

Detection of borderline dosage of malathion intoxication in a rat's brain

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Abstract. – OBJECTIVE: Humans and other animals are liable to expose to low doses of malathion (MAL). However, experimental studies on its toxic threshold dose and toxic low-dose effects have not been conducted. The aims of this study were to detect the initiation of the toxic effects of sub-acute low doses (2.5, 5, and 10 mg/kg) of MAL by immunohistochemical and biochemical parameters in rat brain.

MATERIALS AND METHODS: Twenty-eight rats were randomly assigned into four groups (n=7) including control and three different amounts of MAL-exposed groups (2.5, 5, and 10 mg/kg).

RESULTS: On immunohistochemical examination, the number of caspase-3-positive cells in all MAL-exposed groups was significantly higher than in the control group. Consistent with this, the total antioxidant capacity, total oxidant status, and the levels of superoxide dismutase, malondialdehyde, and paraoxanase activity were significantly different in the 5 and 10 mg/kg MAL-exposed groups compared with the control group. Additionally, the total oxidant status and malondialdehyde levels were significantly higher in the 5 and 10 mg/kg MAL-exposed groups compared with those in the 2.5 mg/kg MAL-exposed group.

CONCLUSIONS: Our results indicate that over 5 mg/kg MAL exposure may result in dose-dependent oxidative stress, increased caspase-3 activity, and launching to the toxic effects in rat brain.

Key Words:

Malathion, Its toxic dosage, Oxidative stress, Rat brain.

control programs such as mosquito, lice and antieradication¹. Therefore, it is the most commonly used organophosphate agent in many fields of most countries. This also leads to exposure with increasing extent and causes the toxicity at different degrees. Even though commonly named as a pesticide, it is still regarded as a therapeutic drug for pediculosis in medicine. This vast consumption gives rise to environmental pollutions due to soil and groundwater contamination. Thus, organophosphates, including MAL remain a serious health hazard².

Incidental or intentional MAL intoxication with an increased mortality rate is a relevant health problem especially in developing countries. The widespread use of MAL may cause acute or chronic toxic effects from high- or low-level exposure^{3,4}. To the best of our knowledge, no work has been yet done to find out its toxic effective doses with regard to caspase-3 activity and biochemical parameters in rat brain. Therefore, this study was planned to investigate the sub-acute toxic effects of low doses of MAL (2.5, 5, or 10 mg/kg, orally and daily for 28 days) using both various biochemical and immunohistochemical parameters.

Materials and Methods

The Ethics Committee performed all procedures used in this study after approval of the protocol. A total of 28 mature female Wistar rats (250±30 g) were randomly assigned into four groups including control and MAL groups (2.5, 5, and 10 mg/kg MAL), and each was comprised of seven animals. MAL (#91481; Fluka, Sigma-

Introduction

Malathion is an organophosphate parasympathomimetic drug, which effects by binding irreversibly to cholinesterase. It is widely used as a pesticide in agriculture, residential landscaping, public recreation areas, and in public health pest

Aldrich Chemie GmbH, Munich, Germany) was administered orally and daily for 28 days. The rats were anesthetized by intraperitoneal (50 mg/kg ketamine and 10 mg/kg xylazine) injection and were sacrificed by decapitation 24 h after the last administration of MAL. Tissue samples were collected from each animal's brains. A portion of the brain tissue was removed at 4°C, washed with ice-cold saline, and stored immediately at -50°C for further biochemical analysis. The remaining brain tissues were placed in 10% formalin for 72 h for pathological examination.

