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Investigation of the toxicity of some organophosphorus pesticides in a repeated dose study in rats

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Abstract

The study aimed to the investigation of the toxicity of organophosphorus pesticides malathion (MLT) and diazinon (DZN) in Wistar rats in a repeated dose study for 35 days. MLT and DZN in corn oil vehicle were oral administered. Body and organs weights, plasma and brain cholinesterase activities, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities, histopathological changes in liver and kidney, and some parameters of the immune function, such as leukocyte formula, spleen weight and cellularity, spleen lymphocytes proliferation in response to concanavalin A (Con A) were investigated; the potential oxidative stress (malondialdehyde in plasma and brain, and blood catalase activity) was also evaluated. No clinical toxicity signs attributed to pesticides were noted; no significant changes in the organ weights have been found. Body weight tends slightly to increase, predominantly in DZN treated rats. The results suggest that plasma cholinesterase is more susceptible than brain cholinesterase to the inhibitory effect of DZN and MLT. Other serum biochemical parameters showed no significant difference. DZN produced a marked increase of the number of spleen lymphocytes without a significant gain of the relative spleen weight. The both pesticides produced an increase of the number of mononuclear cells/weight spleen. The splenic lymphocyte proliferation has not been influenced by MLT or DZN treatment. Histopathological observations identified some changes (vasodilatation, microvacuoles, and granular dystrophy) in the liver, with MLT, inducing macrovacuolar steatosis. The study indicates that repeated exposure, at subclinical doses, to organophosphorus MLT and DZN causes some biochemical, histopathological and immune alterations in rats.

Keywords: organophosphorus pesticides, repeated dose study, biochemical, histopathological and immune alterations.

Introduction

Organophosphorus pesticides act as esterases inhibitors, mainly acetylcholinesterase inhibitors, resulting in severe effects on central or peripheral nervous system. However, both short-term and long-term regulatory studies often reveal non-anticholinesterase effects, such as delayed polyneuropathy, immunotoxic effects, endocrine effects, hepatic injury, genotoxicity, and developmental neurochemical and neurobehavioral impairments.

Recent studies identified reactive oxygen species (ROS) as a cause of the toxic effects exerted by organophosphoric compounds. In subchronic study, MLT was shown to induce oxidative stress and hepatic injuries in rats [1]. Oral administration of MLT for one month in rats resulted in severe alterations of the hematological and biochemical parameters and the damage of the liver and kidney structures [2].

DZN was demonstrated to significantly enhance renal lipid peroxidation in rats, which is accompanied by a decrease in the activities of renal antioxidant enzymes and depletion in the level of reduced glutathione [3]. At high acute doses, DZN induces the production of free radicals and oxidative stress in various rat tissues (brain, heart, and spleen) by alteration of antioxidant enzyme activity, depletion of GSH, and increasing lipid peroxidation [4]. *In vitro* studies demonstrated that chlorpyrifos and diazinon induce lipid peroxidation in erythrocytes and changes of the antioxidant enzymatic systems [5, 6].

In vitro and *in vivo* studies suggest that some organophosphorus pesticides exert immunotoxic effects. *In vivo* animal studies suggest that, in general, low levels of the exposure to organophosphorus compounds seem to have stimulatory effect, while the exposure to high levels suppress the immune function. Suppression of the antibody production following *in vivo* exposure to large doses of malathion, parathion, dichlorphos and chlorfenvinphos was reported [7]. Chronic administration of DZN in mice at doses of 300 mg/kg food for a period of 45 days induced histopathological changes in spleen, thymus and lymph nodes [8]. Rodgers KE and

Ellefson DD [9] showed that at low, non-cholinergic doses, MLT modulate the immune function; consequently, the study indicated that MLT, orally administered to mice at a dose of 0.25 mg/kg, increased respiratory burst of the peritoneal macrophages. Another study demonstrated that oral or dermal administration of MLT at doses of 0.1 mg/kg body weight over a period of 90 days induced the mast cell degranulation and the increase of the macrophages function (measured as respiratory burst, phagocytic capacity and cathepsin D production) [10].

The present study was aimed to the investigation of the toxicity of organophosphorus pesticides MLT and DZN, in terms of biochemical changes, histopathological lesions, and immune answer in a repeated dose study in Wistar rats.

