Cellular and molecular mechanisms of dichlorvos neurotoxicity: Cholinergic, noncholinergic, cell signaling, gene expression and therapeutic aspects

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Inappropriate use of toxic chemicals is common in developing countries, where it leads to excessive exposure and high risks of unintentional poisoning. Risks are particularly high with the pesticides used in agriculture, poor rural populations live and work in close proximity to these compounds and often store these compounds in and around their homes. It is estimated that most of the death from pesticide poisoning occur in developing countries. Organophosphate insecticides have been extensively used in agriculture in developing countries. Dichlorvos is a synthetic insecticide and belongs to a family of chemically related organophosphate pesticides (OP). Toxicity of dichlorvos has been documented in accidental human poisoning, epidemiological studies, and animal models. In this review, molecular mechanisms of dichlorvos neurotoxicity have been described. Usage, biotransformation, environmental levels, general population and occupational exposure, effects on cell signaling receptors, mitochondrial metabolism, oxidative stress and gene expression of dichlorvos have been reviewed. Assessment of acute and chronic exposures as well as neurotoxicity risk for lifetime exposures to dichlorvos have also been considered. In addition special emphasis has been given to describe, the role of dichlorvos in the chronic neurotoxicity and its molecular targets that ultimately lead to neurodegeneration.

Keywords: Behavioral, Dichlorvos, Neurotoxicity, Nicotinic receptors, Organophosphate

Organic derivatives of phosphorus-containing acids are chiefly familiar as organophosphate (OP) pesticides. These compounds are anticholinesterase insecticides, widely used in agriculture, horticulture, veterinary medicine, public hygiene, and also used as nerve agents in chemical warfare. Lack of biopersistance of OPs in comparison with the organochlorines has made most countries to replace the organochlorines with OPs. Consequently their scale of use has increased in recent years. Pesticide poisoning remains a serious public health problem worldwide. According to the World Health Organization’s estimate, 3 million cases of pesticide poisoning occur every year, resulting in more than 250,000 deaths. This number also accounts for a substantial fraction of the almost 900,000 people worldwide who die by suicide every year. However, in developing countries, where the use of OP compounds is particularly widespread because of the hot climatic conditions, the number of deaths may be high. For example, pesticide poisonings are relatively common in countries such as Sri Lanka, Venezuela, Indonesia, South Africa, and Brazil. Acute OP compounds poisoning is an important cause of morbidity and mortality in India. Among the numerous pesticides that can result in death, organophosphate insecticides are the most common culprits because of their high toxicity.

Dichlorvos, an organophosphate, is a direct-acting cholinesterase (ChE) inhibitor. Although first synthesized in the late 1940s, its commercial manufacture started much later, in 1961. The annual production of dichlorvos was as high as 4.2 million pounds (lbs) in the late 1970s, and fell to 992,000 lbs by 1989. More recent estimates are not available, but are likely to be lower due to many recent cancellations of its use. Dichlorvos can be released into the environment as a major degradation product of other OP insecticides, such as trichlorfon, naled, and metrifonate. Dichlorvos can kill insects when ingested, or absorbed through the integument or via spiracles. It is commercially available under the trade names viz., Dedevap, Nogos, Nuvan, Phosvit and Vapona. Some common synonyms are Bayer 19149, DDVF, DDVP, ENT-20738, OMS 14, SD 1750 and C-177. The major mechanism for the action of dichlorvos is inhibition of acetylcholinesterase (AChE), leading to an increase in the level of acetylcholine.
acetylcholine in the synaptic cleft and hence producing both nicotinic and muscarinic signs and symptoms of intoxication in the peripheral and central nervous system like nausea, vomiting, lacrimation, salivation, bradycardia, miosis and finally death may occur due to respiratory failure. In agriculture, dichlorvos has been used to protect stored crops from insect damage. It was used in livestock industry to control external parasites on animals such as fleas and ticks. Dichlorvos was also added in animal feed as an anthelmintic (worming agent) for swine, horses and dogs. It was used in mushroom houses to control flies and insects and was added directly to the water in fish farms, to control fish parasites. The annual agricultural use of dichlorvos was estimated as 248,000 lbs during 1982. Estimates done in late 1980s indicate that 60% of dichlorvos used worldwide was for plant protection, 30% was for public hygiene and vector control, and 10% to protect stored crops. Dichlorvos was among the several organophosphorus pesticides (OP) shipped by the Department of Defense for use during the Gulf War. In water, dichlorvos hydrolyzes into dimethylphosphoric acid and dichloroacetaldehyde (DCA). Dichlorvos has also been reported to penetrate into the waxy layers of plant tissues, where it persists longer and undergoes hydrolysis to DCA. The occurrence of dichlorvos residues in the environment does not necessarily originate from the use of dichlorvos. They may also occur as conversion products of another widely used OP pesticide, trichlorfon. The air concentration of dichlorvos varies according to the method of application (strips, spray cans or fogging), temperature and humidity. Concentration has been reported to be in the range of 0.1-0.3 mg/m³ in the first week after using resin strips (1 strip/30 m³). Food samples, meals and unwrapped ready to eat foodstuffs exposed under practical conditions to dichlorvos generated by resin strips showed residues with in a range of 0.01-0.1 mg/kg. Food and beverages exposed to experimental air concentrations of 0.04-0.58 mg/m³ for 30 min, contained dichlorvos residues in the range of 0.005-0.5 mg/kg, with the exception of margarine, which contained up to 106 mg/kg.

