

Pharmacology, toxicology / Pharmacologie, toxicologie

Biochemical evaluation of hepatic damage in subchronic exposure to malathion in rats: Effect on superoxide dismutase and catalase activities using native PAGE

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Received 15 February 2008; accepted after revision 11 June 2008

Available online 3 July 2008

Presented by Pierre Buser

Abstract

The aim of this study was the evaluation of the hepatic damages following a subchronic exposure to malathion, an organophosphorus (OP) insecticide. Malathion was administered intragastrically in 1 ml corn oil containing 100 mg/kg Body Weight daily for 32 days. Malondialdehyde (MDA) concentration superoxide dismutase (SOD) and catalase (CAT) activities were analysed using a non-denaturing electrophoresis. The serum activities of Pseudocholinesterase (PchE), aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were determined. Malathion exposure leads to a significant decrease in AchE activity, an increase in hepatic MDA, and in serum ASAT and ALAT activities. A positive correlation between serum transaminases levels and hepatic MDA was demonstrated. These results indicate that malathion exposure induced lipid peroxidation LPO, a process of degradation of membrane lipids, involving the deterioration of the cellular integrity. We have recorded a slight increase in antioxidant enzymes activities. This leads us to suggest an insufficient elimination of free radicals, causing cytotoxic effects. **To cite this article: R. Rezg et al., C. R. Biologies 331 (2008).**

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Résumé

Évaluation biochimique des dommages hépatiques induits après une exposition subchronique au malathion chez le rat. Effets sur les activités de la superoxyde dismutase et la catalase en utilisant la technique du native PAGE. Cette étude s'intéresse à l'évaluation des dommages hépatiques induits chez le rat à la suite d'une exposition subchronique à un insecticide organophosphoré, le malathion. Le malathion, solubilisé dans 1 ml d'huile de maïs à raison de 100 mg/kg de poids corporel/jour, est administré par gavage durant 32 jours. À la fin de l'expérience, le foie a été isolé pour la détermination du taux du malondialdéhyde (MDA) et la révélation native par électrophorèse en gel de polyacrylamide (*native PAGE*) des activités de la superoxyde dismutase (SOD) et de la catalase (CAT). Les activités plasmatiques de la *pseudocholinesterase (PchE)*, l'aspartate-amino-transférase (ASAT) et l'alanine-amino-transférase (ALAT) ont été également déterminées. Notre étude montre que l'exposition subchronique au malathion a induit chez le rat une diminution significative de l'activité de la *PchE* et une élévation hautement significative du taux du MDA hépatique, couplée à une augmentation significative des activités sériques des transaminases indicatrices de cytolyse. De plus, une corrélation positive et hautement significative a été démontrée entre l'élévation du taux de MDA et l'augmentation

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concomitante des activités sériques des transaminases. Ceci indique probablement que l'exposition subchronique au malathion entraîne la dégradation des lipides membranaires et donc l'altération de l'intégrité des hépatocytes, provoquant la libération de ces enzymes dans la circulation. Par ailleurs, une faible augmentation des activités des enzymes antioxydantes a été enregistrée, ce qui nous laisse suggérer une élimination insuffisante des radicaux libres, causant des effets cytotoxiques. **Pour citer cet article : R. Rezg et al., C. R. Biologies 331 (2008).**

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Keywords: Malathion; Lipid peroxidation; Transaminases; Cytolysis; Cytotoxicity; Superoxide dismutase; Catalase

Mots-clés : Malathion ; Peroxydation lipidique ; Transaminases ; Cytolyse ; Cytotoxicité ; Superoxide dismutase ; Catalase