Most *in vivo* studies reported in the literature aimed mainly on the effects of the organophosphorus on the humoral and non-specific cellular immune response. In this context, we propose the investigation of some aspects related to the impact of organophosphorus MLT and DZN on the cellular immune response in the secondary lymphoid organs (spleen) in the experimental animal model of repeated dose study in Wistar rats.

Materials and Methods

The control and test groups were mixed, consisting of 10 animals, five males and five females, of 4–6-week-old. The animals were housed in individual cages, with a 12 hours light/dark cycle and free access to special rat food and water *ad libitum*. The temperature was maintained between 21–24^{0}C, while the humidity oscillated between 45–60%. All researches were conducted in accordance with the European Directive 86/609/EEC/24.11.1986, the European Convention on the Protection of Vertebrate Animals (2005) and the Romanian Government Ordinance No. 37/2002 regarding the protection of animals used for experimental and other scientific purposes [11, 12].

MLT and DZN in corn oil vehicle have been oral administered, every second day, at doses of 85 mg/kg body weight (b.w.) (MLT I group), 42.5 mg/kg b.w. (MLT II group), and 20 mg/kg b.w. (DZN group). Corn oil vehicle has been administered to the control group. Behavior, trends in body weight, any signs of cholinergic toxicity, plasma pseudocholinesterase activity (after 14 days treatment) were observed during the study. At the end of the experimental period (35 days), rats were anesthetized with ether and blood samples and organs (liver, kidney, brain, spleen) were collected. Spleens were aseptically removed, weighted to the nearest ±0.1 mg and placed in the sterile container containing 5 mL RPMI 1640 supplemented with antibiotic–antimycotic (Sigma; penicillin 10000 IU/mL, streptomycin 10000 IU/mL and amphotericin B 2.5 µg/mL). Serum AST and ALT activities have been measured using Beckman Coulter analyzer with Beckman Synchron LX® kits. The complete blood cell count was evaluated with CELL-DYN 1700 (Abbott Diagnostics) automatic system. Serum catalase activity was assayed by the decomposition of hydrogen peroxide by the method of Aebi H [13]. Plasma cholinesterase activity was measured by modified Ellman colorimetric methods using the kit Merck-1-Test Cholinesterase, and brain cholinesterase activity was assayed by the method of Voss G and Sachsse K [14]. Malondialdehyde was determined in plasma and brain by the colorimetric method using thiobarbituric acid [6]. Splenic lymphocytes proliferation in response to concanavalin A (Con A) was measured by the tritium-labeled uridine incorporation method, which detects RNA synthesis that requires uridine incorporation *via* the salvage pathway of nucleotide biosynthesis [15]. The isolation of the spleen cells and the assessment of the splenic lymphocytes proliferation have been performed as have been previously described [16]. The uridine incorporation is expressed in counts per minute (cpm).

Histopathology of the liver and kidney implied the Hematoxylin–Eosin (HE) staining for the light microscopy examination and was carried out at the end of the experiment, as it has been described [17]. The tissue samples were fixed in 10% formalin solution, and after 24 hours were passed in a series of graded ethanol, and embedded in paraffin. Paraffin sections were cut into 5-µm thick slices, and stained with HE for the light microscopic examination.

The experimental results were expressed as mean ± standard error (S.E.) for each parameter; statistical differences between the groups of animals were determined by Student *t*-test. Values of p less than 0.05 were regarded as statistically significant.

Results

No clinical toxicity signs attributed to pesticides were noted during the study.

Body and organ weights

DZN administration resulted in an increase of the body weight compared to control group (p_1=0.048), recorded both during the experiment (the periods ranging from the days 7–23 and 29–35) and at end of the study (Table 1).

Table 1 – ***The effects of DZN and MLT on the body and organ weight***

Animal group	Parameter (mean±SE; *n*=10)				
	Body weight [g]	*Liver weight*		*Kidney weight*	
		Absolute [g]	*Relative*	*Absolute [g]*	*Relative*
Control	228.3±19.01	7.07±0.175	0.0351±0.00201	1.89±0.104	0.0093±0.00061
DZN	289.12±28.25	9.12±0.56[a]	0.0327±0.00193	2.33±0.137[d]	0.0083±0.0005
MLT II	274.83±22.74	8.72±0.199[b]	0.0325±0.00216	2.33±0.06[e]	0.0086±0.00047
MLT I	281.12±20.85	8.91±0.387[c]	0.0322±0.00121	2.39±0.055[f]	0.0087±0.00044

[a] p_2=0.0081; [b] p_2=6.66×10^{-5}; [c] p_2=0.0015; [d] p_1=0.0112; [e] p_1=0.00193; [f] p_1=0.00066.

