

# Chlorfenvinphos, an Organophosphate Insecticide, Affects Liver Mitochondria Antioxidative Enzymes, Glutathione and Hydrogen Peroxide Concentration

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Received: 10 September 2003

Accepted: 26 November 2003

## Abstract

Chlorfenvinphos is an organophosphate insecticide widely used alone or in combination in Poland. In the present study, the influence of chlorfenvinphos on the activity of mitochondrial antioxidative system as well as hydrogen peroxide concentration was examined.

The experiments were conducted on male Wistar rats of  $180 \pm 20$ g body weight. The animals were divided into two groups: the control group, which received oil intragastrically by stomach tube and the experimental groups, which received oil solution of chlorfenvinphos in doses of  $0.02 LD_{50}$ ,  $0.1 LD_{50}$  or  $0.5 LD_{50}$ . After 1, 24, 48 hours the livers were quickly removed. Liver mitochondria were isolated as described elsewhere.

Glutathione peroxidase, superoxide dismutase, isocitrate dehydrogenase activity as well as reduced glutathione and hydrogen peroxide concentrations were determined in liver mitochondria using BIOXY-TECH Assay kits produced by OXIS International, Inc., Portland, USA.

The results of this work indicate that chlorfenvinphos induces oxidative stress to rat liver mitochondria. In acute chlorfenvinphos intoxication, we demonstrated that the key role in the oxidative mitochondrial damage play MnSOD and GSH pool, as well as accumulation of hydrogen peroxide.

**Keywords:** chlorfenvinphos, liver, mitochondria, antioxidative enzymes, glutathione, hydrogen peroxide

## Introduction

Many xenobiotics, drugs as well as chemical pollutants, lead to some degree of liver injury. Liver is prone to xenobiotic-induced injury because of its central role in xenobiotics metabolism, and its portal location within the circulation [1, 2]. Liver damage is often caused by oxidative stress and by reactive oxygen species (ROS). The ROS have been implicated in several human diseases as cancers, cardiovascular diseases, and alcohol-mediated liver injury [3, 4, 5]. A large number of studies have associated mitochondrial dysfunction caused by oxidative stress to both necrosis and apoptosis [3, 4, 5, 6]. Mitochondria generate reactive oxygen species as by-pro-

ducts of molecular oxygen consumption in the electron transport chain [3, 6]. As the first, a superoxide anion is generated. Hydrogen peroxide derived from the superoxide anion by the superoxide dismutase is a precursor of hydroxyl radical - the most toxic radical [6]. Being one of the major sources of ROS, mitochondria are highly susceptible to oxidative damage. ROS can directly damage the mitochondrial enzymes as well as change the mitochondrial transmembrane potential, which is indicative of mitochondria membrane integrity [3, 4]. The production of ROS in mitochondria under physiological conditions is strictly regulated by mitochondria antioxidative enzymes that include Mn-superoxide dismutase (MnSOD), classical glutathione peroxidase (cGPx), phospholipid hydroperoxide glutathione peroxidase (PHGPx) [6]. Glutathione (GSH) in mitochondria and glutathione-related mGPx are

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the only defence available to metabolise hydrogen peroxide, because the mitochondria of mammalian cells lack catalase activity [5, 6].

We previously indicated that in the livers of chlorfenvinphos-intoxicated rats, the oxidative stress is generated [7]. Chlorfenvinphos is an organophosphate insecticide widely used alone or in combination in Poland. In the present study, the influence of chlorfenvinphos on the activity of mitochondrial antioxidative system as well as hydrogen peroxide concentration was examined.

### Material and Methods

The experiments were conducted on male Wistar rats of  $180 \pm 10$  g of body weight. The rats were fed standard diet and given water to drink ad libitum.

The animals were divided into two groups: the control group, which received oil intragastrically by stomach tube in the amount of 0.1 ml/100 g, and the experimental groups, which received oil solution of chlorfenvinphos i.e. 2-chloro-1-(2,4-dichlorophenyl) vinyl diethyl phosphate (CVP) in doses of 0.02 LD<sub>50</sub>, 0.1 LD<sub>50</sub> or 0.5 LD<sub>50</sub> (LD<sub>50</sub> = 15 mg/kg b.w.).