**General population exposure to dichlorvos**

No quantitative information is available on the percentage of dichlorvos released to different environmental compartments. Dichlorvos can be released to any or all environmental media (air, surface water, groundwater, and soil). The general population is exposed to dichlorvos primarily through inhalation of contaminated indoor air, either during and/or immediately after application or through the use of polyvinyl chloride resin strips. The second major route of exposure to dichlorvos for the general population is through direct dermal contact with the chemical spray during domestic applications, contact with dichlorvos treated plant materials such as grass or ornamental plants or contact with other treated surfaces (e.g. furniture) in domestic or office buildings. In developing countries dichlorvos may still be applied by professional exterminators for insect control in buildings and in turf grass treatments. These applications create potential for some exposure for the general population through inhalation, dermal contact and oral intake, especially in children. In addition to the individuals occupationally exposed to dichlorvos, several groups within the general population may receive potentially higher inhalation exposures to dichlorvos. These groups include individuals living near factories where dichlorvos is produced or processed and those individuals living near hazardous waste sites where this compound is present. Although dichlorvos is not tightly bound to soil particles, ingestion of dichlorvos contaminated soil or soil where polyvinyl chloride resin strips have been disposed off, might be a route of exposure particularly for children. Ingestion of contaminated groundwater by individuals living in the vicinity of hazardous waste sites may be another possible source of exposure for both adults and children if these individuals use untreated well water as their primary source of drinking water.

**Acute cholinergic toxicity**

Dichlorvos exerts its toxic effects in humans and animals by inhibiting neural acetyl cholinesterase. If this enzyme is inhibited, acetylcholine accumulates in the synapse, resulting in increased firing of the postsynaptic neuron or increased neuroeffector activity. The consequences of increased cholinergic activity in the parasympathetic autonomic nervous system (muscarinic receptors) can include increased salivation, lacrimation, perspiration, miosis, nausea, vomiting, diarrhoea, excessive bronchial secretions etc. The effects of increased neuroeffector activity on skeletal muscles (nicotinic receptors) can include muscle fasciculations, cramps, muscle weakness and depolarization type paralysis. Effects on cholinergic...
synapses in the central nervous system (predominantly muscarinic) can result in drowsiness, fatigue, mental confusion, headache, convulsions and coma. These classical symptoms of organophosphate neurotoxicity increase in severity and rapidity of onset in a dose dependent manner. In addition to this, decreased vigilance, hallucinations, defects in expressive language and cognitive function, impaired memory, depression, anxiety or irritability and psychosis have been well reported after dichlorvos exposure\(^5\).