At the end of the experiment, weight gain was recorded for both groups of the rats treated with MLT, but it is statistically significant compared to the control group only for the MLT I group (p_1=0.040) (Table 1).

Oral administration of DZN and MLT resulted in the statistically significant increase of the absolute weight of the liver, while the relative weight (the ratio liver weight/body weight) was not influenced (Table 1). MLT, and at a lesser extent DZN, significantly increased the kidney weight. The ratio kidney weight/body weight was not affected.

Plasma and brain cholinesterase activities

After 14 days of experiment, as well as at the end of the study, plasma cholinesterase activity (pseudo-cholinesterase) was statistically significant reduced in all treated animal groups compared to the control rats (Table 2).

Table 2 – ***The effects of DZN and MLT on the plasma and brain cholinesterase activities***

Animal group	Cholinesterase activity [% of control]		
	Plasma		*Brain*
	After 14 days study	*At the end of the study*	*At the end of the study*
Control	100	100	100
DZN	26.85[a]	20.08[d]	38.38[g]
MLT II	62.51[b]	32.2[e]	57.02[h]
MLT I	34.01[c]	23.9[f]	43.59[i]

[a]p_2=0.00018; [b]p_2=0.0029; [c]p_2=0.00031; [d]p_2=0.0061; [e]p_2=0.0079; [f]p_2=0.0049; [g]p_2=0.0002; [h]p_2=0.00039; [i]p_2=0.0028.

However, no cholinergic signs of toxicity were observed. At the end of the study, a significant decrease, compared to the control, of the brain cholinesterase levels was observed for all animals groups, with more important reduction in the groups receiving DZN and the higher dose of MLT (Table 2, Figure 1).

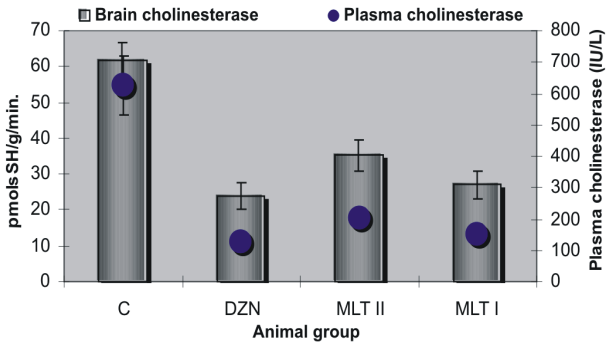

Figure 1 – ***The effects exerted in vivo by DZN and MLT on the plasma and brain cholinesterase activity (C – control group).***

In MLT II group, statistically significant differences compared to the rats receiving DZN were noticed. No correlations between the plasma and brain cholinesterase levels were found. The results suggest that MLT exert lower inhibitory effects; in addition, plasma cholinesterase is more susceptible than brain cholinesterase to the inhibitory action of DZN and MLT.

Other serum parameters

Serum AST and ALT activities, as well as serum catalase activity were not statistically significant influenced by the oral administration of DZN and MLT. There were no statistically significant differences in the plasma and brain malondialdehyde levels of the rats receiving pesticides and the control group (data not shown). These results indicate that, at the dosing regimen in this study, it is unlikely that DZN and MLT to induce oxidative stress and to affect the antioxidant systems.

Immunological parameters

The immunological parameters have been evaluated for DZN and MLT I groups (the higher dose).

Leukocyte formula

The experimental data on leukocyte formula (Table 3) show that the two pesticides induce a marked decrease in the percentage of the peripheral neutrophils compared to the control group (p_2=0.01 for MLT I group and p_2=0.02 for DZN group) (Figure 2). No significant changes of the lymphocytes and monocytes counts were registered (Table 3, Figure 3).

Table 3 – ***Experimental results on the in vivo investigation of the immunotoxic potential of DZN and MLT: the leukocyte formula***

Animal group	Parameter (mean±SE; *n*=10)		
	Lymphocytes	*Monocytes*	*Neutrophils*
Control	82.5±1.45	4.1±0.47	12.1±1.25
DZN	83.5±0.5	5.7±0.7	6.7±1.31*
MLT	85.7±1.84	5.3±0.91	7.2±0.47*

*Statistically different from the control, p<0.05.