1, 24, 48 hours after intoxication with chlorfenvinphos the livers were quickly removed and placed in iced 0.9% NaCl containing 0.16 mg/ml heparin. Liver mitochondria were isolated as described elsewhere [8]. The study was approved by the Local Ethical Committee.

Glutathione peroxidase (mGPx) activity was determined in liver mitochondria using BIOXYTECH GPx-340™ Assay kit produced by OXIS International, Inc., Portland, USA. The GPx assay was based on the oxidation of NADPH to NADP<sup>+</sup>, which is accompanied by the decrease in absorbance at 340 nm. The rate of this decrease is directly proportional to the GPx activity in the sample. The level of liver mitochondria reduced glutathione (GSH) was measured using BIOXYTECH GSH-400™ Assay kit produced by OXIS International Inc., Portland, USA. The method is based on chemical reaction proceeding in two steps. The first leads to the formation of thioethers between reagent and all mercaptans. The second leads to beta-elimination reaction, which specifically transforms the thioethers obtained with GSH into a chromophoric thione which has maximal absorbance wavelength at 400 nm. The activity of liver mitochondrial isocitrate dehydrogenase (ICDH) was measured using Sigma Assay kit produced by Sigma, USA. The method is based on the reduction of NADP to NADPH, which is accompanied by increase in absorbance at 340 nm. The rate of this increase is proportional to the ICDH activity in the sample.

Protein concentration was measured in dilute liver homogenates according to Lowry et al. [9], using bovine serum as a standard. The enzyme activities were expressed as units of enzyme activity per milligram of protein.

The concentration of liver mitochondria hydrogen peroxide was measured using BIOXYTECH H<sub>2</sub>O<sub>2</sub>-560™ Assay kit produced by OXIS International Inc., Portland,

USA. The assay is based on the oxidation of ferrous ions (FeII) to ferric ions (FeIII) by hydrogen peroxide under acid conditions.

Data for the ten groups of animals were compared using one way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison tests. The probability of  $p < 0.05$  was considered significant. The correlations were calculated between the groups.

### Results

Activity of MnSOD in liver decreased after chlorfenvinphos was given at a dose of 0.02 LD<sub>50</sub> - at the 1 and 24 hours, and during the whole examined period after intoxication with the higher doses of insecticide (Table 1). At the 48 h of intoxication with the lowest insecticide dose the MnSOD activity increased compared to earlier period of treatment and returned to the control value. Chlorfenvinphos treatment caused decrease in mGPx activity (Table 1). The lowest value was observed at 24 h of intoxication with chlorfenvinphos at the dose of 0.5 LD<sub>50</sub>. The diminished activity of mitochondrial peroxidase was observed during the whole examination period of intoxication, except the 48 h after treatment at the dose of 0.02 LD<sub>50</sub>. At this time the mGPx activity returned to the control value.

Reduced glutathione level was diminished during the whole examined period after intoxication with two higher doses and at the 1 and 24 h after the lowest (Table 1). After 48 h of treatment with the dose of 0.02 LD<sub>50</sub>, the GSH concentration returned to control value.

The activity of ICDH increased at the 1 and 24 h after treatment with chlorfenvinphos at the lowest dose and returned to the control value at 48 (Table 1). The highest value was observed at the 1st hour. After intoxication of rats with two higher doses of insecticide the increase in ICDH activity, statistically significant in comparison to control, was observed during the whole period of examination.

The hydrogen peroxide concentration increased at 1 and 24 h after treatment with chlorfenvinphos at a dose of 0.02 and 0.1 LD<sub>50</sub>, but at 48 h decreased in comparison to the earlier period (Table 1). At 48 h, the hydrogen peroxide level was still higher in comparison to the control value. After treatment with the highest dose of chlorfenvinphos the hydrogen peroxide level was higher compare to control, during the whole examination period. At the 48 h its concentration has highest value.