Clinical studies of acute and chronic toxicity

The clinical signs and symptoms of dichlorvos are generally attributable to acetylcholine accumulation and are commonly divided into three groups; muscarinic, nicotinic and central. There have been two clinical reports describing four patients suffering from severe poisoning from dichlorvos, taken orally, who survived after treatment and who showed delayed neurotoxic effects. Thus, although the possibility of neuropathy in human beings cannot be excluded, it is likely to occur only after almost lethal oral doses. When dichlorvos was administered orally to human volunteers (single or repeated doses of a slow-release PVC formulation), significant inhibition of red blood cell ChE activity was found at 4 mg/kg body weight or more. At 1 mg/kg body weight or more, plasma ChE activity was significantly inhibited. Daily oral doses of 2 mg dichlorvos/person for 28 days reduced plasma ChE activity by 30\%, but red cell ChE activity was unaffected\(^\text{11}\).

Human volunteers who were exposed to dichlorvos by inhalation for a certain period per day for a number of consecutive days or weeks showed ChE inhibition at a concentration of 1 mg/m\(^3\) or more, but not at 0.5 mg/m\(^3\). These results were confirmed in studies with pesticide operators who came into contact with dichlorvos. Hospitalized patients showed similar results after oral administration or exposure by inhalation. Sick adults and children and healthy pregnant women and babies in hospital wards treated with dichlorvos strips (1 strip/30 or 40 m\(^3\)) displayed normal ChE activity. Only subjects exposed 24 h/day to concentrations above 0.1 mg/m\(^3\) or patients with liver insufficiency showed a moderate decrease in plasma ChE activity. No significant effects on plasma or red blood cell ChE activity were observed in people exposed to the recommended rate of one dichlorvos strip per 30 m\(^3\) in their homes over a period of 6 months, even when the strips were replaced at shorter intervals than that normally recommended. The maximum average concentration in the air was approximately 0.1 mg/m\(^3\). In factory workers exposed to an average of 0.7 mg/m\(^3\) for 8 months, significant inhibition of plasma and red blood cell ChE activity was found\(^\text{11}\).

Delayed neurotoxicity of dichlorvos

Neurotoxic potential of organophosphorus compounds have already been described in details\(^\text{11}\). Several studies have shown that dichlorvos does not produce delayed neurotoxicity in pre-medicated hens, whether it is administered orally or subcutaneously\(^\text{17-19}\). Contradictory to these studies Choudhary et al.\(^\text{20}\) reported, single subcutaneous dose of dichlorvos (200 mg/kg body weight) resulted in marked changes in the dopaminergic neurotransmitter system in terms of increased levels of both dopamine and norepinephrine along with significant increase in the activity of both the catecholamine synthesizing enzymes, tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase. This increase was accompanied with a concomitant decrease in the activity of major degradative enzyme, monoamine oxidase. Scatchard plot analysis revealed a significant decrease in both K(d) and B(max) for dopamine D2 receptors. Administration of nimodipine, a centrally acting calcium channel blocker, along with dichlorvos restricted all these alterations to within control values and could also ameliorate certain behavioral deficits by maintaining the dopaminergic neurotransmitter system. The study underlines the importance of alterations in the dopamine system as a possible causative mechanism behind the behavioral and functional changes associated with delayed neurotoxicity. In addition to this, dichlorvos treatment also led to a consistent increase in the activity of both microtubule associated protein kinases viz. Ca\(^{2+}\)/Calmodulin-dependent and cAMP dependent protein kinases, at all post exposure intervals (day 7, 15 and 21) as compared to that of controls. Autoradiography followed by micro-densitometric studies demonstrated enhanced phosphorylation of 55 and 280 kDa proteins in dichlorvos-exposed animals. These two proteins were confirmed to be tubulin and microtubule associated protein-2 (MAP-2) by western blotting. The hyperphosphorylation of these two proteins was shown to interfere with the assembly of neuronal microtubules as shown by
electron microscopic studies that may eventually lead to possible disruption of neuronal cyto-architecture resulting in axonal degeneration. The inhibition of brain neurotoxic esterase (NTE) without signs of ataxia has been observed. Johnson et al. reported mild signs of ataxia in pre-medicated hens 2 weeks after a single massive subcutaneous dichlorvos dose (100 mg/kg body wt) and severe inhibition of NTE in peripheral nerve, spinal cord, and brain. However, no ataxia was in pre-medicated hens given the same dose through the same route. These hens showed severe inhibition of brain NTE but far less inhibition of spinal cord NTE. It appears that ataxia arises from the inhibition of spinal cord NTE. When the dose was repeated 1-3 days after the first dose, spinal cord NTE inhibition increased and the hens became ataxic.