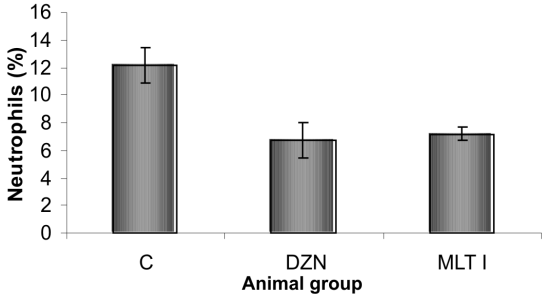

Figure 2 – ***The effects exerted in vivo by DZN and MLT on the neutrophils count [%] (C – control group).***

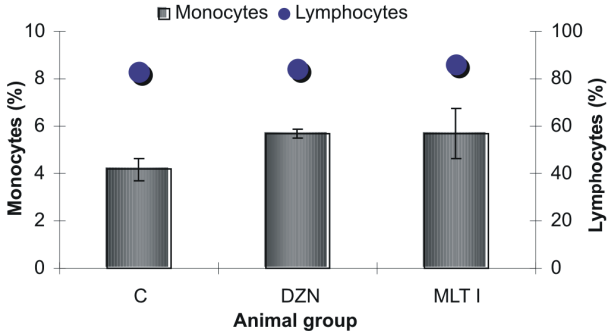

Figure 3 – ***The effects exerted in vivo by DZN and MLT on the monocytes and lymphocytes counts [%] (C – control group).***

Populating of the spleen with mononuclear cells

The results (Table 4) indicate the increase of the spleen weight, statistically significant for the MLT I (p_2=0.020) group compared to the control group (Figure 4).

The increase of the spleen weight is accompanied by an increase in the total number of mononuclear cells isolated from spleen (for DZN group, p_2=0.010, and for MLT group, p_1=0.033). DZN produced a marked increase of the number of splenic mononuclear cells without a significant increase of the spleen weight (Figure 4); DZN group is clearly different from the MLT I group (p_1=0.031).

These results suggested the analysis of a new parameter, the number of the mononuclear cells/spleen weight (Table 4, Figure 5), describing the populating of the spleen with immune cells. This parameter is significantly increased for DZN group compared with control group (p_2=0.005) and, to a lesser extent, for MLT I group (p_1=0.029). DZN group has a higher increase of the cellularity per spleen weight compared with MLT I group (p_2=0.040).

The increase of the parameter number of the mononuclear cells/spleen weight in DZN group is not accompanied by the increase of the relative spleen weight (the ratio spleen weight/body weight) (Figure 5).

Table 4 – ***Experimental results on the in vivo investigation of the immunotoxic potential of DZN and MLT on the secondary lymphoid organ (spleen)***

Animal group	Parameter (mean±SE; *n*=10)						
	Body weight [g]	*Spleen weight [g]*	*Spleen weight/body weight*	*No. of mononuclear cells (×10⁶)*	*No. of mononuclear cells/spleen weight*	*Proliferation (cpm, unstimulated)*	*Proliferation (cpm, stimulated, Con A)*
Control	228.3±19.01	0.46±0.036	2.07±0.12	29.2 ±3.81	65.01±8.83	13858±1550	12124±1112
DZN	289.12±28.25	0.63±0.074	2.29±0.08	136.5±17.4[a]	221.8±25.9[b]	13013±2658	11913±3071
MLT I	281.12±20.5	0.6±0.034[c]	2.24±0.14	79.8±17.4[d]	130.8±23.5[e]	16077±1867	13910±1855

[a]p=0.01; [b]p_2=0.005; [c]p_2=0.020; [d]p_1=0.033; [e]p_1=0.029.

