Caspase 1, Caspase 3, TNF-alpha, p53, and Hif1-alpha gene expression status of the brain tissues and hippocampal neuron loss in short-term dichlorvos exposed rats

**G. Nilufer Yonguc · Yavuz Dodurga ·
Ayse Kurtulus · Bora Boz · Kemalettin Acar**

Abstract Dichlorvos (DDVP) is an organophosphate compound that causes neurotoxicity. Apoptosis plays an important role in neurotoxic cell death in the brain. The aim of this study was to examine caspase 1, caspase-3 and also cell apoptosis related genes as p53, Tumor Necrosis Factor-alpha, Hypoxia Inducible Factor 1-alpha expressions in hippocampus, cerebellum, cortex, and to estimate total hippocampal neuron number in DDVP treated rats. Ten female albino rats were divided into control (n:5) and dose (n:5) groups. In dose group, single dose of DDVP (25 mg/kg) was administered to the animals via oral gavage. A week later, brains were removed and total neuron number was estimated in the left hippocampus using by optical fractionator method. The right part of the brain was used for gene expression analysis. In dose group, total hippocampal neuron number was significantly decreased compared to control group ($p = 0.008$). Caspase 1 and TNF-alpha gene expression were increased in all brain tissues and p53 gene expression was decreased in only hippocampus tissue in dose group. Short-term exposure to dichlorvos leads to neuronal loss in hippocampus and TNF-alpha rapidly and potently induces apoptosis and also several caspases as possible participants in the apoptotic cascade.

G. Nilufer Yonguc
Department of Anatomy, İzmir University School
of Medicine, İzmir, Turkey

Y. Dodurga (✉)
Department of Medical Biology, Pamukkale University
School of Medicine, Denizli, Turkey
e-mail: yavuzdodurga@gmail.com

A. Kurtulus · B. Boz · K. Acar
Department of Forensic Medicine, Pamukkale University
School of Medicine, Denizli, Turkey

Introduction

Organophosphates (OP) execute a high level of pesticides control ability combined with low degree of environmental toxicity. Among common pesticides, OPs are commonly used in medicine, industry, and agriculture [1]. Dichlorvos (DDVP or 2,2-dichlorovinyl dimethyl phosphate) is an OP pesticide, being used throughout the world to protect stored products and also as a public health insecticide. DDVP has been reported to use their primary toxicological and pharmacological effects through the inhibition of acetylcholinesterase (AChE) and also it has been required for the transmission of impulse across the cholinergic synapse [2]. The acute effects of DDVP due to AChE inhibition are well reported; although significant data gaps obtain about the adverse effects and the mediating biochemical events following chronic low level exposure to DDVP [3]. A mechanistic explanation of possible long-term neuro-behavioral consequences of low level exposure is largely lacking at present. The molecular mechanisms for the permanent signs underlying chronic intoxication induced by DDVP have not been elucidated yet. There are many areas concerning with chronic DDVP neurotoxicity other than AChE inhibition, which need to be discovered like their ability to induce apoptosis in brain at low level exposure.

Apoptosis is a form of programmed cell death and inappropriate apoptosis may contribute to various neurodegenerative disorders. Recent studies suggest that environmental toxic agents may contribute to the development of several neuronal disorders [4–6]. In the developing brain, changes of apoptosis after exposure to toxicants

could cause structural changes which lead to the developmental defects in brain functioning. Therefore, mechanistic studies with OP (including DDVP) induced apoptosis may provide new important information regarding the molecular basis of OP-induced neurodegeneration.

Brain is apart from the other body organs because of its high metabolic rate and near complete dependence on glucose for the maintenance of neural activity [7]. Even though the variety of death promoting stimuli, cell death is considered to occur by only two main mechanisms: necrosis and apoptosis, which can be separated by their characteristic morphological and biochemical situations. These features of apoptosis are observed in brain tissue from animal models, and while there is widespread necrosis occurring within the area of injury, apoptosis is also play an important role in neurotoxic cell death in the brain [8]. For instance; work with various animal models has shown that apoptosis plays an important role in neuronal death. The central feature of apoptosis is a cascade produced by several apoptosis-regulatory genes. Among these genes, cysteinyl aspartate specific protease (caspase) family genes are the most effective apoptotic regulators. Caspases are cysteine proteases that play a central role in apoptotic cell death. It may also be the case that the underlying complexities are associated with the morphological and biochemical variability of signs of apoptotic cell death in the brain and with the absence of any strictly specified criteria for identifying and detecting these signs. Thus, no studies haven't been studied involving the training of animals with simultaneous analysis of multiple measures of apoptosis in the mature brain in vivo, yet [9, 10].

