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Effect of short-term exposure to dichlorvos on rat hepatocyte: molecular and histopathological approach

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Abstract: Background: Organophosphate (OP) insecticides are the most widely used in both agricultural and landscape pest control. The mortality and morbidity rate of OP poisoning is high. The aim of the present study is to investigate the effect of acute organophosphate exposure on hepatocyte and to examine caspase 1 and caspase-3 gene expression, and cell apoptosis related genes as p53, Tumor Necrosis Factor-alpha, Hypoxia Inducible Factor 1-alpha expression changes in rat hepatocyte.

Material and Methods: 10 adult Wistar Albino female rats weighing 250-300 g were divided into control (n=5) and experiment (n=5) groups. In experimental group, rats were treated 25 mg/kg of dichlorvos (Bayer DDVP EC 550, Bayer) in corn oil by 16 gauge oral gavage tube. In control group, rats were treated only 2.5 ml corn oil by oral gavage. After seven days, all of the rats were sacrificed by cervical dislocation under anesthesia. The liver was removed and divided into fragments. Hepatocyte density and histopathological examination were performed in fixed liver tissues. For this purpose, sections were taken and stained with hematoxylin-eosin. A part of the liver was used for gene expression analysis. Total RNA was extracted from the liver tissue using an RNA isolation reagent via manufacturer's instruction. Changes in mRNA levels, detected using semi-quantitative reverse transcription–polymerase chain reaction, were calculated as the proportion of the target gene amplification products to the amplification products of the housekeeping gene GAPDH.

Results: Hepatocyte density were decreased in experimental group compared to control group ($p<0.05$). The histopathological changes, such as portal inflammation and picnosis were observed in liver sections of experimental group. According to molecular genetics analysis, Caspase 1, Caspase 3, and p53 gene expression were increased in liver tissue after dichlorvos treated rats compared with the control group. There were no expression changes for TNF-Alpha and Hif1-Alpha gene expression level among groups.

Conclusion: Acute organophosphate exposure leads to loss in hepatocyte. Correlation with histopathological results, OP compound-induced cytotoxicity may be modulated through multiple sites including caspase1-3 pathway and also changes in the quantitative criteria of molecular markers of apoptosis in the rat hepatocytes on formation of behavioral skills were characterized by increased in caspase expressions in the hepatocyte.

Key Words: Organophosphate, intoxication, liver, hepatocyte density, apoptosis.

Organophosphate (OP) insecticides are phosphoric acid esters or thiophosphoric acid esters and are the most widely used in both agricultural and landscape pest control [1, 2]. These potent chemicals may harm people by accidental exposure, either during their application to crops, or due to incorrect or careless storage.

Another major source of human poisoning is through self-administration, when the easily available substances are used for suicide [3]. The mortality rate of OP poisoning is high.

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Most of OP compounds are highly lipid-soluble agents and are well absorbed from the skin, oral mucous membranes, conjunctiva and gastrointestinal and respiratory tracts [4]. The organophosphate insecticides have relatively short biological half-lives and are fairly rapidly metabolized and excreted [5].

The primary mechanism of action of OP insecticides is inhibition of carboxyl ester hydrolases, particularly acetylcholinesterase (AchE) that degrades the neurotransmitter acetylcholine (ACh) into choline and acetic acid [6,7]. However, recent studies showed that oxidative stress could be an important component of the mechanism of OP compounds toxicity including apoptotic cell death [4,8,9].

Dichlorvos (DDVP) is an OP compound that is used to control household, public health, and stored product insects. The EPA has classified it as toxicity class I-highly toxic. The oral LD50 for DDVP is 25 to 80 mg/kg in rats. DDVP is broken down rapidly in the liver [10].

Death of hepatocyte is a characteristic feature of liver diseases as diverse as cholestasis, ischemia/reperfusion, viral hepatitis, and drug/toxicant-induced injury. Apoptosis is a form of cell death as known programmed cell death. Apoptosis is a normal cell phenomenon which depends on the expression of genes capable of inducing or inhibiting this type of cell destruction. But apoptosis can also be triggered by many external factors and has been described in many diseases [11].