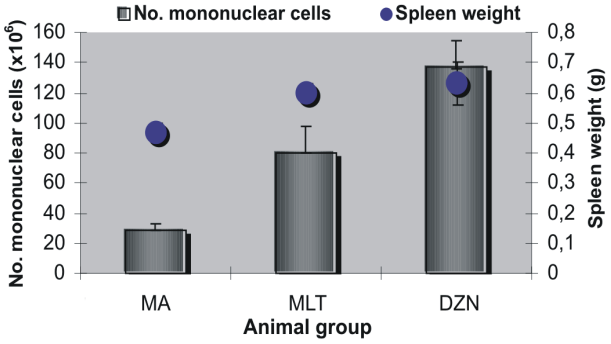

Figure 4 – ***The effects exerted in vivo by DZN and MLT on the parameters number of the mononuclear cells (×10⁶) and spleen weight [g] (C – control group).***

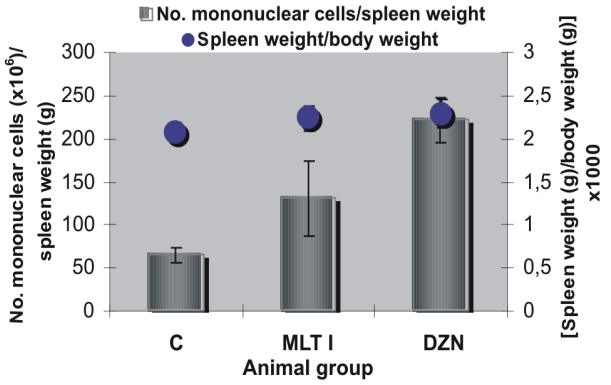

Figure 5 – ***The effects exerted in vivo by DZN and MLT on the parameters number of the mononuclear cells/spleen weight and spleen weight/body weight (C – control group).***

Proliferative capacity of the splenic mononuclear cells

The experimental data (Table 4, Figure 6) indicate that the treatment with MLT or DZN did not induce the suppression of the lymphocyte function; there are no statistically significant differences between the treated groups and control group in terms of the proliferative capacity of the splenic mononuclear cells mitogen activated (Con A).

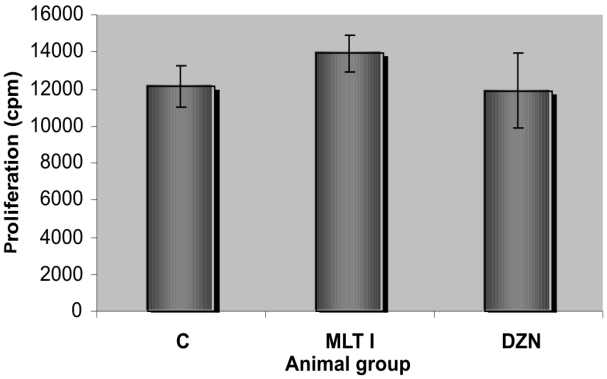

Figure 6 – ***The effects exerted in vivo by DZN and MLT on the proliferative capacity (counts per minutes, cpm) of the splenic mononuclear cells, activated ex vivo with concanavalin A (Con A) (C – control group).***

Histopathological observations

No gross-pathology of the liver and kidney was observed.

Histopathological changes in the liver

Liver sections of the control rats revealed no changes, while those of the rats treated with pesticides indicate discrete, moderate (reversible) to severe changes.

Liver damage was stronger at the higher doses of MLT, while in DZN group, mild and moderate injuries were found.

In MLT I group (the higher dose), the following

changes have been observed: three cases with moderate dystrophic lesions, with the prevalence of the macrovesicular steatosis (Figure 7) and pericentrilobular vasodilatation, one case with the ballooning and apoptotic nuclei; in the other four animals marked granulovacuolar dystrophy lesions were recorded. Only two animals were found with severe injuries such as necrosis (Figure 8).

The lower dose of MLT (42.5 mg/kg body weight) resulted in predominantly mild lesions (one case) and moderate (seven cases) with predominance of the pericentrilobular vasodilatation, microvesicular steatosis and pericentrilobular ballooning (Figure 9); one animal showed relatively normal aspects and in the other two animals severe damage, such as necrosis, were found.

Histopathological changes in the liver were predominantly mild to moderate in animals that received DZN. Given the incidence and the severity of injuries, we can say that DZN behaved like MLT at the low dose; two cases of discrete granular dystrophy, six cases of moderate vacuolar injuries and only two animals were observed with severe damage (necrosis).

Histopathological changes in the kidney

Histological examination of the kidney of control rats revealed normal histological features.

Various changes were observed in the kidney of the pesticides treated rats. It should be noted that there was no evidence of severe injuries such as necrosis in the kidney cells.