We also reported delayed neurotoxicity potential of dichlorvos. In vitro, dichlorvos caused a concentration and time-dependent decrease in the activity of NTE. The Ki of dichlorvos for NTE was calculated to be $1.28 \times 10^3 \text{ M/min}$. In vitro reactivation and ageing studies revealed that dichlorvos-inhibited NTE became refractory to activation by potassium fluoride after 5 min in the ageing medium, thus indicating the formation of an aged complex between dichlorvos and NTE. In vivo also, dichlorvos (200 mg/kg body wt) given as a single subcutaneous dose inhibited NTE in brain at various intervals after exposure (24 h, 10 days, and 21 days). The delayed neurotoxicity potential of dichlorvos was finally confirmed by the rota rod test, which revealed severe motor deficit in all the exposed animals.

**Chronic neurotoxicity**

Previous years have seen a continuous interest in studies concerning the effects of sub lethal acute exposure to various OPs or chronic contact with organophosphate pesticides. There is increasing evidence that OPs may also cause a long-term, persistent chronic neurotoxicity following either a single acute high-dose exposure or repeated exposures to low-level, subclinical doses of OPs. The clinical and epidemiological data in support of chronic OP neurotoxicity presents with pathological lesions in both the PNS and CNS, but it is the latter that is primarily responsible for presenting neurologic symptoms and changes in neurobehavioral performance, reflecting cognitive and psychomotor dysfunction. The most sensitive manifestation of chronic OP neurotoxicity is a general malaise lacking in specificity and related to mild cognitive dysfunction, similar to that described for Gulf War syndrome. The mechanisms underlying these effects are not known, and the role of AChE inhibition is controversial and may vary depending on the exposure parameters. Chronic neurotoxicity subsequent to a single acute exposure to OPs may be triggered by AChE inhibition. In fact, acute sub lethal doses of OPs were shown to have long-term effects in humans. Oral administration of dichlorvos to rat (70 mg/kg) inhibited not only AChE but also hexokinase, phosphofructokinase, lactate dehydrogenase and glutamate dehydrogenase. Dichlorvos administration also caused significant depletion in the brain glycogen content along with increased glycogen phosphorylase activity. Repeated administration of 50% of LD50 (i.e., 40 mg/kg body wt per day for 10-21 days), caused myelin pallor and micro-vacuolation of the white matter. It seems likely that primary degeneration of axons and secondary myelin sheath abnormalities caused the spongy tissue loosening observed under the electron microscope.

In studies by Ali et al., male rats were given 3 mg dichlorvos/kg per day intraperitoneally for 10 days. Following perfusion-fixation, sections of cerebellum and spinal cord were studied with the electron microscope. An abnormal increase in the number of mitochondria in the spinal cord was found. Myelin degeneration was detected in the spinal cord and myelin figures were occasionally noted within oedematous dendrite profiles. Another study of dichlorvos neurotoxicity involved the investigation of lipid peroxidation. This entails the direct reaction between oxygen and lipids to form free-radical intermediates and semi-stable peroxides. Major cellular components, such as membranes and subcellular organelles, are damaged by these free radicals. Dose-dependent increase in the rate of lipid peroxidation in various regions of the brain of the rat after ip administration of dichlorvos (at concentrations ranging from 0.6 to 3 mg/kg body wt, daily) for 10 days have been reported. Also, there was an increased incidence of lipofuscin-like pigment in the Purkinje cells of the cerebellar cortex. Julka et al. observed dichlorvos exposure (5 mg/kg body wt, ip) cause significant increase in the activities of the antioxidant enzymes superoxide dismutase (SOD) and catalase. Dichlorvos exposure also resulted in a significant decrease in glutathione...
peroxidase activity. The decreased levels of both reduced and oxidized glutathione as observed on dichlorvos exposure affected the GSH/GSSG ratio. These results indicate that the enzymes SOD and catalase may enhance the disposal of potentially toxic radicals. Furthermore, the decrease in GSH levels may be a mechanism for the detoxification of dichlorvos in the brain. Maslinska et al.\textsuperscript{36} found that dichlorvos (dose levels of 4-8 mg/kg body weight for 10 days) affected the phospholipid-protein balance in the brain of rabbits. The animals were exposed during the postnatal "critical" life period, which constitutes a turning point in the development of the brain. At this time, the neurons have already undergone considerable arborization, and myelination and vascularization are expanding rapidly. In addition, the overall oxygen consumption is reaching its steepest rate of increase. In the myelin sheaths under formation, several phospholipids are deposited. The authors found changes in the phospholipid-protein ratio which correlated well with the observed delay in myelin sheath formation. Ultrastructural changes in certain subcellular organelles may be connected with the change in this ratio, since it is crucial to the structural and functional properties of the membranes and enzymes bound to them.