1. Introduction

The widespread use of pesticides in public health and agricultural programs has resulted in the pollution of water, air, and food that has led to severe acute and chronic cases of human poisonings [1–6]. The World Health Organization estimates that the incidence of pesticide poisoning in developing countries has doubled between 1987 and 1997 [7]. However, the use of organophosphorus pesticides (OP) has increased considerably due to their low potency and durability as compared to organochlorine pesticides [8], in spite of their risks to human health and the environment. The OP compound malathion [S-1,2(bis ethoxycarbonyl) ethyl O,O dimethyl phosphorodithioate] is used extensively throughout the world, especially in developing countries, to control major arthropods in public health programs, animal ectoparasites, human head and body lice, household insects and to protect grain in storage [9–12]. It kills insects by contact or vapour action. OP compounds are primarily recognized for their ability to induce toxicity in mammals through the inhibition of acetylcholinesterase (AChE), leading to the accumulation of acetylcholine and subsequent activation of cholinergic muscarinic and nicotinic receptors [13]. Also, a study suggests that OP compounds may exert direct action on muscarinic and nicotinic receptors, when their concentration in the circulation exceeds micromolar levels [14]. The toxicity of OP agents may be due, at least in part, to the formation of reactive oxygen species (ROS), leading to lipid peroxidation LPO, which is generally assessed by an increase in the levels of thiobarbituric acid reactive substances (TBARS) [13, 15, 16]. In addition, malathion is a lipophilic substance; it may enhance LPO by directly interacting with the cellular plasma membrane [13]. It is evident that LPO is accompanied by an alteration (inhibition or activation) in the antioxidant defence system in different organs following acute, subchronic and chronic exposure to OP compounds [13, 17–22]. Nevertheless, recent evidence suggests that exposure to different stress models and

concentrations (acute, repeated and chronic restraint stress) have an important implication in the alteration of the antioxidant defence system [23]. The liver is the major metabolizing and the most active organ for mediating bio-activation of thiono-organophosphates [24]. It is considered among the primary targets for malathion toxicity [25]. Thus, the aim of the present investigation was the evaluation of liver damage after subchronic exposure to malathion and the estimation of the antioxidant status using non-denaturing electrophoresis followed by activity-staining [26].

2. Materials and methods

2.1. Chemicals

Malathion (fyfanon 50 EC 500 g/l) of commercial grade was used in this study. 2-thiobarbituric acid (TBA); 2,6-di-tert-butyl-4-methylphenol (BHT); trichloroacetic acid (TCA); Acetylthiocholine iodide; 5,5-dithio bis(2-nitrobenzoic acid)(DTNB); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenazine methosulfate (PMS), magnesium chloride ($MgCl_2 \cdot 6H_2O$); potassium iodide; sodium thiosulfate ($Na_2S_2O_3$) were obtained from sigma-Aldrich Co (Germany).

2.2. Animals and treatment

Adult male *wistar* rats (170–180 g), procured from Tunisian Society of Pharmaceutical Industries, were kept in clean plastic cages and allowed to acclimatize in the laboratory environment from 7 days. The ambient temperature was ($22 \pm 1^\circ C$) with a 12-h dark/light cycle. Balanced food and drinking water were given to the animals ad-libitum.

Animals were randomly divided into two groups of 12 animals and treated for 32 days. The first group received orally 1 ml corn oil via gavage. The second received instead 1 ml corn oil containing 100 mg Mal/kg body weight per day. The test concentration

of malathion is calculated directly from commercial grade. Body weight and food intake were recorded daily throughout the experiment. All the procedure was in accord with the Guidelines for Ethical Conduct in the Care and Use of Animals. At the end of the treatment, 24 hours after the last dose of malathion [26], animals were killed by decapitation, without preliminary anaesthesia, and arteriovenous blood was taken quickly for centrifugation ($3000\text{ g} \times 10\text{ min}$) at 4°C . The liver was removed by transverse abdominal incision and kept frozen at -80°C .

2.3. Pseudocholinesterase PchE activity assay

The pseudocholinesterase activity of plasma was estimated by the method of Ellman [27], using acetylthiocholine iodide. The rate of hydrolysis of acetylthiocholine iodide is measured at 412 nm through the release of the thiol compound which, when reacted with DTNB, produces the color-forming compound TNB.

2.4. Aspartate aminotransferase ASAT and alanine aminotransferase ALAT assay

The serum activities of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) were determined spectrophotometrically at 340 nm using established clinical chemical methods. The rate of reduction in the concentration of NADH, is directly proportional to the ASAT or ALAT activity in the sample [28]. These values are reported in units per litre (UI/l).

2.5. Lipid peroxidation

Each liver was homogenised separately in Tris Buffered Saline (TBS) pH 7.4. The homogenates were centrifuged at 4°C at 3000 g and the supernatants were stored at 4°C for determining lipid peroxidation. Each supernatant was mixed with the (BHT)-TCA solution, and centrifuged at 1000 g for 10 min. Malonaldehyde (MDA) is an end product of the oxidation of polyunsaturated fatty acids and can react with TBA to produce a complex that can be determined spectrophotometrically at 530 nm. So, the supernatant was mixed with 0.5 N HCl and 120 mM TBA in 26 mM Tris, and heated at 80°C for 15 min. After cooling, the absorbance was determined using a BIORAD UV-visible spectrophotometer. Lipid peroxidation in homogenates was assessed in terms of thiobarbituric acid reactive substances (TBARS) produced [29]. The protein content in liver homogenates was quantified by the method

of Bradford [30], using bovine serum albumin as a standard.