Examination of kidney sections from animals treated with MLT revealed a distinct aspect from the other two substances, since chronic interstitial inflammatory infiltrate (Figure 10) was observed in two animals in MLT I group (receiving the high dose) and one animal in the MLT II group (receiving the low dose). In MLT I group, the inflammatory infiltrate was accompanied by the moderate tubular dystrophic lesions. For both MLT doses, one case of moderate dystrophic lesions and the rest with slight dystrophic lesions were recorded.

DZN had a similar behavior in terms of the severity of renal lesions produced; only three cases with moderate dystrophic lesions (Figure 11) and seven cases with slight lesions were recorded.

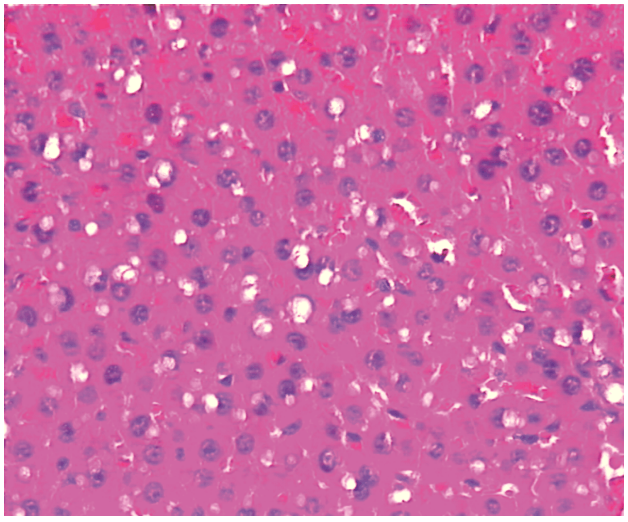

Figure 7 – ***Liver with macrovacuolar steatosis (MLT I group, HE staining, ob. 32×).***

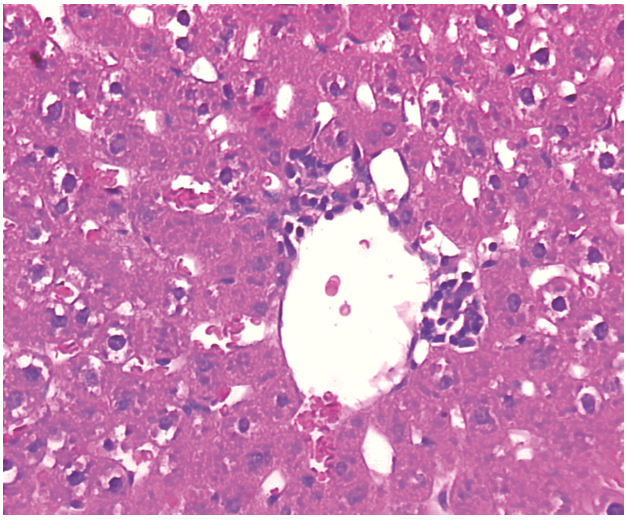

Figure 8 – ***Liver with necrosis and granulovacuolar dystrophy (MLT I group, HE staining, ob. 32×).***

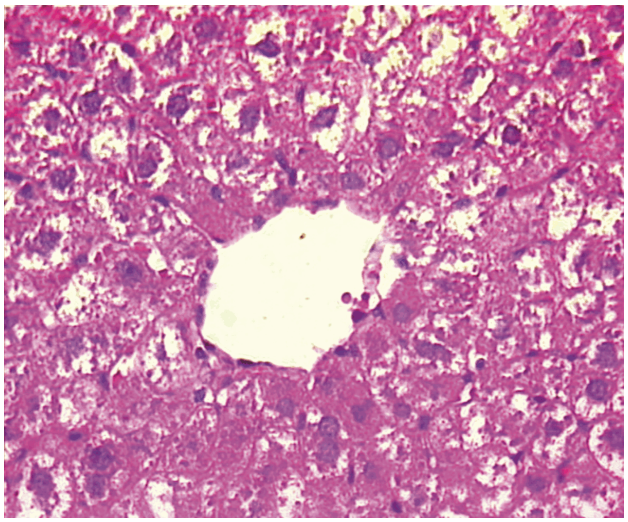

Figure 9 – ***Liver with pericentrilobular ballooning (MLT II group, HE staining, ob. 32×).***