Kobayashi et al.\textsuperscript{37,38} investigated the concentration of total, free, labile-bound and stable-bound ACh in the brain of rats given single or multiple subcutaneous injections of dichlorvos (0.2-4 mg/kg body weight). The results suggest that alterations in the mobilization and storage of ACh in the central cholinergic nerves may be induced. The time course for ACh accumulation was measured in rat brain regions after intravenous treatment with 15 mg dichlorvos/kg body weight\textsuperscript{39}. The striatum had the highest rate of accumulation and the cerebellum the lowest. The calculated turnover time for the different regions of the brain was between 0.9 and 5.6 min.

In studies by Ali and Hasan\textsuperscript{40} and Ali et al.\textsuperscript{34} rats were given ip 3 mg dichlorvos/kg body weight per day for 10 or 15 days. The concentrations of dopamine, norepinephrine, and 5-hydroxytryptamine (5-HT) were significantly decreased in different parts of the brain, and 5-HT was significantly increased in the spinal cord. A single dose or short-term (12 weeks) treatment of rats with high concentrations of dichlorvos, which produced brain ChE inhibition, resulted in decreased norepinephrine levels in the brain\textsuperscript{41}. From these studies, it was suggested that the metabolism of catecholamines and 5-HT may be disturbed by dichlorvos.

**Dichlorvos and cell signaling receptors**

Due to the ubiquitous distribution of both nicotinic and muscarinic cholinergic receptors throughout the body, exposure to OP compounds has widespread toxic consequences in several target organs. Virtually all cholinergic synapses can be affected by exposure to anticholinesterase compounds such as OPs. These include autonomic postganglionic parasympathetic nerve endings, sympathetic and parasympathetic ganglia, motor end plates of skeletal muscle, and, of course, various regions of the CNS. Hyperactivity at these synapses due to accumulation of ACh causes a variety of symptoms mediated by overstimulation of muscarinic and nicotinic receptors.

Density of receptors with a stereo specific binding site for nicotine in the mammalian brain is only 1% of that of muscarinic receptors\textsuperscript{42}. In the brain, the highest concentrations of nicotinic receptors are found in the thalamus, cortex, superior colliculus, and striatum, whereas the lowest concentrations occur in the piriform cortex and hippocampus. Thus, the distribution of nicotinic receptors in the CNS clearly differs from that of muscarinic receptors. It is also quite evident that most of the cholinergic effects of OPs in the CNS are mediated via muscarinic rather than nicotinic receptors. This is important because the most dramatic toxic actions of OPs are mediated via their effects on cholinergic receptors in the CNS and subsequent stimulation of other neurotransmitter systems in the brain, as well as via cholinergic receptor stimulation in other target organs, subsequent to the initial effects of OPs on AChE and the cholinergic systems.