2.6. Analysis of antioxidant enzyme activities using native PAGE

2.6.1. Non denaturing gradient gel electrophoresis

500 mg of liver were homogenized with the extract buffer [31] added with benzamidine (0.1 M) for inhibiting proteases. The homogenate was centrifuged at 4000 g for 30 min at 4°C , and the pellet discarded. Protein concentration was determined according to the method of Bradford [30]. The equivalent of $25\text{ }\mu\text{g}$ of each sample protein were mixed with an equal volume of sample buffer containing glycerol, 0.1 M Tris-HCl, pH 6.8, 0.1% and blue bromophenol. The mix of each sample was loading, respectively, per lane on the gel realized on 8% polyacrylamide. At the end of the electrophoretic migration, gels were incubated with staining solution to identify the enzymes' activities.

2.6.2. Catalase (CAT)

Native PAGE was realized on 8% polyacrylamide gel at 200 V and 4°C . The gel was incubated in the first step in sodium thiosulfate solution (60 mM) with 3% H_2O_2 for 30 to 60 seconds. Secondly, the first solution was discarded and the gel was incubated in a second solution containing 90 mM KI and acid acetic (glacial). Catalase activity is indicated by the presence of achromatic bands [32].

2.6.3. Superoxide dismutase (SOD)

Native PAGE was realized on 10% polyacrylamide gel at 200 V and 4°C . Enzymatic activities were developed in staining solution containing 50 mM Tris-HCl (pH, 8.5), 10 mg MTT, 6 mg PMS, 15 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in daylight. The enzyme bands are seen as pale zones on a dark blue background [32].

2.6.4. Quantitative estimate

For the quantitative estimate we have to use an application software image J that is a special Plugin for convenient densitometry of 1D gels and also dot blots.

2.7. Statistical analysis

All data are expressed as mean \pm SEM by factorial ANOVA, followed by Student test. The relationships between MDA levels and serum transaminases (ASAT and ALAT) activities were assessed by linear regression analysis. Differences between groups were considered significant when $p < 0.05$.

Table 1

Effect of subchronic treatment with malathion on TBARS level measured in liver, on plasma PChE activity, and on transaminases activities (ASAT, ALAT) of rats

Treatment	MDA nmol/mg protein	PChE nmol/min/mg protein	ASAT UI/l	ALAT UI/l
Control ($n = 12$)	5.5 ± 0.24	12.877 ± 0.259	47.25 ± 2.78	19.32 ± 1.25
Malathion ($n = 12$)	$7.05 \pm 0.24^{***}$	$4.918 \pm 1.496^{***}$	$70.10 \pm 7.40^*$	$27.92 \pm 1.55^{***}$

Values are presented as the mean \pm SEM, n : number of rats used.

* $p < 0.05$ as compared with control.

*** $p < 0.001$ as compared with control.

3. Results

3.1. Physical observation

No signs of toxicity were observed in malathion-treated animals until the end of the experiment. In addition, our results showed that malathion administration caused no significant decrease in mass gain between controls and malathion-treated animals.

3.2. Blood estimations and lipid peroxidation

Malathion at dose of 100 mg/kg/day increased significantly hepatic MDA and serum activities of aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) and decreases *PchE* activity – see Table 1.

3.3. Relationship between serum ASAT and ALAT activities and hepatic MDA

There was a significant positive correlation between serum ASAT and ALAT activities and hepatic MDA; see, respectively, Figs. 1 and 2.

3.4. Hepatic antioxidant enzyme activities

In native PAGE, our results showed a slight increase in antioxidant enzymes activities CAT and SOD, respectively (16%) (Fig. 3(a), (b)) and (11%) (Fig. 4(a), (b)).