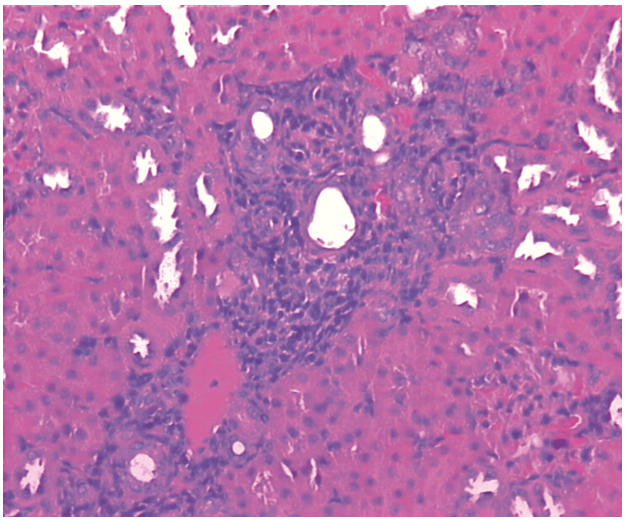

Figure 10 – ***Kidney with inflammatory interstitial infiltration (MLT I group, HE staining, ob. 25×).***

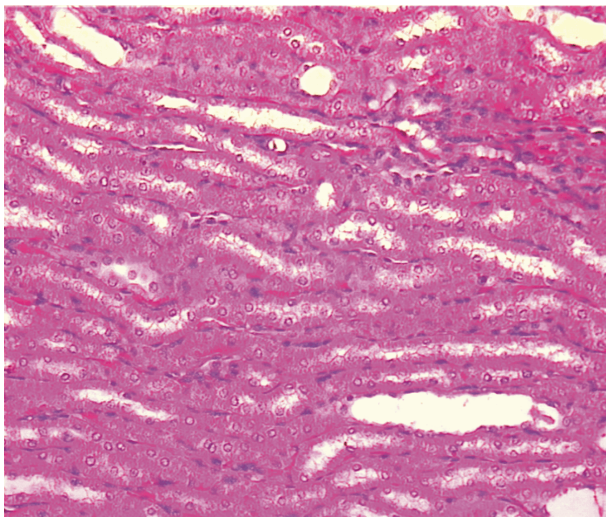

Figure 11 – ***Kidney with granulovacuolar dystrophy (DZN group, HE staining, ob. 20×).***

Discussion

This study aimed to the evaluation of the toxicological profile of two widely used organophosphate pesticides, DZN and MLT, in a repeated dose study in rats. Some relevant parameters for the immune function, such as leukocytes formula, spleen weight and cellularity, splenic lymphocyte proliferation in response to concanavalin A, were also assessed in the study. Therefore, the administered doses were selected so that not induce any acute signs of cholinergic toxicity, since some studies reported in the literature showed that the immunotoxic effects were caused by the cholinergic stress induced by organophosphorus compounds. The organophosphorus compounds were orally administered in doses equivalent to 1/16 of DL_{50} (DZN and MLT I groups) and 1/32 of DL_{50} (MLT II group).

The evolution of the body weight during the study was a parameter indicating the general clinical status of the animals, and, surprisingly, a trend of the increasing of the body weight was noticed for the both pesticides, but more pronounced in DZN group. The tendency of DZN to increase the body weight has been also reported in the literature in experiment on Sprague–Dawley rats at doses of 15 mg/kg b.w. [18]. The significance of the weight gain induced by the low oral doses of MLT and DZN is not clear, but may be associated with metabolic disturbances caused by the two pesticides. Recent research highlights the new insights into the metabolic abnormalities caused by early-life organophosphate (such as DZN and parathion) exposure and the interaction with other factors that are relevant to human obesity and diabetes [19].

While the organophoshates had no influence on the relative organ weights, the increase of the absolute liver weight was recorded. The increase of the liver weight after DZN administration in rats is reported in the literature in the experimental studies with doses similar to those in our study. Thus, dietary administration of DZN in Sprague–Dawley rats at doses equivalent to 15 mg/kg resulted in the increase of the absolute and relative liver weights, as well as the central liver hypertrophy [20]. On the other hand, dietary administration of MLT in mice, in doses of 21 mg/kg b.w. over a period of 3–21 weeks induced the increase of the relative liver weight [20]. The published data also indicate the increase in the absolute and relative kidney weight at doses of MLT of 359 mg/kg diet over a long period (two years) [21].

