000.

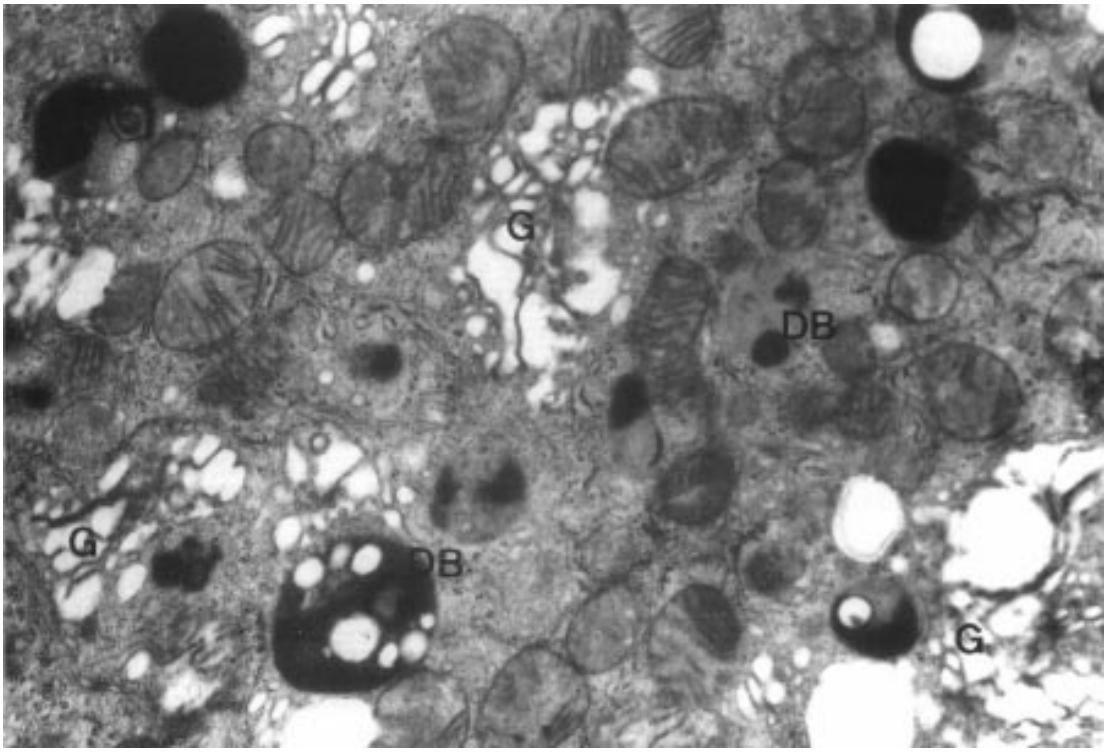

Figure 8. Kidney of a mouse after per os application alpha-cypermethrin (1/2 LD_{50}). A considerable accumulation of electron dense bodies (DB) and a strong widening of the Golgi structures (G) was noted. EM, × 13000.

LD_{50} for 28 days results in an increase in the phagocytic activity, while the phagocytic activity index for the dose 1/5 LD_{50} of the preparation is slightly higher than for the dose 1/2 LD_{50}. The alpha-cypermethrin doses applied resulted in a lower bactericidal activity in all experimental groups; however, the lowest NBT index, compared to the control group, was noted in female mice poisoned with 1/2 LD_{50} of the preparation.

Studies by Santoni *et al.* [32], indicate that the administration of cypermethrin during the prenatal period may affect the differentiation of thymocytes and impair their function. Prenatal cypermethrin exposure induced a significant decrease in the absolute number of all thymocyte subsets during the first 30 days after birth, with the double negative CD4- CD8-, single positive CD4 and CD8 T cells being preferentially affected. Later, on days 60 and 90, the double positive CD4+ CD8+ and single positive thymocytes gradually recovered, while the total number of CD4 CD8 cells was increased. Thymocytes from rats prenatally exposed to cypermethrin showed an impaired ability to proliferate in response to different doses of concanavalin A (Con A) and to produce and/or release IL-12 [32].

Young CD4 cells developing into multipotential Th0 cells in the later process of development become differentiated from Th1 or Th2. Numerous studies showed that IL-12 is one of the main agents inducing Th1 lymphocytes [29].

Based on the profile of lymphokines produced, two subsets of CD4+ helper lymphocytes, Th1 and Th2 have been described [1]. Th1 cytokines (IL-2, IFN-γ, TNF-α, TNF-β) favour delayed type hypersensitivity responses, whereas Th2 type cytokines (IL-4, IL-6, IL-10) favour antibody production and allergy associated responses [5].

Th1 response is significant in the elimination of virus infections and cancerous cells. Th2 type cytokines favour the development of effective defence mechanisms leading to the elimination of non-cellular pathogens [33]. The coordinating effect of IL-12 and IFN-γ is necessary for the development of young T CD4+ cells into Th1, while the presence of TGF-γ has an inhibitory effect on this process [6]. Receptors for the IL-12 heterodimer are found on activated CD4+ T cells, CD8+ T cells and NK cells [4, 7]. Mature DC cells are capable of loosely bonding the antigen and acquire an increasing ability to stimulate T cells, which enhances the IL-12 production [13, 24]. Snijders *et al.* found that in LPS-stimulated monocytes, p70 and p40 production is enhanced by IFN-γ and inhibited by IL-10 and IL-4 [34].

In the present study it has been found that in male mice poisoned per os with 1/5 LD_{50} alpha-cypermethrin a mobilization of the leukocytic system took place, manifested by a statistically significant increase in the number of leukocytes. This increase was particularly clear for monocytes and lymphocytes. In this group of animals, which possessed considerably more cells capable of IL-12 p70 synthesis, compared to the control group, the observed level of IL-12 p70 in blood plasma was, however, lower than that in the control group, 2.2 *vs* 3.3 pg/ml respectively.

In female mice administered alpha-cypermethrin per os the number of monocytes increased, compared to the control group - 147.2 and 93.8 respectively, while the level of IL-12 p70 in blood plasma was significantly lower, 0.2 *vs* 8.3 pg/ml respectively. The obtained results suggest that in Swiss mice poisoned with alpha-cypermethrin the release of IL-12 p70 into the blood plasma is inhibited.

Histologic studies confirmed the presence of lymphoid infiltrations in the liver and kidneys of animals administered both doses cypermethrin, as well as parenchymatous degeneration after higher dose. Similar histologic changes were observed in Wistar rats administered dermally a higher dose of alpha-cypermethrin (1/2 LD_{50}) [20].

CONCLUSIONS

1. Subacute poisoning of mice with alpha-cypermethrin in doses 1/2 LD_{50} (25 mg/kg b.m.) and 1/5 LD_{50} (10 mg/kg b.m.) results in decreased bactericidal activity of neutrophils.
2. Alpha-cypermethrin administered in the dose 1/5 LD_{50} has a stronger stimulatory effect on phagocytic activity than when administered in the dose 1/2 LD_{50}.
3. Activation of the leukocytic system, manifested by an increase in the number of monocytes and lymphocytes, is observed in male Swiss mice poisoned with 1/5 LD_{50} alpha-cypermethrin.
4. In subacute poisoning with alpha-cypermethrin lower levels of IL-12 p70 in the blood plasma are noted, compared to the control groups.
5. Histopathologic changes observed in the liver and kidneys have the form of microfocal lymphatic infiltrations and of parenchymatous degeneration of hepatocytes.
6. On the ultrastructural level, pathologic changes were noted in the liver and kidneys.

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