Biochemical Analyses

The excised brain tissue samples were weighed and immediately stored at -50°C. The tissues were homogenized in five volumes of ice-cold 1.15% KCl (w/v). Assays were performed on the supernatant of a homogenate that was prepared by centrifugation at 14,000 rpm for 30 min at 4°C. The protein concentration of the tissues was measured using the Lowry et al⁵ method. SOD activity was measured according to the method described by Fridovich⁶. The lipid peroxidation level in the cerebrum is expressed as the MDA level and was measured by the method of Ohkawa⁷. PON-1 activity was measured spectrophotometrically using the modified Eckerson method⁸. The TAC and TOS of the supernatant fractions were evaluated using a novel automated and colorimetric measurement method developed by Erel^{9,10}. The results are expressed as nmol Trolox equivalent/mgprotein.

Histopathological and Immunohistochemical Analyses

Coronal brain sections from the fronto-temporal region were prepared to assess the level of caspase-3 activity using an immunohistochemical staining procedure. During the this examination, the number of caspase-3-positive brain tissue cells without vascular structures was counted in ten randomly selected microscopic fields for each rat (five gray matter fields and five white matter fields), and the average number of stained cells was calculated for statistical analysis. Due to a possible aberrant staining, the vascular structures were excluded from analysis^{11,12}.

Statistical Analysis

The normality of the distribution was tested using the Kolmogorov-Smirnov test. Because the distributions were normal, they were analyzed by one-way ANOVA with a least-squares difference post-hoc test. The data are expressed as the mean $\pm$ standard deviation. A p -value < 0.05 was considered to be statistically significant.

Results

Biochemical Results

As shown in Table I, SOD and TAC activity was not significantly decreased in the 2.5 mg/kg MAL-exposed group compared with the control group ($p > 0.05$). The TOS and MDA levels in rat brain were not significantly in-

Table I. Biochemical and immunohistochemical results (mean $\pm$ Standard deviation)

Groups	TAC (mmol Trolox Eq./g protein)	TOS (mmol H ₂ O ₂ Eq./g protein)	PON-1 (U/L)	SOD (U/gr protein)	MDA (nmol/gr protein)	Caspase-3 positive cell numbers (meanvalue/ 10HPF)
Control (I)	0.56 $\pm$ 0.07	153.9 $\pm$ 31.9	15.3 $\pm$ 3.1	3.72 $\pm$ 0.63	252.3 $\pm$ 23.6	12.8 $\pm$ 2.1
Malathion 2.5 mg/kg (II)	0.47 $\pm$ 0.17	168.7 $\pm$ 46.5	11.4 $\pm$ 2.7	2.73 $\pm$ 0.75	308.7 $\pm$ 72.1	21.2 $\pm$ 2.9
Malathion 5 mg/kg (III)	0.41 $\pm$ 0.07	198.8 $\pm$ 25.0	10.2 $\pm$ 2.2	2.19 $\pm$ 1.55	384.7 $\pm$ 79.6	29.5 $\pm$ 3.6
Malathion 10 mg/kg (IV)	0.37 $\pm$ 0.07	372.3 $\pm$ 49.8	8.7 $\pm$ 1.6	1.80 $\pm$ 0.71	443.2 $\pm$ 68.5	33.6 $\pm$ 4.2
<i>p</i> values						
I – II	0.16	0.49	0.006*	0.071	0.12	0.001*
I – III	0.017*	0.045*	0.001*	0.008*	0.001*	0.001*
I – IV	0.003*	0.001*	0.001*	0.001*	0.001*	0.001*
II – III	0.28	0.17	0.38	0.32	0.038*	0.001*
III – IV	0.50	0.001*	0.28	0.47	0.10	0.028*
II – IV	0.086	0.001*	0.058	0.093	0.001*	0.001*

*Statistically significant values ($p < 0.05$); TOS: total oxidant status; TAC: total antioxidant capacity; MDA: malondialdehyde; SOD: superoxide dismutase; PON-1: paraoxonase-1; HPF: high power field.

creased in the 2.5 mg/kg MAL-exposed group compared with the control group ($p > 0.05$). However, the decreased levels of SOD and TAC, and increased levels of TOS and MDA, were statistically significant ($p > 0.05$) in the 5 and 10 mg/kg MAL-exposed groups compared with the control group. Additionally, PON-1 activity was significantly decreased in the 2.5, 5, and 10 mg/kg MAL-exposed groups compared to the control group ($p > 0.05$).