Although in our study, serum parameters, except for the pseudocholinesterase activity, were not significantly influenced by pesticides, there are some published studies that provide contrary evidence. The increase of the transaminases (AST and ALT) activities has been found in rats treated with DZN at doses of 1/30 LD_{50} for three weeks [22]. It was reported that DZN induced significant increases in the level of serum malondialdehyde and the activity of lactate dehydrogenase in female rats [23]. DZN was shown to induce significant increases in the level of MDA in rat brain [24]. MLT significantly increased MDA levels in the rat liver and kidney tissues [25].

Our results indicate some alterations in the leukocyte formula and in the populating of the spleens with mononuclear cells. Comparing the effects exerted by the pesticides on the concentrations of the peripheral leukocyte populations, a negative correlation between the number of granulocytes and lymphocytes was noticed, mainly for the rats in MLT I group (r=-0.957). These experimental data show the existence of the mechanisms regulating the population of the peripheral granulocytes and lymphocytes, so that the low concentrations of granulocytes are balanced by the increased levels of lymphocytes. The decrease of the nonspecific immunity mediated by granulocytes (at least as the number of these cells) is compensated by the intensification of the specific immune responses associated with lymphocyte by increasing the concentration of this peripheral leukocyte subpopulation.

DZN produced a marked increase of the number of spleen lymphocytes without a significant gain of the relative spleen weight. The both pesticides produced an increase of the number of mononuclear cells/weight of spleen. It is possible that DZN to trigger an immune response that causes recruitment of the mononuclear cells from the circulation into secondary lymphoid organs.

The proliferative capacity of the splenic mononuclear cells mitogen activated (Con A) was not affected by the pesticide treatment. The result confirms the observations reported for MLT by Johnson VJ *et al.* [26]; the authors showed that MLT oral administrated to the SJL/J females mice at doses of 0.018 to 180 mg/kg b.w. over a period of 28 days, alternating (a day dosing, a day pause) did not affect the blastogenesis of T-lymphocytes stimulated with Con A; in addition, MLT did not affect the ratios body organ weight/body weight.

Histopathological observations revealed some moderate to severe changes (vasodilatation, microvacuoles, and granular dystrophy) in the liver of the rats treated with pesticides. At the higher dose, MLT induced predominant macrovacuolar steatosis; severe injuries, such as necrosis, have been rarely observed. Moderate kidney injuries have been noticed (*i.e.*, granulovacuolar dystrophy). In the rats treated with MLT, a distinctive aspect in the

kidney was shown, namely, interstitial inflammatory infiltrate.

A number of literature data indicate various histopathological changes in rats caused by oral administration of organophosphate pesticides.

In a study for 13 weeks in Sprague–Dawley rats with DZN at doses of 250 and 2500 ppm in the diet (equivalent to 15 mg/kg b.w. and 168 mg/kg b.w.), hepatic centrilobular hypertrophy has been showed [18]. At 10–15% of the LD_{50} dose, DZN was reported to induce histopathological changes in the rat liver [27].

In Wistar rats, treated for 28 days with oral 1/50 of LD_{50} doses of MLT, histopathological changes in the liver, such as fine subcapsular infiltration and diffuse parenchymal degeneration, were observed in 80% of animals [28].

Our observation of the interstitial inflammatory infiltrate in the kidney induced by MLT is consistent with the results reported by Tós-Luty S *et al.* in a study in Wistar rats at MLT doses of 1/50 from LD_{50}, administered orally for 28 days [28]. In all animals, histopathological changes in the kidney, such as parenchymal degeneration, hyperemia and inflammatory infiltrate in the cortical area were observed.

Conclusions

The study indicates that repeated exposure, at subclinical doses, not inducing acute cholinergic toxicity signs, organophosphorus MLT and DZN cause some biochemical, histopathological and immune alterations in rats. The plasma pseudocholinesterase seems to be more susceptible than the brain cholinesterase to the inhibitory action of DZN and MLT. At the dose levels tested, MLT and DZN lowered granulocyte counts, indicating possible consequences in the unspecific immune defense. DZN may trigger an immune response resulting in the recruitment of the mononuclear cells into secondary lymphoid organs. Histopathological observations identified some changes, predominantly in the liver, while the kidney were moderate affected by the pesticide treatment.

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