The aim of the present preliminary study was to examine some of the molecular aspects of apoptosis in DDVP treated rats. For this purpose, we evaluated caspase 1 and caspase-3 gene expressions, and also cell apoptosis related genes as p53, tumor necrosis factor alpha (TNF-alpha), Hypoxia-inducible factor 1 (HIF1-alpha) expression in different parts of the brain as cerebellum, cortex, hippocampus, and to estimate total hippocampal neuron number (using the optical fractionator method) in DDVP treated rats.

Materials and methods

Dichlorvos was purchased from Bayer (DDVP EC 550, Bayer)

Animals and care

A total of 10 female adult Wistar Albino rats weighing 250–300 g (Pamukkale University Experimental Animal Laboratory, Denizli, Turkey) were housed in groups of five rats per cage in a room with a controlled temperature (23 ± 2 °C) and relative humidity (60 ± 5 %) with a 12-h light–dark cycle. Food and water were available ad libitum. This study was approved by the Pamukkale University Animal Ethics Committee (12nd November 2007/056).

Experimental design

The animals were divided into following two groups

Control group (*C*) In this group, animals received 2.5 ml corn oil via single oral gavage
Dichlorvos group (*D*) In this group, animals received 25 mg/kg dichlorvos (LD50 oral dose) dissolved in corn oil, via single oral gavage.

Estimation of the total number of neurons

A week later, all animals were sacrificed by cervical dislocation under anesthesia with Xylazine (10 mg/kg, i.p.) and Ketamine (90 mg/kg, i.p.). The brains were removed and frozen in a cryostat (Leica CM3050 S) at −50 °C. Frozen brains were cut in horizontal plane of 150 μm thickness at −15 °C in the cryostat. Sections stained with cresyl violet. Microscopic images obtained from pyramidal cell layers in CA1, CA2 and CA3 regions of the hippocampus using a microscope (Olympus CX31; 100× oil objective; Numerical Aperture = 1.25). The images were transferred to a monitor (Sony LCD monitor LMD-2010) using a video camera (Exwave HAD Color Video Camera SSc-DC88P).

The total pyramidal neuron number in the pyramidal cell layer of the left hippocampus CA1, CA2 and CA3 regions) was estimated using by the optical fractionator method—a stereological method (Fig. 1).
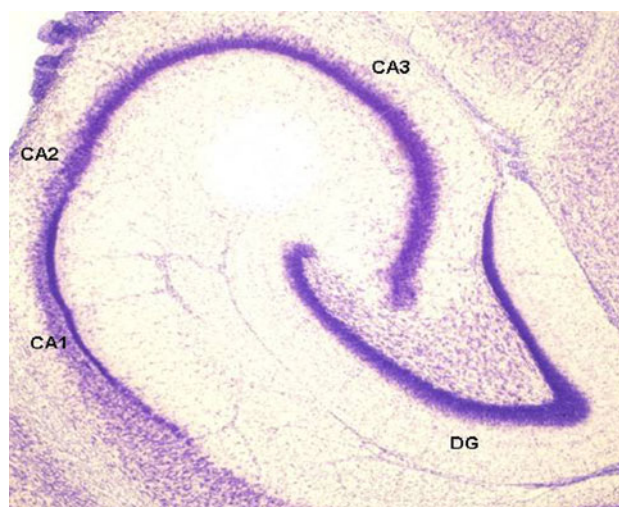
Fig. 1 A representative image for rat hippocampus CA1, CA2 and CA3. *CA1* Hippocampus CA1 region, *CA2* Hippocampus CA2 region, *CA3* Hippocampus CA3 region. *DG* Dentate Gyrus, Cresyl violet staining, ×4 magnification

This method includes the systematic random sampling strategy, the fractionator and optical dissector. The sampling scheme applied was the following: The first section in the series to be analyzed was chosen randomly from the first 3 sections. This section and every third section thereafter were stained with cresyl violet for use in the analysis. So section sampling fraction (ssf) is determined as 1/3.