Histopathological and Immunohistochemical Results

On microscopic examination, the H&E-stained brain tissues showed no noticeable pathological changes in and between the groups. However, an immunohistochemically significant increase in the number of caspase-3-positive brain cells in the gray and white matter of the MAL-exposed

tissues was revealed compared with the controls (Figure 1a-d). The caspase-3-positive cell numbers in the brain tissues of the groups are demonstrated in Table I. Accordingly, compared with the control group, the 2.5, 5, and 10 mg/kg MAL-exposed groups showed a significant and dose-dependent increase in the number of caspase-3-positive brain cells ($p = 0.001$ for each).

Discussion

Recent studies regarding MAL toxicity have focused on chronic intoxication and environmental contamination. Some diseases arising from the pesticides appear its effects in long-term and delayed health problems in agricultural workers and in populations exposed to environmental sources¹³⁻¹⁵. To understand the pathophysiology

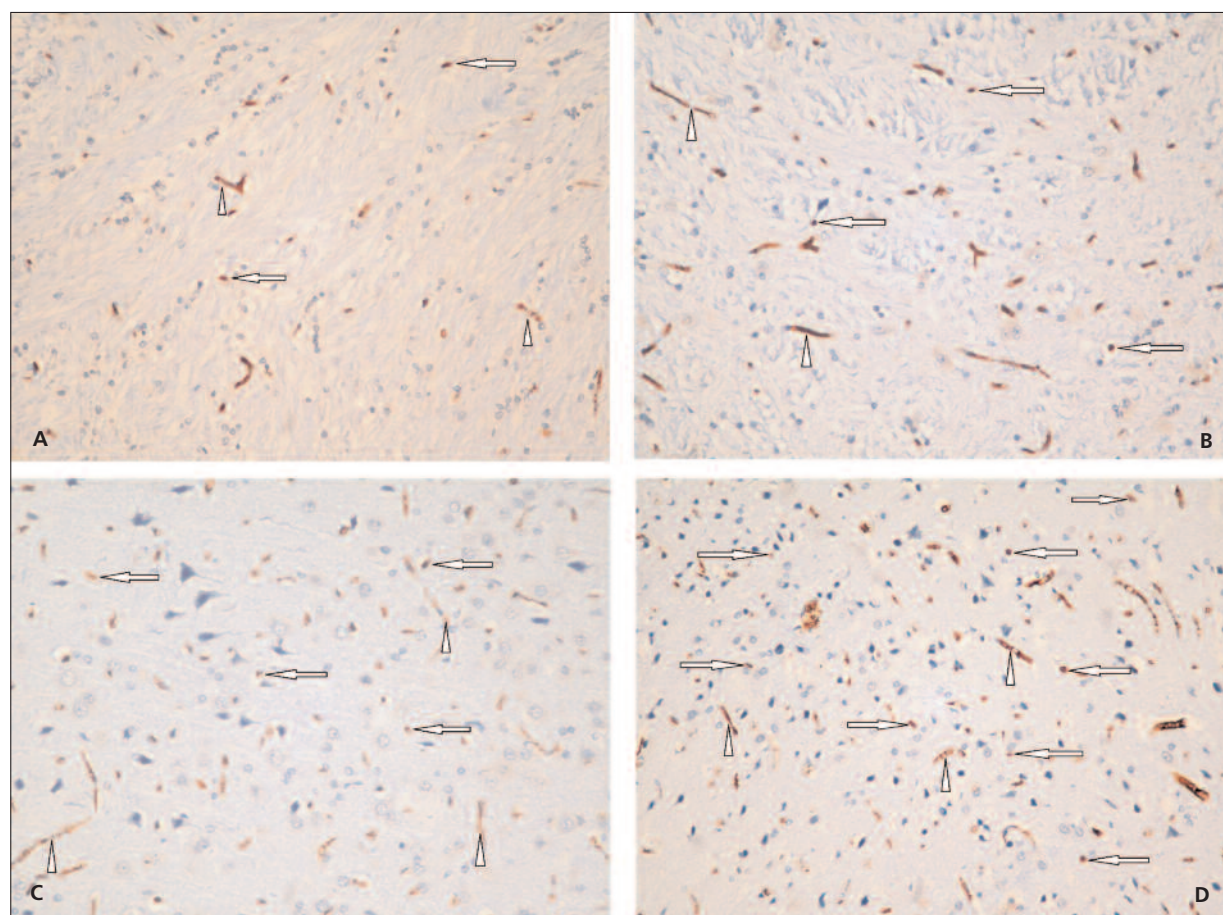


Figure 1. Caspase-3-positive cells in the rat brain tissue. **A**, Control group. **B**, Malathion 2.5 mg/kg-exposed group. **C**, Malathion 5 mg/kg-exposed group. **D**, Malathion 10 mg/kg-exposed group. A dose-correlated increase in the number of caspase-3-positive glial cells in the different groups can be observed in **(A-D)** (immuno peroxidase staining, $\times 200$), (arrows, caspase-3 positive glial cells; arrowheads, caspase-3 positive vascular endothelial cells).

of these non-cholinergic effects, *in vitro* and *in vivo* investigations have been performed using concentrations of MAL comparable to their exposure levels^{11,14}. Our study was designed to investigate the oxidative stress imposed by sub-acute exposure to low doses of MAL (2.5, 5, and 10 mg/kg) in terms of free radical production and lipid