In addition to their indirect effects on muscarinic receptors through AChE-mediated changes in ACh levels, many OPs can affect their expression and function directly. Paraoxon, dichlorvos, and tetraethyl pyrophosphate (TEPP) were found to be non-competitive antagonists of muscarinic receptors in bovine caudate nuclei labeled with \(^{[3]H}\) quinuclidinyl benzilate (\(^{[3]H}\)-QNB) at concentrations that had no effect on AChE activity. Direct effects of OP compounds on muscarinic receptors were studied by using rat brain membranes or cultures of human neuroblastoma N1E-115 cells\textsuperscript{43}. Recently, it has been reported that dichlorvos exposure causes significant reduction in the expression of \(M_1\), \(M_2\) and \(M_3\)
muscarnic receptor subtypes in high dose group animals whereas, in low dose group animals where AChE is not inhibited, the expression of only M2 receptor was found to be reduced significantly. QNB binding to the muscarinic receptors were carried out in the synaptic plasma membranes of the rat brain in the presence of varying concentrations of [3H] QNB (0–2 nM). Non-specific binding was carried out in the presence of atropine (1 µM). The Bmax for the 6.0 mg/kg body wt. dichlorvos exposed group was 26% and for 1.0 mg/kg body wt. dichlorvos exposed group was 22% lower than the control group (982.8 fmol/mg) animals. The affinity constants (Kd) for control, low and high dose group animals were 0.23, 0.23 and 0.022 nM, respectively. So, marked reductions in [3H] QNB binding were seen immediately after the cessation of the exposure, indicating a marked reduction in muscarinic receptor numbers.

Effects of mitochondrial metabolism and oxidative stress

Reactive oxygen species and their destructive nature is known for a long, but their diverse pathophysiological effects on vital organs are still of great interest. Oxidative stress can be defined as the imbalance between the production of free radicals capable of causing peroxidation of the lipid layer of cells and the body’s antioxidant defense. Free radicals are defined as atoms or molecules that contain one or more unpaired electrons. The toxicity of many xenobiotics is associated with the production of free radicals, which are not only toxic themselves, but are also implicated in the pathophysiology of many diseases. For example, there is extensive evidence for oxidative stress as an important mechanism of neurodegeneration in Alzheimer’s disease. Other diseases include Parkinson’s disease, cataracts, atherosclerosis, neoplastic diseases, diabetes, chronic inflammatory diseases of the gastrointestinal tract, aging of skin, asthma, and many others.

Pesticide induced oxidative stress has also been a focus of toxicological research for the last decade as a possible mechanism of toxicity. Several studies have been conducted to determine whether oxidative stress in humans or animals results from various agents in this group and is associated with their toxic effects. To understand the exact nature of oxidative stress, it is necessary to describe the principles of free radical production and the body’s normal defense system.

The balance between the production of free radicals and antioxidant defenses in the body has important health implications: if there are too many free radicals or too few antioxidants for protection, a condition of oxidative stress develops, which may cause chronic and permanent damage. Pesticides may induce oxidative stress, leading to generation of free radicals and alteration in antioxidants, oxygen free radicals, the scavenging enzyme system and lipid peroxidation.

There is growing evidence supporting the involvement of ROS and reactive nitrogen species in excitotoxicity injury. Excessive activation of cholinergic and glutamatergic receptor is thought to be responsible for excitotoxicity. During normal respiration, small amounts of ROS are produced as by products of the ETC process. However, perturbations in mitochondrial respiration can lead to excessive ROS generation and inundate cellular antioxidant capacity, leading to DNA damage, lipid peroxidation, protein modification, and eventually cell death.