Sectional area

In each of the sections to be sampled, neurons were counted with optical dissectors at regular predetermined x, y axis within the CA1, CA2 and CA3 regions. Stepping on microscopic images was performed according to Adiguzel et al. [11]. "x" step was predetermined 500 μm and "y" step was predetermined 50 μm. Microscopic images obtained from pyramidal cell layers in CA1, CA2 and CA3 regions of the left hippocampus using 100× oil objective (NA = 1.25) were transferred to a monitor. An unbiased counting frame (Gundersen's unbiased counting frame) was then superimposed on the monitor image of the section [12]. The area of the counting frame of the dissector, a (frame), was known (20 μm × 20 μm = 400 μm^2) relative to the area associated with each x, y movement, a (x, y step). Thus, the real sampling fraction (asf) = a (frame)/a (x,y step).

Section thickness

At each step in the pyramidal cell layers to be sampled the neuronal nuclei were first observed under the frame, and then the plane of focus was moved 5 μm into the section. The counting frame was then focused through 30 μm of the thickness of the section and the number of neuronal nuclei was counted with unbiased counting rules (Q^-). With the optical dissectors it is only necessary to determine the first recognizable profile of the nucleus to come into focus within the counting frame. The height of the dissector was 30 μm for this study (h = 30 μm).

At each step in the pyramidal cell layers to be sampled the distance between the positions of the stage where the neuronal nuclei of first layer came into focus from above and below the section (i.e., the top and bottom surfaces) was determined. The distance between the top and the bottom surfaces was measured. The mean thickness of the section was calculated for each of the section used in the analysis. Thus, the fraction of the section thickness sampled, here referred to as the thickness sampling fraction (tsf) = h (the height of the dissectors)/t (the mean of the section thickness).

Neurons were directly counted in a known fraction of CA1, CA2 and CA3 regions of the hippocampus. The total number of neurons in the CA1, CA2 and CA3 regions of the hippocampus (N) was estimated as [12, 13];

$$N = \Sigma Q^- \times (1/ssf) \times (1/asf) \times (1/tsf)$$

where ΣQ^- is the total number of neurons counted in the dissectors on the sampled sections, ssf is the section sampling fraction or the fraction of the sections sampled, asf is the areal sampling fraction is then a (frame)/a (x, y step), tsf is the section thickness sampling fraction is then h/t, h is the height of the disector (30 μm), t is the mean thickness of the sections

Coefficient of error (CE) was also estimated in this study as described in the literature [12].

Statistical analysis

Statistical analyses for neuron counts were performed using non-parametric Mann–Whitney U test. A p value < 0.05 was taken as statistically significant. Since random sampling method was used during neuron count, these results were expressed as mean ± standard error of the mean (SEM).

RNA isolation and semiquantitative RT–PCR analysis

All the animals in whole groups were anaesthetized via intramuscular injection of xylazine (5 mg/kg) and ketamine (90 mg/kg). Cerebellum, hippocampus, and cortex were then immediately isolated from the brain tissue on an ice-cold glass stage. For this purpose, right part of the brain was used in this method. 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 mixture of Ominiscript and Sensiscripts reverse transcriptases and Hot Star Taq DNA polymerase. Gene expression was presented as the yield of PCR products from target sequences relative to the yield of PCR products from the *GAPDH* gene. In each instance, the amount of reverse transcription (RT)-PCR product for the gene of interest was normalized to the amount of *GAPDH* in the same sample. The experiments were repeated two times using duplicates in each group. 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.
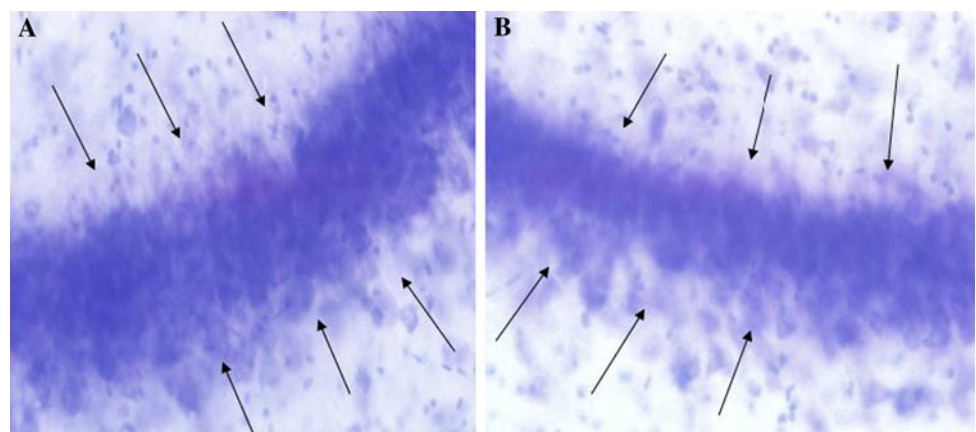
Table 1 Primers used for one-step RT-PCR