The very different conditions where programmed cell death occurs suggest that the mechanisms leading to the activation of apoptosis controlling genes are variable. Hepatocyte apoptosis can be triggered either in vivo or in vitro by many toxic agents. Apoptosis, the programmed death of cells is linked intimately with both physiology as well as pathology in variety of cellular systems. It may be either caspase dependent or caspase independent.

The key mediators of apoptosis are caspases, intracellular cysteine proteases that cleave various substrates including structural proteins. Apoptotic cells may be recognized by the genes and proteins that mediate the apoptotic process such as caspases and apoptosis related genes. Studies of these genes are essential, however, to elucidate their specific roles in the apoptotic processes [12,13].

For these purposes, in this present study we aimed to investigate the effects of short term dichlorvos exposure on hepatocyte and to analyze caspase 1 and caspase-3 gene expression, and cell apoptosis related genes as p53, Tumor Necrosis Factor-alpha (Tnf-alpha), Hypoxia Inducible Factor 1-alpha (Hif1-alpha) expression changes in rat hepatocyte.

Materials and methods

Animals

In our study, we used 10 adult Wistar Albino female rats weighing 250-300 g which obtained from Pamukkale University Experimental Animal Laboratory (Denizli, Turkey). This study was approved by the Pamukkale University Animal Ethics Committee (12th November 2007/056). The rats were placed into special cages with plastic at the bottom and wire at the top. Throughout the study, all rats were kept in room temperature (23±2°C), at 60±5% humidity, and in an environment with 12-hour light-darkness cycle and easily accessed food and water. The rats were randomly divided into control (n=5) and dichlorvos treatment (n=5) groups.

In dichlorvos treatment group, rats were treated 25 mg/kg of dichlorvos (Bayer DDVP EC 550, Bayer) in corn oil by 16 gauge oral gavage tube. In control group, rats were treated only 2.5 ml corn oil by oral gavage. At the end of seven days, all of the rats were sacrificed by cervical dislocation under anesthesia (via intramuscular injection of 5 mg/kg xylazine and 90 mg/kg ketamine). The livers were removed and separated into several minor fragments.

Histomorphological evalution

Several fragments of livers were fixed in 10% neutralise formaldehyde for 48 h at room temperature and embedded in paraffin (Sigma Co., St. Louis, MO, USA), according to standard procedures. Paraffin block of livers were cut in coronal plane of 5 µm thickness by the rotary microtome (Leica RM2125 RT, Germany) with disposable microtome blades (Feather C35, Germany). Obtained serial sections were stained with hematoxylin-eosin [14].

Estimation of the hepatocyte density

Microscopic images obtained from liver using x40 with a microscope (Nicon Eclipe E 600) were transferred to a monitor (Sony Trinitron Color Video Monitor PVM- 14N1MDE) using a video camera (Hitachi OSP Color Video Camera VK - C220E). The counting frame (20000 µm2) was randomly placed three times on the image at the monitor. The hepatocyte was counted within the counting frame, or touching the inclusion lines, and not touching the forbidden lines or their extensions.

The density of hepatocytes was estimated by counting cells within a defined disector volume (Vdis). The disector volume was calculated as the area of a counting frame [a(frame)] multiplied by the height of the dissector (h).

$$N_V = \left(\sum Q^-\right) / \left(\sum V_{\text{dis}}\right)$$

Further binucleated hepatocyte densities were estimated in the same disector volume [15,16].

Histopathological examinations were performed on the same sections at light microscopy.