4. Discussion

The results of this study indicate that malathion in subchronic exposure at a dose of 100 mg/kg per day decreases significantly *PchE* activity. In addition, malathion exposure increases significantly the hepatic TBARS level of malathion-exposed rats. These results are somewhat similar to those found in the liver of rats exposed to malathion acutely [13] or subchronically [21]. Elevated levels of TBARS in liver can be an indicator of increased lipid peroxidation (LPO) in

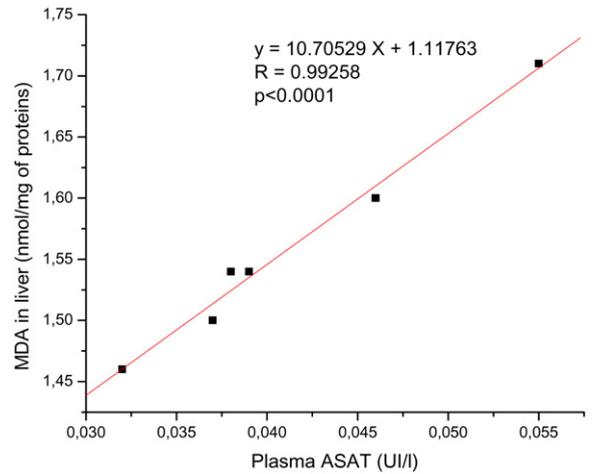


Fig. 1. The relationships between the levels of hepatic MDA and plasma ASAT activity. There is positive correlation between hepatic MDA and plasma ASAT activity. y : Equation of the linear fit; r : correlation factor.

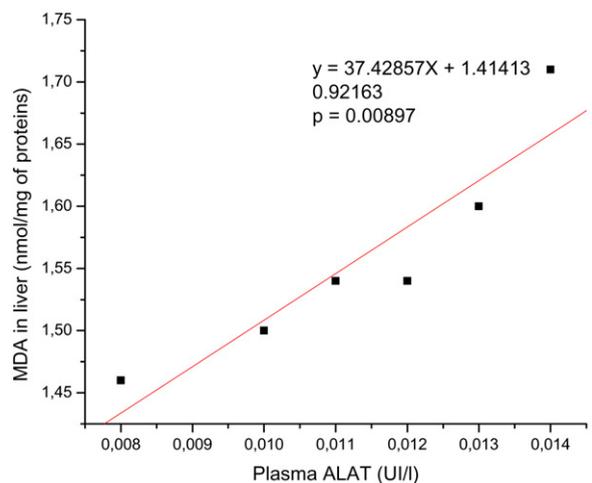


Fig. 2. The relationship between the levels of hepatic MDA and plasma ALAT activity. There is positive correlation between hepatic MDA and plasma ALAT activity. y : Equation of the linear fit; r : correlation factor.