Primer name	Sequence	Annealing temperature (°C)	Amplicon size (bp)
rCaspase l	F: 5′-CCACTCCT TGTTTCTCTC-3′	52	189
rCaspase l	R: 5′-CCTTCCTTG TATTCATGTC-3′		
rCaspase 3	F: 5′-TGAGCATTGA CACAATACAC-3′	52	349
rCaspase 3	R: 5′-AAGCCGAA ACTCTTCATC-3′		
rTNF alpha	F: 5′-TACTGAACTTCGG GGTGATTGGTCC-3′	63	295
rTNF alpha	R: 5′-CAGCCTTGTCCC TTGAAGAGAACC-3′		
rp53	F: 5′-GCACAAACAC GCACCTCAAAGC-3′	57	494
rp53	R: 5′-CTTGCATTCT GGGACAGCCAAG-3′		
rHifl-alpha	F: 5′-CCACCGCAA CTGCCACCACT-3′	57	392
rHifl-alpha	R: 5′-AGGGGCAC GGTCACCTGGTT-3′		
rGAPDH	F: 5′-TCATCTCCGC CCCTTCCGCT-3′	57	549
rGAPDH	R: 5′-GAGCAATGCC AGCCCCAGCA-3′		

Results

Total number of pyramidal neurons changes

Pyramidal neurons were counted using by the optical fractionator method—a stereological method, which is believed to be a more unbiased, reliable, and effective method compared to non-stereological methods [12]. In addition, a "CE" value lower than 10 % is in acceptable range [14]. In our study, CE values were calculated for all animals. Mean CE values were below the 10 % for groups.
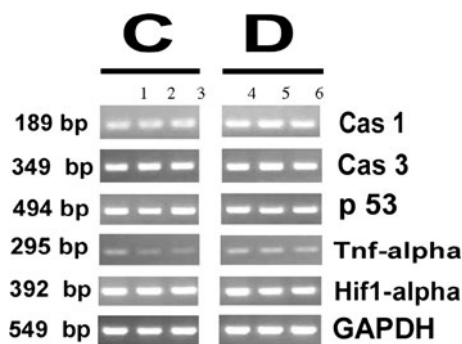
Analysis of the mean total pyramidal neuron number revealed a significant decrease in the Dichlorvos group (mean ± SEM = 219.726 ± 8.180) compared to the Control group (mean ± SEM = 354.461 ± 14.129; Mann–Whitney-U test, $p = 0.008$; Figs. 2, 3).

Caspase 1, Caspase 3, TNF-alpha, p53, and Hif1-alpha mRNA expressions in the cerebellum, cortex and hippocampus

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 Fig. 3. Changes in mRNA levels, detected using semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR), were calculated as the proportion of the target gene amplification products to the amplification products of the housekeeping gene (Glyceraldehyde-3-phosphate dehydrogenase) GAPDH. Caspase 1 and Tnf-alpha gene expression were increased in brain tissues and p53 gene expression was decreased in only hippocampus tissue after dichlorvos exposed rats than the control group
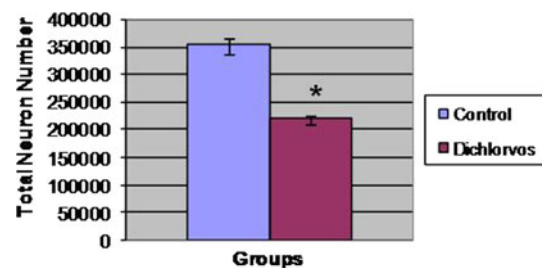
Fig. 3 Means of total hippocampal pyramidal neuron numbers in groups. Total neuron number was significantly decreased in dichlorvos group compared to control group (*$p = 0,008$; Mann–Whitney U)

Fig. 2 Photograph of the pyramidal cell layer in rat hippocampus stained with cresyl violet. *Arrows* indicate the borders of the pyramidal cell layer studied. **a** Control group, **b** dichlorvos group, ×40 magnification

Fig. 4 Expression analysis of Caspase 1, Caspase 3, p53, Tnf-alpha and Hif1-alpha in brain tissues

(Fig. 4). There were no expression changes in other genes among three brain tissues.