peroxidation. Like other OP compounds, MAL inhibits AChE activity. This effect is thought to underlie the neurotoxicity formed by these compounds¹⁶. In addition to inhibiting AChE activity, some studies have shown that oxidative stress could be an important component in the mechanism of OP toxicity. The brain has the propensity to suffer significant oxidative injury due to its lipid composition, which is easily oxidized due to its high oxygen consumption rate and relatively low concentration of antioxidant substances. The brain levels of oxidants can be measured separately in the laboratory. However, measurement of the TOS accurately reflects the oxidative status of tissues, indicating the level of all free oxidant radicals caused by MAL-related oxidative stress^{10,17}. Lipid peroxidation, a well-established mechanism of cellular injury in animals, was used as an indicator of oxidative stress in the present study by measuring the production level of MD¹⁸. Several authors have found increased lipid peroxidation end-products after acute, sub-acute, or chronic MAL exposure^{18,19}. Acute exposure to MAL (250 mg/kg, intraperitoneally) has been clearly demonstrated to increase the levels of lipid peroxidation end-products in rat brain¹⁸. Additionally, Fortunato et al²⁰ showed MAL-induced oxidative stress in rat brain in acute and chronic protocols at doses of 25, 50, 100, and 150 mg MAL/kg. Our findings indicate an increase of MDA and TOS levels in all brains of MAL-exposed rats, but not significantly increased in the 2.5 mg/kg MAL-exposed group compared with the control group. However, the TOS and MDA levels in rat brain were significantly and dose-dependently increased in the 5 and 10 mg/kg MAL-exposed groups compared with the control group in the present study. These results suggest that MAL causes oxidative stress and toxic effects on brain tissue in rats at a dose of ≥ 5 mg/kg.

