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Mitochondrial energy metabolism impairment and liver dysfunction following chronic exposure to dichlorvos

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ABSTRACT

Although the effects of acute pesticide poisoning are well known but, hardly any data exists regarding the health effects after long-term low-level exposure. Major unresolved issues include the effect of moderate exposure in the absence of poisoning. The present study elucidates a possible mechanism by which chronic organophosphate exposure (dichlorvos 6 mg/kg b.wt., s.c. for 12 weeks) causes liver dysfunction. Mitochondria, a primary site of cellular energy generation and oxygen consumption represent a likely target for organophosphate poisoning. Therefore, the objective of the current study was planned with an aim to investigate the effect of chronic dichlorvos exposure on liver mitochondrial electron transport chain (ETC), mitochondrial calcium uptake and its implications on the induction of liver enzymes and liver dysfunction in rodent model. Our results indicated decreased mitochondrial electron transfer activities of cytochrome oxidase along with altered mitochondrial complexes I and II activity. This decrease in the activities of electron transport complexes in turn affected the ATP synthesis and ATP levels adversely in the mitochondria isolated from dichlorvos (DDVP) treated rat liver. Mitochondrial preparation from DDVP treated rat liver demonstrated significant increase in mitochondrial Ca^{2+} uptake and increase ROS levels. The alterations in the mitochondrial calcium uptake, mitochondrial electron transfer enzyme activities and increase ROS levels in turn might have caused an increase in liver enzymes (ALT, AST and ALP). The electron micrographs of liver cells depicted morphological changes in mitochondria as well as nucleus following 12 weeks of exposure to DDVP. These studies provide an evidence of impaired mitochondrial bioenergetics which may lead to liver dysfunction after chronic exposure to dichlorvos.

1. Introduction

For centuries, pesticides have been used in agriculture to enhance food production by eradicating unwanted insects and controlling disease vectors. Among common pesticides: organophosphorus compounds (OP) are widely used in agriculture, medicine and industry. The extensive international use of pesticides (mainly organophosphorus compounds, OPs) results in numerous acute intoxications each year. Pesticides cause approximately 3 million poisonings and 200,000 deaths annually (Jeyaratnam, 1990). Organophosphate (OP)-based pesticides are widely used and have emerged as a major contributor to ill health associated with pesticides. These irreversible inhibitors of acetylcholinesterase (AChE) are a leading cause of death in agricultural countries globally (Karalliedde, 1999). In 1996, the American Association of Poison Control Centers reported 86,914 human exposures to pesticides in the United States only (Fengsheng et al., 1998). There were 50,000–70,000 thousand cases of acute pesticide poisoning reported from 27 provinces of China each year during the 1990s (Litovitz et al., 1996).

Organophosphates, including dichlorvos have been reported to exert their primary pharmacological and toxicological effects through the inhibition of an enzyme acetylcholinesterase (AChE), required for the transmission of impulse across the cholinergic synapse (Kaur et al., 2007). The acute effects of dichlorvos due to acetylcholinesterase inhibition are well documented; however significant data gaps exist regarding the adverse effects and the intervening biochemical events following chronic exposure to dichlorvos.

As a primary site of cellular energy generation and oxygen consumption, the mitochondrion presents itself as a likely target for organophosphate poisons which may explain noncholinergic toxicity of organophosphates (Kaur et al., 2007). Mitochondrial oxidative damage is a major factor in many human disorders, including mitochondrial hepatopathies, chronic hepatitis C, steatosis, early graft dysfunction after liver transplantation, ischemia–reperfusion