In the rat liver mitochondria the activity of MnSOD and mGPx were positively correlated ( $r=0.429$ ,  $p=0.0001$ ) as well as GSH level and activity of MnSOD ( $r=0.32$ ,  $p=0.004$ ) and mGPx ( $r=0.47$ ,  $p=0.0001$ ).

### Discussion

Organophosphate insecticide causes their effects by inhibiting AChE and thus leading to ACh accumulation. The rapidity of this accumulation depends on dose of the insecticide. In the present study we observed the symptoms of

Table 1. Activity of superoxide dismutase, glutathione peroxidase and izocitrate dehydrogenase (U/g protein) and concentration of reduced glutathione (nmol/mg protein) and hydrogen peroxide concentration ( $\mu\text{mol/g}$  tissue) in rat liver mitochondria after acute intoxication with chlorfenvinphos (CVP).

	MnSOD	GPx	GSH	ICDH	H <sub>2</sub> O <sub>2</sub>
<b>control</b>	221.01±20.79 (n=11)	23.25±3.71 (n=11)	11.36±1.26 (n=10)	2.55±0.40 (n=10)	53.63±10.22 (n=10)
<b>CVP-0.02LD<sub>50</sub></b>					
<b>1 h</b>	166.85±21.59 <sup>a</sup> (n=8)	17.38±2.19 <sup>a</sup> (n=8)	9.53±1.15 <sup>a</sup> (n=8)	7.72±2.13 <sup>a</sup> (n=8)	107.17±14.81 <sup>a</sup> (n=8)
<b>24 h</b>	147.57±23.32 <sup>a</sup> (n=8)	18.31±2.17 <sup>a</sup> (n=8)	9.15±1.32 <sup>a</sup> (n=8)	4.73±0.39 <sup>ab</sup> (n=8)	114.18±13.20 <sup>a</sup> (n=8)
<b>48 h</b>	230.65±21.07 <sup>bc</sup> (n=8)	27.41±3.22 <sup>bc</sup> (n=8)	10.64±0.96 <sup>c</sup> (n=8)	2.44±0.35 <sup>bc</sup> (n=8)	76.43±13.70 <sup>abc</sup> (n=8)
<b>CVP-0.1LD<sub>50</sub></b>					
<b>1 h</b>	150.53±23.68 <sup>ad</sup> (n=8)	15.14±3.15 <sup>ad</sup> (n=8)	6.18±1.14 <sup>abcd</sup> (n=8)	4.78±0.52 <sup>abd</sup> (n=8)	118.63±15.37 <sup>ad</sup> (n=8)
<b>24 h</b>	134.81±22.04 <sup>ad</sup> (n=8)	18.93±2.67 <sup>ade</sup> (n=8)	6.02±1.40 <sup>abcd</sup> (n=8)	5.61±0.66 <sup>abcd</sup> (n=8)	123.68±17.89 <sup>ad</sup> (n=8)
<b>48 h</b>	135.25±23.36 <sup>ad</sup> (n=8)	17.41±1.89 <sup>ad</sup> (n=8)	7.73±1.51 <sup>abd</sup> (n=8)	3.87±0.60 <sup>abdef</sup> (n=8)	82.28±14.83 <sup>abcef</sup> (n=8)
<b>CVP-0.5LD<sub>50</sub></b>					
<b>1 h</b>	170.62±21.65 <sup>ade</sup> (n=8)	14.31±2.74 <sup>acd</sup> (n=8)	5.06±1.04 <sup>abcdg</sup> (n=8)	4.58±0.58 <sup>abdfg</sup> (n=8)	106.42±15.27 <sup>a</sup> (n=8)
<b>24 h</b>	175.15±27.37 <sup>ade</sup> (n=8)	12.59±2.01 <sup>abcdfg</sup> (n=8)	6.01±0.90 <sup>abcdg</sup> (n=8)	6.04±1.12 <sup>acdegh</sup> (n=8)	130.97±20.73 <sup>adg</sup> (n=8)
<b>48 h</b>	149.34±28.76 <sup>ad</sup> (n=8)	17.05±2.02 <sup>adhi</sup> (n=8)	6.83±0.31 <sup>abcdhi</sup> (n=8)	5.76±1.24 <sup>acdegh</sup> (n=8)	153.80±22.35 <sup>abcdegh</sup> (n=8)