Conversely, the brain contains many antioxidant molecules that prevent and/or inhibit harmful free radical reactions²¹). SOD is an antioxidant enzyme and its activity limits the accumulation of peroxides in tissues, contributing to antioxidant protection against oxidative cell injury²². By

contrast, TAC reflects the total antioxidant status of tissues^{9,23} and may be an important factor in protecting the brain from neurological damage caused by MAL-related oxidative stress. Considering the extensive data showing increased lipid peroxidation as a general finding after MAL exposure, it is clear that the antioxidant system is not coping with the oxidative challenge^{20,22}. One possible explanation for this increased lipid peroxidation is the parallel impairment in antioxidant defenses, as presented in the current study. The decrease in TAC and SOD activity in the brain indicates reduced antioxidant defenses after 5 and 10 mg/kg sub-acute MAL exposure, which is in-line with the increased levels of MDA. However, some studies have indicated that the activities of antioxidant enzymes are unchanged in the brains of animals poisoned with MAL^{16,24}. Additionally, Dos Santos et al²⁴ reported that biochemical parameters related to oxidative stress (glutathione peroxidase, glutathione reductase, and catalase activity, as well as lipid peroxidation) were not affected in the brain of animals treated with a single dose of MAL (1.25 mg/kg). However, in the present study, MAL was shown to cause a significant and dose-dependent decrease in SOD and TAC activity. Thus, these data support pro-oxidative action as the mechanism for MAL neurotoxicity.

PON-1, which hydrolyzes aromatic carboxylic acid esters and OPs, is synthesized by the liver²⁵. The detoxification of lipid peroxidation and antioxidant property of PON-1 has been proposed²⁶. Sufficient evidence has revealed that serum PON-1 activity can be inhibited by OPs²⁷. Thus, this may decrease the protective ability of PON-1 against free radicals²⁸. In the present report, a marked negative relationship between PON-1 activity and lipid peroxidation levels was found in rat brain exposed to MAL. Compared with the control group, PON-1 activity was found to be significantly lower in MAL-exposed rat brain tissue in a dose-dependent manner (2.5, 5, and 10 mg/kg). Our results also show that all biochemical parameters were consistent with the effects of MAL-correlating with the dose quantities-on rat brain tissue. Thus, at higher doses, MAL may cause more oxidative stress and toxic effects on brain tissue.

Apoptosis, or programmed cell death, is one of the most important signaling pathways triggered by cytotoxic stresses such as oxidative stress; it induces the activation of executioner caspases such as caspase-3, leading to cellular destruc-

tion^{29,30}. Although the cytotoxicity of MAL has been shown to involve several biochemical pathways, only one study demonstrated apoptosis following exposure to this compound in the literature. Accordingly, low-dose exposure to MAL has been shown to be effective in apoptosis in a time- and dose-dependent manner, and non-cholinergic doses of MAL induce caspase-mediated apoptosis¹¹. In our study, immunohistochemically significant increase in the number of caspase-3-positive brain cells in the gray and white matter of the MAL-exposed tissues was revealed. And compared with the control group, the 2.5, 5, and 10 mg/kg MAL-exposed groups showed a significant and dose-dependent increase in the number of caspase-3-positive brain cells ($p = 0.001$ for each). To date, according to our literature search, this is the first report to demonstrate caspase-3 immunoreactivity in the brain tissue of MAL-exposed rats. Additionally, these findings suggest that low doses of MAL induce caspase-mediated apoptosis in rat brain tissue.

As a result, when we evaluated all of the biochemical parameters in our study (SOD, TAC, TOS, MDA, and PON-1), significant differences were found in the 5 and 10 mg/kg MAL-exposed groups compared with the control group ($p < 0.05$); however, a significant difference was not found in the 2.5 mg/kg MAL-exposed group compared with the control group ($p > 0.05$; except for PON-1). In particular, TOS and MDA levels were significantly increased in the 5 and 10 mg/kg MAL-exposed groups compared with the 2.5 mg/kg-exposed group. Additionally, our immunohistochemical results are consistent with our biochemical results and indicate that low doses, including ≥ 5 mg/kg of MAL, can lead to oxidative stress and increased caspase-3 immunoreactivity in rat brain tissue.