RNA Isolation and Semi-quantitative RT–PCR Analyses

Several fragments of livers were immediately placed on an ice-cold glass stage. Total RNA was extracted from the tissues using an RNA isolation reagent, Tri-Reagent (Sigma, St. Louis, MO, USA). The single-tube one-step RT-PCR was standardized using the one-step RT-PCR kit (Qiagen, USA). Briefly, one-step RT-PCR was carried out in a 25-µL reaction mixture containing 1 µg total RNA, 10 pmol each primer, 5 µL 5X buffer (12.5 mM $MgCl_2$) 1 µL dNTPs mix (containing 10 mM of each dNTP), and 1 µL of a mixure of Ominiscript and Sensiscripts reverse transcriptases and Hot Star Taq DNA polymerase. The primer sequences used in this study and cycling conditions are summarized in Table 1. The RT-PCR products were analyzed by electrophoresis using 2% Molecular Screening Agarose gel (Roche Diagnostics, GmbH, Mannheim, Germany) and visualized by UV light.

Statistical Analysis

SPSS Version 10.0 for Windows was used for statistical analyses. Differences between the control and experimental groups were evaluated with Mann-Whitney U test. $P< 0,05$ was considered as statistically significant.

Results

The hepatocyte density and the binucleated hepatocyte density in each group were presented in table 1. The hepatocyte density was significantly decreased in dichlorvos treatment group compared to control group ($p<.05$, Mann Whitney U test). The binucleated hepatocyte density was significantly decreased in dichlorvos treatment group compared to control group ($p<.05$, Mann Whitney U test).

The histopathological changes in liver of experimental groups were shown in figure 1. Portal inflammation was seen in liver sections of all dichlorvos treated rats. Nuclear alterations such as picnosis were also seen in liver sections of all dichlorvos treated rats.

Table 1. Primers used for one-step RT-PCR.

Primer name	Sequence	Annealing temperature (°C)	Amplicon size (bp)
rCaspase1	F: 5’-CCACTCCTTGTTTCTCTC -3’	52	189
rCaspase1	R: 5’-CCTTCCTTGTATTCATGTC -3’		
rCaspase3	F: 5’- TGAGCATTGACACAATACAC-3’	52	349
rCaspase3	R: 5’- AAGCCGAAACTCTTCATC-3’		
rTNFalpha	F: 5’- TACTGAACTTCGGGGTGATTGGTCC-3’	63	295
rTNFalpha	R: 5’- CAGCCTTGTCCCTTGAAGAGAACC-3’		
rp53	F: 5’- GCACAAACACGCACCTCAAAGC-3’	57	494
rp53	R: 5’-CTTGCATTCTGGGACAGCCAAG-3’		
rHif1-alpha	F: 5’- CCACCGCAACTGCCACCACT -3’	57	392
rHif1-alpha	R: 5’- AGGGGCACGGTCACCTGGTT -3’		
rGAPDH	F: 5’TCATCTCCGCCCCTTCCGCT3’	57	549
rGAPDH	R: 5’GAGCAATGCCAGCCCCAGCA3’		

Table 1: Density of hepatocyte and binucleated hepatocyte in dichlorvos treatment and control groups.

	Hepatocytes density (mean± SEM)	Binucleated Hepatocytes density (mean± SEM)
Control group (n=5)	86,8 ± 3,34	6 ± 0,67
Dichlorvos treatment group (n= 5)	56,07 ± 1,64	2,56 ± 0,5

P<0.05; Mann-Whitney U test

Caspase 1, Caspase 3, TNF-Alpha, p53, and Hif1-Alpha mRNA expression in the Liver

The quality of RNA samples was confirmed by electrophoresis of RNA through a 2% agarose gel stained with ethidium bromide. The A260/A280 ratio was between 1.9 and 2.0. The effect of dichlorvos on all gene expression is shown in Figure 2. Changes in mRNA levels, detected using semi-quantitative reverse transcription–polymerase chain reaction (RT–