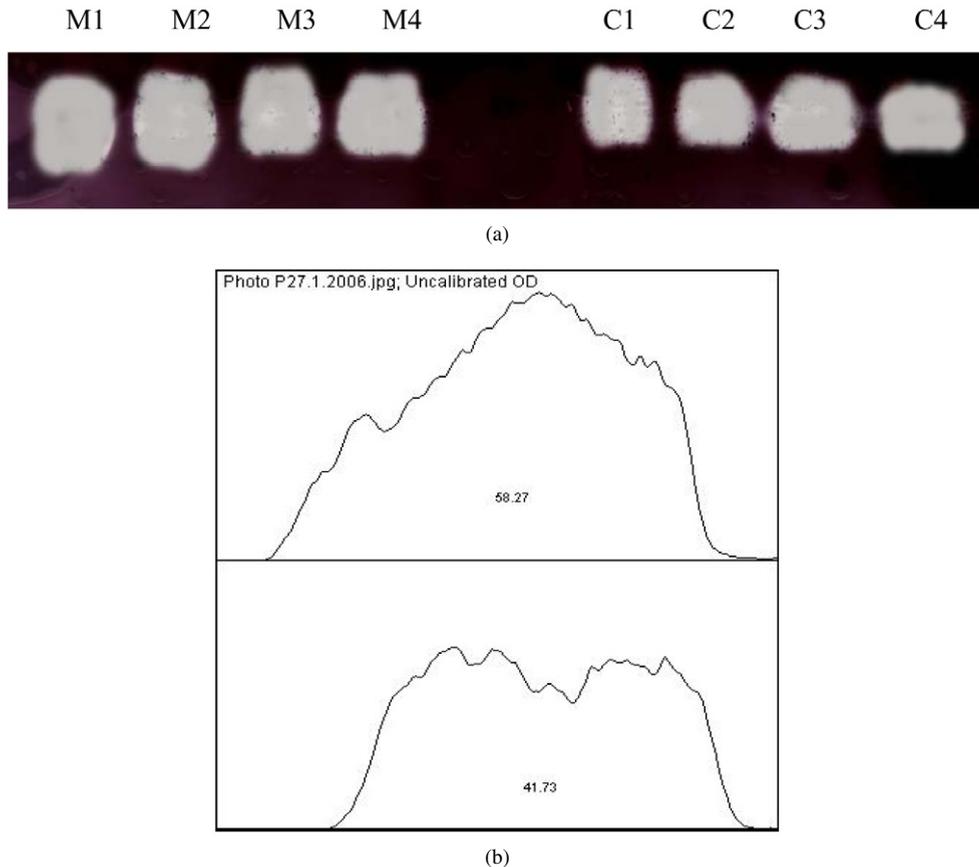


Fig. 3. (a) Effect of malathion on hepatic catalase (CAT) activity using native PAGE ($n = 4$); (b) quantitative estimation of hepatic (CAT) activity. The increase of CAT activity in treated rats (M) was about 16% compared to control rats (C).

hepatocyte membranes that suggests the participation of free-radical induced oxidative cell injury in mediating the toxicity of (OPs) pesticides. On the other hand, our study shows a significant increase in serum activities of ASAT and ALAT considered like markers enzymes of hepatocyte cytolysis. Indeed, ASAT and ALAT are two hepatic enzymes which will be released in blood in the event of cellular destruction proportionally to the intensity of the cellular aggression [33]. The recorded increase of ASAT and ALAT levels may be due to increased LPO. Indeed, we have seen a high positive correlation between serum rates of ASAT and ALAT and hepatic MDA level. The formation of MDA in liver is a sign of lipid membrane degradation that involving the deterioration of cellular integrity and the release of these enzymes in blood (see Figs. 3 and 4). In the same way, Nemcsok [34] has noted a tissular necrosis indicated by the rise in the serum activities respectively of the ASAT and the ALAT following the exposure of fish (carp) to an OP compound. Additionally, histological examinations of the hepatic tissues of

malathion-exposed rats revealed degenerative changes in the liver in the form of parenchymatous degeneration. The areas of cytoplasm were observed to be deprived of normal cellular organelles, with an increased number of lipid vacuoles. These changes confirm the focal degeneration of the cytoplasm [35]. Among the possible explanations for this cytolysis, is the leakage of hepatocyte's biomembrane, causing efflux of K^+ , influx of Na^+ and concomitant influx of water into the cell that can cause cytotoxic edema [13]; it is well elucidated as one of the most important effects of LPO. Indeed, OP pesticides are known to affect the activity of membrane-bound ATPases [36–38]. Also, the lipophilicity of organophosphorus insecticides favors their incorporation in membranes [39,40]. The incorporated compounds elicit physical and chemical perturbations and presumably a dysfunction of the membranes in their phospholipids bilayer structure. Therefore, the primary effects of insecticides may result from physicochemical changes at the level of membrane lipid structure and organization [39].