Conclusions

These results indicate that ≥ 5 mg/kg of MAL may dose-dependently cause oxidative stress, increased caspase-3 immunoreactivity, and toxic effects on rat brain tissue. However, additional studies of the brain effects of repeated low doses of MAL (including doses of < 5 mg/kg) should be conducted.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

- 1) KAMANYIRE R, KARALLIEDDE L. Organophosphate toxicity and occupational exposure. *Occup Med (Lond)* 2004; 54: 69-75.
- 2) ALP H, AYTEKIN I, HATİPOĞLU NK, ALP A, OGUN M. Effects of sulforaphane and curcumin on oxidative stress created by acute malathion toxicity in rats. *Eur Rev Med Pharmacol Sci* 2012; 16 (3 Suppl): 144-148.
- 3) NIGG HN, KNAAK JB. Blood cholinesterases as human biomarkers of organophosphorus pesticide exposure. *Rev Environ Contam Toxicol* 2000; 163: 29-111.
- 4) RANJBAR A, GHAHREMANI MH, SHARIFZADEH M, GOLESTANI A, GHAZI-KHANSARI M, BAEERI M, ABDOLLAHI M. Protection by pentoxifylline of malathion-induced toxic stress and mitochondrial damage in rat brain. *Hum Exp Toxicol* 2010; 29: 851-864.
- 5) LOWRY OH, ROSEBROUGH NJ, FARR AL, RANDALL RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265-275.
- 6) FRIDOVICH I. Superoxide dismutases. *Adv Enzymol Relat Areas Mol Biol* 1974; 41: 35-97.
- 7) OHKAWA H, OHISHI N, YAGI K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351-358.
- 8) LIANG B, LI YH, KONG H. Serum paraoxonase, arylesterase activities and oxidative status in patients with insomnia. *Eur Rev Med Pharmacol Sci* 2013; 17: 2517-2522.
- 9) EREL O. A new automated colorimetric method for measuring total oxidant status. *Clin Biochem* 2005; 38: 1103-1111.
- 10) EREL O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* 2004; 37: 277-285.
- 11) MASOUD L, VIJAYASARATHY C, FERNANDEZ-CABEZUDO M, PETROIANU G, SALEH AM. Effect of malathion on apoptosis of murine L929 fibroblasts: a possible mechanism for toxicity in low dose exposure. *Toxicology*. 2003;185:89-102.
- 12) TUNCEL N, KORKMAZ OT, TEKIN N, SENER E, AKYUZ F, İNAL M. Antioxidant and anti-apoptotic activity of vasoactive intestinal peptide (VIP) against 6-hydroxy dopamine toxicity in the rat corpus striatum. *J Mol Neurosci* 2012; 46: 51-57.
- 13) SHAYEGHI M, KHOOBDEL M, VATANDOOST H. Determination of organophosphorus insecticides (malathion and diazinon) residue in the drinking water. *Pak J Biol Sci* 2007; 10: 2900-2904.
- 14) BŁASIAK J, JALOSZYŃSKI P, TRZECIAK A, SZYFTER K. *In vitro* studies on the genotoxicity of the organophosphorus insecticide malathion and its two analogues. *Mutat Res* 1999; 445: 275-283.
- 15) TSATSAKIS AM, TZATZARAKIS MN, TUTUDAKI M. Pesticide levels in head hair samples of Cretan population as an indicator of present and past exposure. *Forensic Sci Int* 2008; 176: 67-71.