values expressed as means  $\pm$  SD; n- the number of rats in the group; statistically significant in comparison with: a -control, b- 0.02LD<sub>50</sub>-1h, c- 0.02LD<sub>50</sub>-24h, d- 0.02LD<sub>50</sub>-48h, e- 0.1LD<sub>50</sub>-1h, f- 0.1LD<sub>50</sub>-24h, g- 0.1LD<sub>50</sub>-48h, h- 0.5LD<sub>50</sub>-1h, i-0.5LD<sub>50</sub>-24h,

toxic effects: such as weakness, fasciculation, salivation, after intoxication with chlorfenvinphos at a dose of 0.5 LD<sub>50</sub>. Insecticide intoxication also leads to liver injury. The liver damage is often caused by oxidative stress.

Mitochondria efficiently reduce free radicals under normal conditions predominantly through antioxidant mechanisms, including superoxide dismutase as well as glutathione and glutathione-dependent enzymes [10]. In examinations of cellular damage there are suggestions that mitochondria can play a key role.

In the present study we investigated the influence of chlorfenvinphos on the oxidative status of mitochondria by the examination of the activity of MnSOD, mGPx, ICDH as well as concentrations of GSH and H<sub>2</sub>O<sub>2</sub>. We found diminished activity of MnSOD in rat liver mitochondria after intoxication with chlorfenvinphos at two higher doses during the whole examination period and at the 1 and 24 h after intoxication with the lowest. Under normal conditions, MnSOD balances the production of excess of superoxide anion from electron transport [10]. The decrease in the activity of MnSOD was accompanied by an increase in the concentration of hydrogen peroxide. H<sub>2</sub>O<sub>2</sub> is a product of the superoxide dismutase reaction, and the former may be inhibited by the product of its own reaction. In this study we can suppose that a high concentration of H<sub>2</sub>O<sub>2</sub> influences the MnSOD activity. The activity of MnSOD increased in the liver mitochondria

of rats in which the enhancement of mGPx activity was observed, as it has been observed at 48 h after intoxication with chlorfenvinphos at a dose of 0.02 LD<sub>50</sub>. These data strongly support the hypothesis that MnSOD is inhibited by H<sub>2</sub>O<sub>2</sub>. Recently, it has been shown that decrease of mitochondrial SOD by about 50% results in a functional decline of oxidative phosphorylation, an increase in oxidative stress, and an increase in the rate of apoptosis [10]. The increase in MnSOD activity is protective in acute liver damage [11]. This data suggests that MnSOD plays an important role for balances of mitochondria redox status.

In the acute intoxication with chlorfenvinphos the mitochondria GSH level diminished during the whole examined period (except 48 h after dosing of 0.02 LD<sub>50</sub>). The diminished GSH level was accompanied by decreased activity of mGPx. GSH is known to be synthesized in the cytosol and transported into the mitochondria [3, 5, 12, 13]. Oxidized glutathione disulfide can not be exported from mitochondria into cytosol. For this reason the role of mitochondria glutathione reductase (mGR) as well as reducing the equivalent for this reaction is very important. Chlorfenvinphos depletes mitochondrial glutathione levels, which is consistent with the hypothesis that this insecticide causes oxidative stress in mitochondria. Chlorfenvinphos probably inhibits the mitochondria glutathione transporter that shuttles GSH into mitochondria from cytosol. This transport is energy-dependent. Chlorfenvinphos influences mitochondria energy