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injury, ageing and inflammatory damage (Fiskum and Starkov, 2003). Oxidative damage accumulates more in mitochondria than in the rest of the cells because electrons continually leak from the respiratory chain to form damaging reactive oxygen species (ROS) (Smith et al., 1999). This oxidative damage may modify mitochondrial proteins, DNA and lipids which may lead to mitochondrial bioenergetics failure leading to necrotic or apoptotic cell death (Smith et al., 1999). Further, it has been demonstrated that the sub cellular events that initiate cytotoxicity in the processes like apoptosis and necrosis are connected with structural and functional alterations in mitochondrial membranes exposed to organophosphate compounds (Carlson and Ehrich, 1999). The concern about major adverse health effects of low-level exposure to dichlorvos in general seem entirely unwarranted on the basis of currently available data, as it remains to be envisaged if chronic dichlorvos exposure in rats causes liver degeneration by impairing mitochondrial bioenergetics. The mechanism by which mitochondria transduce cell death signals likely involves interaction between mitochondrial reactive oxygen species and Ca^{2+}. The kinetic characteristic of mitochondrial calcium uptake and efflux mechanism dictate that above a certain threshold concentration of cytosolic Ca^{2+}, mitochondria rapidly accumulate and slowly release it (Murphy et al., 1996). We have already shown in our lab that chronic dichlorvos intoxication results in raised brain intracellular Ca^{2+} levels, and decline in Ca^{2+}–Mg ATPase activity (Raheja and Gill, 2002). In response to increase in cytosolic Ca^{2+} and mitochondrial Ca^{2+}, may cause Ca^{2+}-induced respiratory impairment, potentiating free radical generation, inflicting structural damage to mitochondria and ultimately apoptotic cell death. In addition to this, we have also reported that potential exposure of chronic dichlorvos can disrupt cellular antioxidant defense system which in turn triggers the release of cytochrome c from mitochondria to cytosol as well as caspase-3 activation in dichlorvos treated rat brains (Kaur et al., 2007). These studies provide an evidence of impaired mitochondrial bioenergetics and apoptotic neuronal degeneration after chronic low-level exposure to dichlorvos. There is a plenitude of published literature on acute toxicity in vitro, however, it remains to be envisaged the role of chronic organophosphate exposure in vivo in liver mitochondria and its implications on liver damage. Therefore, the present study was designed with an aim to carry out an investigation to find out the possible perturbations in liver mitochondrial Ca^{2+} levels, mitochondrial electron transport chain functions and apoptosis to broaden our understanding on the molecular mechanisms by which chronic low-level exposure to dichlorvos causes liver disfuction in rodent model that may help in designing of better therapeutic measures to combat organophosphate toxicity.

2. Materials and methods

Dichlorvos (2,2-dichlorovinyl dimethyl phosphate) was purchased from Hindustan Ciba Geigy Ltd., Mumbai, India. ATP, EDTA, EGTA, DTT and Tris–HCl, were purchased from Sigma Chemicals Co., St. Louis, MO, USA. All other chemicals used were of highest-grade commercial products.

2.1. Animals and their care

Male albino rats (Wistar strain) in the weight range of 100–120 g, at the beginning of the experiments were procured from the institute animal house. The animals were housed in polypropylene cages, and kept in well ventilated rooms on a 12-h light–dark cycle (lights on 7:00 a.m.). Animals were provided standard rat pellet diet (Hindustan lever Ltd., Mumbai, India) and water ad libitum. Rats used in this study were maintained and treated in accordance with the guidelines established by the Ethical and Practical Principles of the Use of Laboratory Animals and all procedures used in the present study complied with the Guide for the Care and Use of Laboratory Animals.

The animals were divided into following two groups (5 animals in each group):

2.1.1. Control group

Animals received an equal volume of corn oil (vehicle) as administered to the animals of dichlorvos treated group.

2.1.2. Dichlorvos treated group

Animals received 6 mg/kg b.wt./day dichlorvos dissolved in corn oil, s.c. for 12 weeks. After the completion of treatment, animals were fasted overnight and sacrificed by decapitation using sodium pentathol. The livers were removed, rinsed in ice cold physiological saline (0.9% NaCl). Ethical clearance for killing of animals was duly obtained from Institute's Animal Ethical Committee. The dose used in the present study was the same as was used in our previous studies (Kaur et al., 2007; Verma et al., 2009).