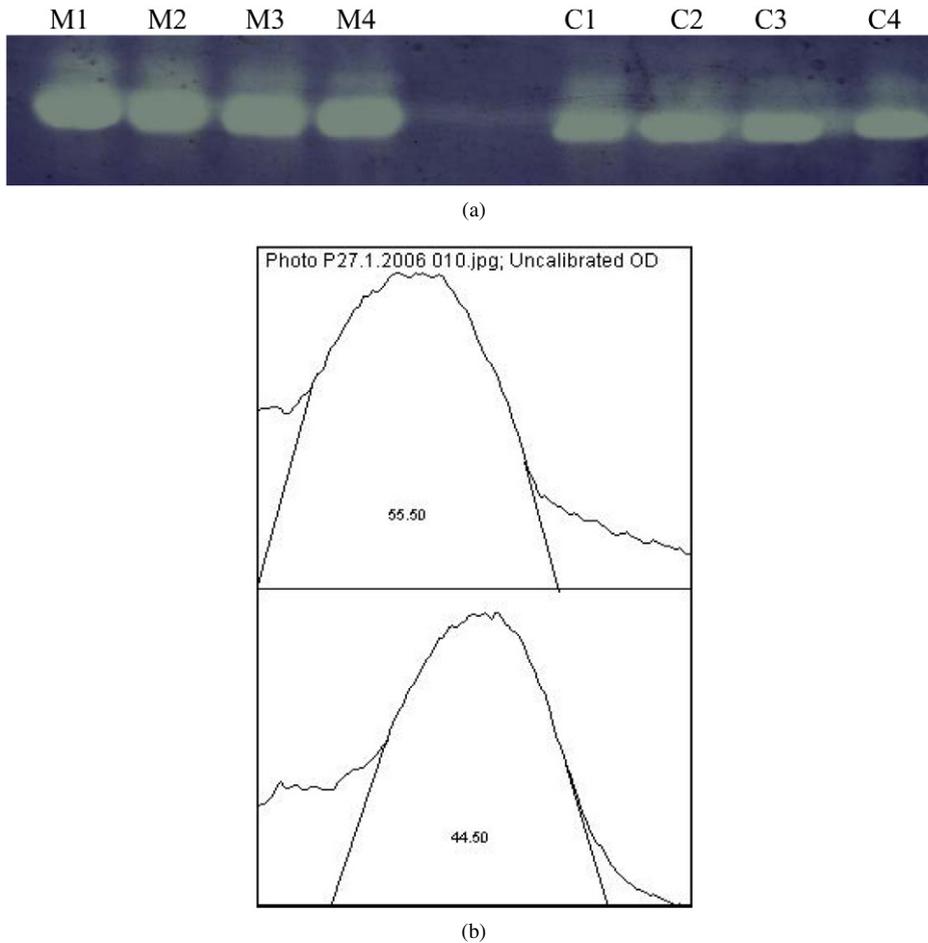


Fig. 4. (a) Effect of malathion on hepatic superoxide dismutase (SOD) activity using native PAGE ($n = 4$); quantitative estimation of hepatic (SOD) activity. The increase of SOD activity in treated rats (M) was about 11% compared to control rats (C).

On the other hand, some studies have reported, in acute malathion exposure [13], a decrease in detoxifying enzymes like glutathione S-transferases (GST), which produce less toxic forms by conjugation [41] and carboxylesterases, responsible for the rapid degradation of malathion and malaoxon in mammals [42,43]. Thus, it is obvious that this leads to insufficient detoxification of malathion in the liver. Indeed, it is interesting to note that Blasiak et al. [44] reported that malathion has an acute cytotoxic effect leading to cellular death in human lymphocytes, without causing damage to the DNA; but its active metabolites, malaoxon and isomalathion act on DNA, breaking its chains. Each mole of undetoxified xenobiotic or its metabolites can potentially produce multiple moles of active oxygen species in redox-cycling. Thus, we can suggest an accumulation of active oxygen species that can exceed the organism's capacity to neutralize them and consequently, may produce cytotoxic effects. This result might be due to

time and concentration-dependent mechanisms. Indeed, Gultekin et al. [45] confirmed in vitro that MDA values increased with increasing organophosphorus compound concentration and incubation period, but SOD and CAT activities decreased with increasing its concentration and incubation period. In the same way Rosety et al. [46] have demonstrated a time course variation of antioxidant enzyme activities in fish exposed to a sublethal concentration of malathion for 24, 48, 72 and 96 h. They reported that superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities were altered significantly from 24 h onward. Supporting this idea, we can suggest that hepatic SOD and CAT activities pass by two phases during the subchronic treatment with malathion. Firstly, a gradual increase, reaching a maximum, considered like an initial adaptive response to increased oxidative stress. Secondly, a gradual decrease when free radicals concentrations exceed their capacity to neutralize them. Related to our

work (subchronic protocol and high dose) we can suppose that enzymes in our zymograms were in the second phase of enzymatic activity. This explanatory assumption needs to be elucidated *in vivo* by the investment of the kinetics of antioxidant enzymes activities and their mRNAs after oral exposure to malathion in subchronic treatment. Generally, the accumulation of free radicals can involve cytotoxicity and enormous damage to DNA, as well causing an alteration in the expression of genes including those of the antioxidant system.

In conclusion, our evaluation shows that malathion in subchronic exposure disrupted the cellular integrity of hepatocytes; thus more attention is needed to limit the use of OP insecticides, particularly malathion, which is contained in most agricultural pesticides used in the developing countries, as much as possible and reduce their entrance into the human food cycle via environmental pollution.

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