Discussion

In worldwide, OP compounds have a vital role in controlling home, industrial, agricultural, and public health pesticides. DDVP primarily acts by irreversibly inhibiting AChE at cholinergic junctions of the nervous system [15]. There is evidence suggesting that diverse stimuli can trigger programmed cell death (apoptosis) and the activation of the caspase family enzymes. Moreover, it is possible that separate pathways may be responsible for activating caspases or the other apoptosis related genes. The known mechanism of DDVP toxicity is the inhibition of cholinesterase activity resulting in blocking the hydrolysis of neurotransmitter acetylcholine at the central and peripheral neuronal synapses. This preliminary study has been examined the total hippocampal neuron number and gene expression changes of Caspase 1, Caspase 3, p53, TNF-alpha and HIF 1-alpha in DDVP rat model.

DDVP is taken into the human body very rapidly by the lungs, stomach, or skin. DDVP not only has toxic effects on mammals but also has toxic effects on fish, birds, honey bees, and non-target invertebrates [16]. Okamura was determined the oral LD_{50} dose of DDVP is 80 mg/kg for rats [17]. Oral et al. [18] gave 4 mg/kg DDVP orally to rats and observed endometrial damage. In the present study, dichlorvos was given 25 mg/kg at LD_{50} oral dose; however, no rats died during the experimental period.

The neuronal cell number of the pyramidal layer of the hippocampus has recently been estimated using the stereological methods. The results of our cell counting process by using optical fractionator method revealed loss of the pyramidal neurons in three subdivisions, as CA1, CA2, CA3, of the hippocampus after DDVP treatment. Our results showed that neuron number of hippocampus in

DDVP treated group decreased according to the control group ($p = 0.008$). These results agree with previous studies that reported OP toxicity in the brain region. There is no study to calculate neuron number in hippocampus with the optical fractionator method—a stereological method—in DDVP. The hippocampus is a suitable area to evaluate the effects of neurotoxic agents. There are some studies to support of our results that are evaluated showing neuronal damage in the brain due to different OP exposure with different methods [10, 19, 20]. These types of stereological studies are usually focused on the other various rat models. In support of this, there are studies showing neuronal loss in the hippocampus due to alcohol, copper, formaldehyde etc exposure [21]. These studies were showed the same results as hippocampus neuron number was reduced.

Programmed cell death is an important form of neuronal death modulated by a number of gene expression changes. There is evidence suggesting that diverse stimuli may trigger cell death and the activation of the caspase family enzymes. Additionally, it is possible that separate pathways may be responsible for activating the caspases. Previous studies suggest that small doses of OP (including DDVP) compounds cause neuronal cell death. And also OPs that cause mitochondrial damage, cause depletion of adenosine triphosphate (ATP) and increased generation of reactive oxygen species which results in oxidative stress [22]. Reactive oxygen species cause fatal depletion of mitochondrial energy (ATP), induction of proteolytic enzymes and DNA fragmentation, leading to apoptotic death. Kaur et al. [23, 24] have shown that DDVP induced ROS production triggers caspase dependent cell death in rat brain. In this preliminary study we have examined the expression of Caspase 1, Caspase 3 and apoptosis related genes as p53, Tnf-alpha and Hif1-alpha gene expression levels in dichlorvos rat model for the first time. In our experiment, DDVP exposure causes decreasing of the number of hippocampal neurons. In the fatal DDVP exposure, the optical fractionator method—a stereological method—diagnosis to determine the neuron loss of hippocampus.

According to molecular analysis, caspase 1 and TNF-alpha gene expressions were increased in cerebellum, cortex and hippocampus, and p53 gene expression was decreased in only hippocampus tissue after DDVP exposed rats. Therefore, cell apoptosis may play an important role in the regulation of cell quantities within the brain.

Finally, we think that TNF-α rapidly and potently induces apoptosis and also several caspases as possible participants in the apoptotic cascade. Also present preliminary study will be the first research for correlating with molecular genetics approaches and stereology and we think that this article will contribute to further studies for new researches.