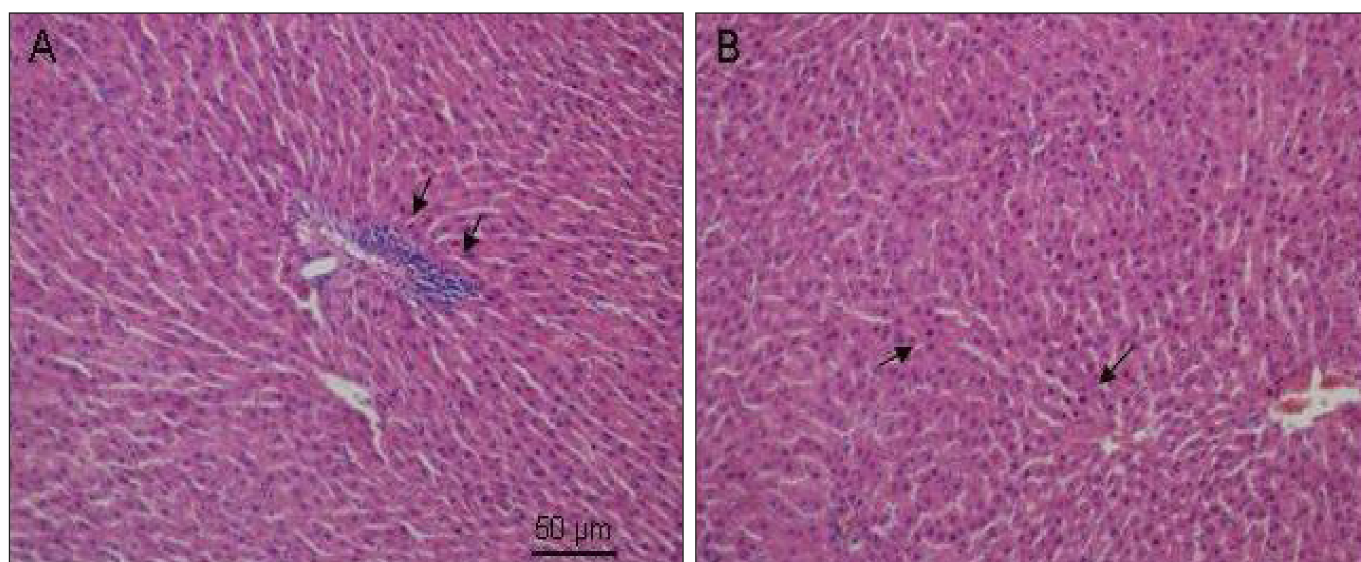

Figure 1. Liver sections of dichlorvos treatment group. Arrows indicate portal inflammation (A) and nuclear chromatin condensation 'pycnosis' (B). X20 magnification, stained with hematoxylin-eosin.

PCR), were calculated as the proportion of the target gene amplification products to the amplification products of the housekeeping gene GAPDH.

Caspase 1, Caspase 3, and p53 gene expression were increased in liver tissue after dichlorvos treated rats compared with the control group Figure 2. There were no expression changes for TNF-Alpha and Hif1-Alpha gene expression level among groups.

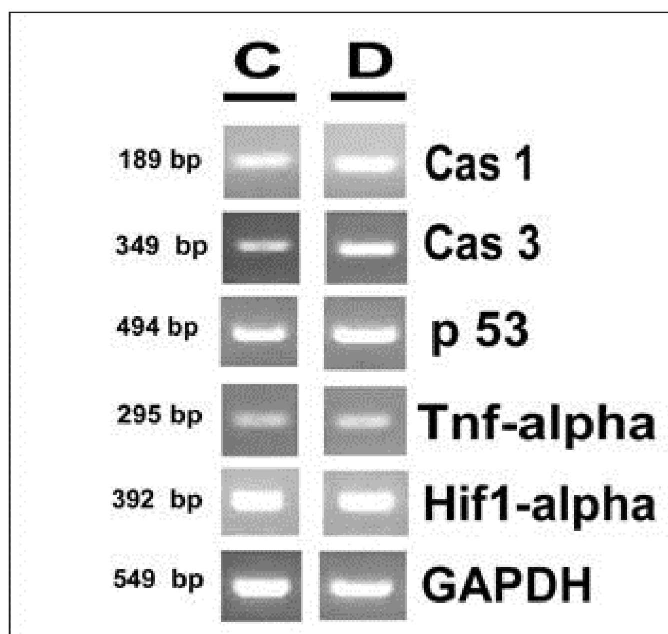

Figure 2. Expression Analysis of Caspase 1, Caspase 3, p53, Tnf-alpha and Hif1-alpha in liver tissues. C:Control group D: Dichlorvos treated group