- 16) DA SILVA AP, MEOTTI FC, SANTOS AR, FARINA M. Lactational exposure to malathion inhibits brain acetylcholinesterase in mice. *Neurotoxicology* 2006; 27: 1101-1105.
- 17) ASILTÜRK Z, NAZLIĞÜL Y, YILDIZ M, KÜÇÜKAZMAN M, BULUR O, TEZER A, ÇİZMECI Z, AKIN KO. Serum oxidative stress status in CagA positive *Helicobacter pylori* infection. *J Clin Exp Invest* 2011; 2: 202-206.
- 18) BROCARDO PS, PANDOLFO P, TAKAHASHI RN, RODRIGUES AL, DAFRE AL. Antioxidant defenses and lipid peroxidation in the cerebral cortex and hippocampus following acute exposure to malathion and/or zinc chloride. *Toxicology* 2005; 207: 283-291.
- 19) TREVISAN R, ULIANO-SILVA M, PANDOLFO P, FRANCO JL, BROCARDO PS, SANTOS AR, FARINA M, RODRIGUES AL, TAKAHASHI RN, DAFRE AL. Antioxidant and acetylcholinesterase response to repeated malathion exposure in rat cerebral cortex and hippocampus. *Basic Clin Pharmacol Toxicol* 2008; 102: 365-369.
- 20) FORTUNATO JJ, AGOSTINHO FR, REUS GZ, PETRONILHO FC, DAL-PIZZOL F, QUEVEDO J. Lipid peroxidative damage on malathion exposure in rats. *Neurotox Res* 2006; 9: 23-28.
- 21) DRINGEN R. Oxidative and antioxidative potential of brain microglial cells. *Antioxid Redox Signal* 2005; 7: 1223-1233.
- 22) FRANCO JL, POSSER T, MATTOS JJ, TREVISAN R, BROCARDO PS, RODRIGUES AL, LEAL RB, FARINA M, MARQUES MR, BAINY AC, DAFRE AL. Zinc reverses malathion-induced impairment in antioxidant defenses. *Toxicol Lett* 2009; 187: 137-143.
- 23) GÜRBÜZ DG, ULA T, PAKSOY F, AKGÜN ÖK, TURSUN I, ÇAKIR A, DAL MS, BORLU F. The effect of intravenous iron therapy on total antioxidant capacity in patients with iron deficiency anemia. *J Clin Exp Invest* 2011; 2: 287-291.
- 24) DOS SANTOS AA, DOS SANTOS DB, RIBEIRO RP, COLLE D, PERES KC, HERMES J, BARBOSA AM, DAFRE AL, DE BEM AF, KUÇA K, FARINA M. Effects of K074 and pralidoxime on antioxidant and acetylcholinesterase response in malathion-poisoned mice. *Neurotoxicology* 2011; 32: 888-895.
- 25) COSTA LG, COLE TB, VITALONE A, FURLONG CE. Measurement of paraoxonase (PON1) status as a potential biomarker of susceptibility to organophosphate toxicity. *Clin Chim Acta* 2005; 352: 37-47.
- 26) ABDOLLAHI M, DONYAVI M, POURNOURMOHAMMADI S, SAADAT M. Hyperglycemia associated with increased hepatic glycogen phosphorylase and phosphoenolpyruvate carboxykinase in rats following subchronic exposure to malathion. *Comp Biochem Physiol C Toxicol Pharmacol* 2004; 137: 343-347.
- 27) AKGUR SA, ÖZTÜRK P, SOZMEN EY, DELEN Y, TANYALCIN T, EGE B. Paraoxonase and acetylcholinesterase activities in humans exposed to organophosphorous compounds. *J Toxicol Environ Health A* 1999; 58: 469-474.
- 28) SHIH DM, GU L, XIA YR, NAVAB M, LI WF, HAMA S, CASTELLANI LW, FURLONG CE, COSTA LG, FOGELMAN AM, LUSIS AJ. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature* 1998; 394: 284-287.
- 29) ANNUNZIATO L, AMOROSO S, PANNACCIONE A, CATALDI M, PIGNATARO G, D'ALESSIO A, SIRABELLA R, SECONDO A, SIBAUD L, DI RENZO GF. Apoptosis induced in neuronal cells by oxidative stress: role played by caspases and intracellular calcium ions. *Toxicol Lett* 2003; 139: 125-133.
- 30) KOSOVA F, ARI, Z. The relationship between prostate cancer and apoptosis. *J Clin Exp Invest* 2011; 1: 124-131.