References

1. Storm JE, Rozman KK, Doull J (2000) Occupational exposure limits for 30 organophosphate pesticides based on inhibition of red blood cell acetylcholinesterase. Toxicology 150(1–3):1–29

2. Kobayashi H, Yuyama A, Chiba K (1986) Cholinergic system of brain tissue in rats poisoned with the organophosphate, 0,0-dimethyl 0-(2,2-dichlorovinyl) phosphate. Toxicol Appl Pharmacol 82(1):32–39

3. Balali-Mood M, Shariat M (1998) Treatment of organophosphate poisoning. Experience of nerve agents and acute pesticide poisoning on the effects of oximes. J Physiol Paris 92(5–6):375–378

4. Giasson BI, Lee VM (2000) A new link between pesticides and Parkinson's disease. Nat Neurosci 3(12):1227–1228

5. Levin ED, Addy N, Baruah A et al (1995) Prenatal chlorpyrifos exposure in rats causes persistent behavioral alterations. Neurotoxicol Teratol 24(6):733–741

6. Terry AV Jr, Stone JD, Buccafusco JJ et al (2003) Repeated exposures to subthreshold doses of chlorpyrifos in rats: hippocampal damage, impaired axonal transport, and deficits in spatial learning. J Pharmacol Exp Ther 305(1):375–384

7. Kanatani T, Mizuno K, Okada Y (1995) Effects of glycolytic metabolites on preservation of high energy phosphate level and synaptic transmission in the granule cells of guinea pig hippocampal slices. Experientia 51(3):213–216

8. Li Y, Chopp M, Jiang N et al (1995) Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 15(3):389–397

9. Reed JC (2000) Mechanisms of apoptosis. Am J Pathol 157(5):1415–1430

10. Graeber MB, Moran LB (2002) Mechanisms of cell death in neurodegenerative diseases: fashion, fiction, and facts. Brain Pathol 12(3):385–390

11. Adiguzel E, Duzcan SE, Akdoggan I et al (2003) A simple low-cost method for two dimensional microscopic measuring and stepping on the microscopic plate. Neuroanatomy 2:6–8

12. West MJ, Slomianka L, Gundersen HJ (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Rec 231(4):482–497

13. Hosseini-Sharifabad M, Nyengaard JR (2007) Design-based estimation of neuronal number and individual neuronal volume in the rat hippocampus. J Neurosci Methods 162(1–2):206–214

14. Gundersen HJ, Jensen EB (1987) The efficiency of systematic sampling in stereology and its prediction. J Microsc 147(Pt 3):229–263

15. Petroianu GA, Hasan MY, Nurulain SM et al (2006) Ranitidine in acute high-dose organophosphate exposure in rats: effect of the time-point of administration and comparison with pyridostigmine. Basic Clin Pharmacol Toxicol 99(4):312–316

16. Ural MS, Koprucu SS (2006) Acute toxicity of dichlorvos on fingerling European catfish, Silurus glanis. Bull Environ Contam Toxicol 76(5):871–876

17. Okamura A, Kamijima M, Shibata E et al (2005) A comprehensive evaluation of the testicular toxicity of dichlorvos in Wistar rats. Toxicology 213(1–2):129–137

18. Oral B, Guney M, Demirin H et al (2006) Endometrial damage and apoptosis in rats induced by dichlorvos and ameliorating effect of antioxidant vitamins E and C. Reprod Toxicol 22(4):783–790

19. Grady MS, Charleston JS, Maris D et al (2003) Neuronal and glial cell number in the hippocampus after experimental traumatic brain injury: analysis by stereological estimation. J Neurotrauma 20(10):929–941

20. Binukumar BK, Bal A, Kandimalla RJ et al (2010) Nigrostriatal neuronal death following chronic dichlorvos exposure: crosstalk between mitochondrial impairments α synuclein aggregation, oxidative damage and behavioral changes. Mol Brain 3:35

21. Miki T, Harris SJ, Wilce PA et al (2004) Effects of age and alcohol exposure during early life on pyramidal cell numbers in the CA1-CA3 region of the rat hippocampus. Hippocampus 14(1):124–134

22. Murphy AN, Fiskum G, Beal MF (1999) Mitochondria in neurodegeneration: bioenergetic function in cell life and death. J Cereb Blood Flow Metab 19(3):231–245

23. Gupta RC, Milatovic D, Dettbarn WD (2001) Depletion of energy metabolites following acetylcholinesterase inhibitor-induced status epilepticus: protection by antioxidants. Neurotoxicology 22(2):271–282

24. Kaur P, Radotra B, Minz RW, Gill KD et al (2007) Impaired mitochondrial energy metabolism and neuronal apoptotic cell death after chronic dichlorvos (OP) exposure in rat brain. Neurotoxicology 28(6):1208–1219