2.2. Isolation of mitochondria

Rat liver mitochondria were isolated by the method of Berman and Hastings (1999) as described previously (Kumar et al., 2008). A portion of liver was homogenized in isolation buffer with EGTA (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, pH 7.2). Homogenate was centrifuged at $1300 \times g$ for 5 min at 4 °C. Pellet was resuspended in isolation buffer with EGTA and spun again at $13{,}000 \times g$ for 5 min. The resulting supernatant was transferred to new tubes and topped off with isolation buffer with EGTA and again spun at $13{,}000 \times g$ for 10 min. Pellet containing mitochondrial rich fraction was resuspended in isolation buffer without EGTA.

2.3. Mitochondrial NADH dehydrogenase (complex I) activity

NADH dehydrogenase activity was measured spectrophotometrically by the method of King and Howard (1967), as described previously (Kaur et al., 2007). The method involves catalytic oxidation of NADH to NAD^+ with subsequent reduction of cytochrome c. The reaction mixture contained 0.2 mM glycyl glycine buffer pH 8.5, 6 mM NADH in 2 mM glycyl glycine buffer and 10.5 mM cytochrome c. The reaction was initiated by addition of requisite amount of solubilized mitochondrial sample and followed spectrophotometrically at 550 nm for 2 min. Enzyme activity was calculated on the basis of absorbance index of cytochrome c (reduced–oxidized), 19.2 mM^{-1} cm^{-1} and results expressed as nmol NADH oxidized/(min mg) protein.

2.4. Mitochondrial succinate dehydrogenase (complex II) activity

Succinate dehydrogenase activity was measured spectrophotometrically as per the method of King (1967), described previously (Kaur et al., 2007). This method involves oxidation of succinate by an artificial electron acceptor, potassium ferricyanide. The reaction mixture contained 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid, and 0.03 M potassium ferricyanide. The reaction was initiated by the addition of mitochondrial sample and change in absorbance was followed at 420 nm for 2 min.

2.5. Mitochondrial cytochrome oxidase (complex IV) activity)

Cytochrome oxidase activity was assayed in liver mitochondria according to the method of Sottocassa and Ernester (1967). The assay mixture contained 0.3 mM reduced cytochrome c in 75 mM phosphate buffer. The reaction was started by the addition of solubilized mitochondrial sample and change in absorbance was recorded at 550 nm for 2 min.

2.6. ATP synthesis

ATP synthesis was measured using a glucose/hexokinase trap system as described by Griffiths et al. (1977). Phosphorylation was determined in terms of disappearance of inorganic phosphorus. The assay mixture (1.1 ml) contained 0.25 M sucrose, 10 mM Tris–HCl, 22 mM glucose, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 0.5 mM EDTA, 20 U of hexokinase (EC units, 1 μmol substrate/min), 2 mM ADP (pH 7.4), 20 mM succinate and appropriate amount of mitochondrial protein. The reaction was terminated by addition of 10% trichloroacetic (TCA) acid after 20 min of incubation at 30 °C. The supernatant was assayed for inorganic phosphorus.

2.7. Mitochondrial ATPase (ATP hydrolysis)

ATPase was assayed using the method of Griffiths and Houghton (1974). The method involves measurement of inorganic phosphorus liberated following catalytic hydrolysis of ATP to ADP. The reaction mixture (1.0 ml) containing 5 mM ATP, 2 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.5) and appropriate amount of mitochondrial protein were incubated for 5 min at 30 °C. The reaction was terminated by the addition of 10% TCA and the supernatant assayed for inorganic phosphorus.

2.8. ATP levels

ATP levels were determined luminometrically using ATP Bioluminescence assay kit (Sigma, St. Louis, MO, USA) according to the provided protocol. Mitochondrial