Discussion

In general, OPs are neurotoxic in nature by acting as inhibitors of neuronal cholinesterase activity. However it has also been linked to liver damage. Some studies reported that exposure to organophosphate induced histopathological changes in liver, including infiltration in mononuclear cells at paranchymal tissue, sinusoidal dilatation, focal necrotic areas, granular degeneration and picnotic nuclei in the hepatocytes [6,17]. In the present study, portal inflammation and nuclear alterations, such as picnosis, were also seen.

Recent studies indicate that toxic manifestations induced by OPs may be associated with an enhanced production of reactive oxygen species and lipid peroxides [4,6,8,18,19]. Reactive oxygen species are included superoxide, hydrogen peroxide, and hydroxyl radical.

The major intracellular source of oxygen radicals is the mitochondrial electron transport chain where superoxide is produced by transfer of one electron to O2 from the stable semiquinone produced during reduction of ubiquinone by complexes I and II of the electron transport chain. Superoxide anion (02-), (either spontaneously or through a reaction catalysed by superoxide dismutases), produces hydrogen peroxide (H2O2), and O2. Glutathione peroxidase reduces hydrogen peroxide by converting to water or hydroxy lipits and in the process, glutathione is converted to oxidized glutathione.

Catalase converts hydrogen peroxide water and oxygen [20,21]. Hydroxyl radical is generated by Fenton reaction the most dangerous free radical as it is involved in reactions such as lipid peroxidation and generation of other toxic radicals including nitric

oxide (NO•). One significant consequence of lipid peroxidation is increased membrane permeability leading to an influx of Ca2+ and other ions with subsquent swelling of the cell. Similar increases in permeability of organelle membranes may also result in maldistribution of ions and result in intracellular damage. Accumulation of excessive amounts of Ca2+ in mitochondria may trigger apoptosis [20].

Kromer et al reported ROS generation is considered to be one of the key signals for oxidative stress-induced apoptosis [22]

It was shown organophasphate is targeted in mitochondria and reduced amount and activity of superoxid dismutase, glutathione peroxidase, and glutathion. Thus, reactive oxygen species (ROS) that can not be ineffective lead to peroxidation of cell membrane lipids, and a destruction nucleic acids, proteins and carbonhydrates [18].

Abou-Donia et al are also reported that other mechanism of action of organophosphates is apoptotic cell death and involve oxidative stress [9]. In present study, we have also shown decreased hepatocytes density in the organophosphate treated group compared with the control group. p53, Caspase 1 and Caspase 3 gene expression were increased in liver tissue after dichlorvos exposed rats.

Our results have shown that organophosphate may be caused p53 dependent apoptotic hepatocyte death. One of the most dramatic responses to p53 activation is the induction of apoptosis. In hepatocytes, as well as in many other cell types, apoptosis occurs through either one of two major pathways described as either the intrinsic mitochondrial or extrinsic death receptor pathway [23].

Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways [20,23]. Cells committed to die via p53-dependent apoptosis typically follow the mitochondrial pathway, although p53 can also modulate cell death through death receptors [23]. Both the extrinsic and the intrinsic pathways to apoptosis ultimately lead to cell shrinkage, chromatin condensation, nuclear fragmentation (which is frequently accompanied by internucleosomal DNA fragmentation), blebbing, and phosphatidylserine exposure on the surface of the plasma membrane [22].

Short term dichlorvos exposure leads to hepatocyte loss and histopathological changes in rat liver. Dichlorvos induced cytotoxicity may be modulated through multiple sites including caspase1-3 pathway and also changes in the quantitative criteria of molecular markers of apoptosis in the rat hepatocyte on formation of behavioral skills were characterized by increased in caspase expressions in the hepatocyte.

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