production. Reduced mitochondria GSH levels lead to  $H_2O_2$  accumulation that can cause lipid peroxidation and cell injury [13, 14]. Indeed, in the present work enhanced level of  $H_2O_2$  is observed. The diminished pool of mitochondria glutathione resulted in decreased activity of mGPx. The lowest activity of mGPx and the lowest level of mGSH were observed at the same time. GSH is required for the activity of mGPx, as it is known [3, 15]. [15] has reported decreased activity of GPx in the liver and mitochondria after diminished level of GSH as a result of inhibited activity of gamma-glutamylcysteine. The activity of mGPx and GSH level was positively correlated as it has been observed in acute intoxication with chlorfenvinphos. Direct evidence that activity of mGPx dependent on GSH levels comes from the fact that the increase in the GSH pool also increased mGPx activity, as it has been observed at 48 h after dosing of 0.02 LD<sub>50</sub> of chlorfenvinphos. Decrease in the mGPx activity is very dangerous, because catalase, which metabolises  $H_2O_2$ , is not present in mitochondria of most mammalian cells [3, 5, 14]. Thus mitochondria glutathione peroxidase plays a key role in metabolizing  $H_2O_2$  [5, 10, 13].

In the present work the activity of mitochondria ICDH was also estimated. As mentioned above, the mitochondria GSH system reducing hydrogen peroxide is the main mechanism protecting mitochondria against oxidative stress. This fact suggests the importance of mitochondria NADPH as a necessary reducing equivalent for the regeneration of GSH from GSSG by the activity of mGR [3]. The major source of cellular NADPH is glucose-6-phosphate dehydrogenase since it reduces cellular oxidative stress by increasing the GSH level [3, 16, 17]. But this enzyme is absent in mitochondria. Jo et al. [3] proposed that key role in the maintenance of an adequate NADPH level in the mitochondria plays ICDH. The reactive oxygen species induce the activity of this enzyme. In the acute intoxication with chlorfenvinphos the activity of ICDH rapidly increased at the 1 and 24 h after dosing 0.02 LD<sub>50</sub> and after 48 h returned to control value. After treatment with higher doses, the ICDH activity increased during the whole examined period but this fact has no influence on the GSH pool. The interpretation of these results is difficult. The decreased level of mitochondria GSH in spite elevated activity of ICDH, may be a result of diminished transport and/or synthesis of GSH in cytosol, direct use of GSH as a scavenger of free radicals or as result of diminished pool of total glutathione (GSH+GSSG). The low level of mitochondria GSH may also be a result of decreased activity of mGR since the high level of GSSG saturated the mGR [14]. Such phenomenon was observed after treatment of rats with chlorfenvinphos at the dose of 0.1 and 0.5 LD<sub>50</sub>. In these experimental groups the GSH level was diminished by 60-70% and thus probably the level of oxidized glutathione rapidly increases. In this study, the activity of mGR was not investigated. After intoxication with chlorfenvinphos at the lowest dose the mGSH level decreased only by approximately 20%. At 48 h its concentration returned to control value probably due to increased activity of mGR.

As mentioned above, the activity of ICDH increased as a response to enhancement of ROS level [3]. In the chlorfenvinphos treatment the ICDH activity increased at 1 h after dosing 0.02 LD<sub>50</sub> and this increase was higher, but after dosing the insecticide at the dose of 0.1, and 0.5 LD<sub>50</sub>, the highest values of ICDH activity were observed after 24 h of intoxication. Therefore, our data suggest that an oxidative stress was generated earlier after dosing of chlorfenvinphos at the lowest dose. The enhancement of ICDH activity in the liver mitochondria has been suggested, that NADPH level increased also. However, in acute intoxication with chlorfenvinphos, this nucleotide is not used as reducing equivalent of GSSG since GSH level is maintained at a low level. Exceptionally, the GSH pool returned to control value after intoxication with chlorfenvinphos at the dose of 0.02 LD<sub>50</sub>. At the same time, the activity of MnSOD also returned to control value, as well as the concentration of hydrogen peroxide decreased compare to the earlier period of intoxication. In the groups of rats in which the MnSOD remain decreased the GSH level was diminished, and the hydrogen peroxide level was still high and even increased (48 h, 0.5 LD<sub>50</sub>). These data suggest that changes in MnSOD activity have a significant impact on the antioxidant status of mitochondria.

The results of this work indicate that chlorfenvinphos induces oxidative stress to rats liver mitochondria. In acute chlorfenvinphos intoxication, we demonstrated that the key role in the mitochondria damage play MnSOD and GSH pool, as well as accumulation of  $H_2O_2$ .

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