Common initiating mechanism of excitotoxicity is thought to be frequent stimulation of nicotinic acetylcholine receptor at the mammalian neuromuscular junction and muscaranic, nicotinic and glutamatergic receptor in brain. It has been hypothesized that increased ACh levels following AChE inhibition activate glutamatergic neurons causing the release of glutamate, which ultimately results in excitotoxicity via increased intracellular calcium and activation of nitric oxide synthase following NMDA receptor activation. There is a support for the proposed mechanism of chronic OP neurotoxicity in that it has been demonstrated that nitric oxide synthesis inhibitors block OP-induced seizures. However, the role of AChE inhibition in this sequence of events has yet to be established. In contrast, chronic OP neurotoxicity induced by repeated exposures to subclinical OP doses has been reported to occur in the absence of AChE inhibition, suggesting that mechanisms other than anticholinesterase activity mediate the neurotoxic effects elicited by this exposure scenario. However, what these mechanisms are has yet to be established. Our results also indicated decreased mitochondrial electron transfer activities of cytochrome oxidase (complex IV) along with altered mitochondrial complex I, and complex II activity, which might have resulted from elevated mitochondrial calcium uptake. The alterations in the mitochondrial calcium uptake and mitochondrial...
electron transfer enzyme activities in turn might have caused an increase in malondialdehyde, protein carbonyl and 8-hydroxydeoxyguanosine formation as a result of enhanced lipid peroxidation, and as well as protein and mtDNA oxidation. All this could have been because of enhanced oxidative stress, decreased GSH levels and also decreased Mn-SOD activity in the mitochondria isolated from dichlorvos treated rat brain. In addition to disruption of cellular antioxidant defense system, chronic dichlorvos exposure also triggers the release of cytochrome c from mitochondria to cytosol as well as activates caspase-3 also. Low-level long-term dichlorvos exposure finally resulted in oligonucleosomal DNA fragmentation, a hallmark of apoptosis. These studies provide an evidence of impaired mitochondrial bioenergetics and apoptotic neuronal degeneration after chronic low-level exposure to dichlorvos, (Fig. 1). These results are also in accordance with other’s results and suggest an intriguing possibility that repeated exposures to sublethal or subclinical doses of OPs increases apoptotic neuronal death via oxidative stress.

The experimental evidence supports the hypothesis that OPs modulate intracellular signaling pathways downstream of receptors and suggests that the diverse neurotoxic effects of many OPs may reflect their influence on multiple intracellular signaling pathways. Functional studies examining the effects of dichlorvos on signaling events downstream of muscarinic receptor activation further support the hypothesis that dichlorvos can interact directly with M2 receptors. Activation of M2 and M4 receptors generally reduces the activity of adenylyl cyclase, which decreases cAMP production, whereas activation of M1, M3, or M5 receptors increases phosphoinositide-specific phospholipase C activity, which increases release of inositol trisphosphate,
Chronic dichlorvos exposure (6 mg/kg body wt./day) for a period of 8 weeks caused significant reduction in both high affinity (HA) and low affinity (LA) choline uptake (CU), with maximal effect being observed in the brain stem followed by cerebellum and cerebrum. Muscarinic receptor binding was significantly decreased in brain stem and cerebellum as reflected in the decreased receptor number (Bmax), without any change in the binding affinity [K(d)] of the receptors. Dichlorvos treatment caused marked inhibition in cAMP synthesis as indicated by decreased adenylate cyclase activity as well as cAMP levels in cerebrum, cerebellum and brain stem. These studies show that dichlorvos may interact with muscarinic receptor-linked second messenger system and this could be a potential mechanism for the neurotoxic effects observed after repeated exposure to low levels of dichlorvos, which are unexplainable on the basis of cholinergic hyperactivity. Interestingly, the mechanism of action for the OP steroidogenesis inhibitor, diethylumbelliferyl phosphate, is also believed to be mediated through an interaction with the cAMP/PKA pathway. OPs can activate CaM kinase II. Raheja and Gill have also reported that chronic dichlorvos administration caused significant rise in the intrasynaptosomal calcium levels. The activity of major calcium expelling enzyme i.e. Ca\(^{2+}\)-ATPase was found to be reduced. Also, the depolarization induced calcium uptake via voltage operated calcium channels increased significantly. Concomitant to the increase in intrasynaptosomal calcium, calpain activity was found to be increased. Dichlorvos could mediate through modifications in the intracellular calcium homeostasis which may lead to impaired neuronal function.

**Effects on signaling and gene expression**

Signaling pathways identified as potential targets in OP neurotoxicity can modulate gene expression via alterations in the expression levels or activational status of transcription factors. One transcription factor of considerable interest in OP neurotoxicity is Ca\(^{2+}\)/cAMP response element binding protein (CREB), which is activated via phosphorylation by a variety of signaling pathways, including cAMP/PKA, MAP kinase/ERK, p38, and CaM kinase II. Numerous studies have indicated that CREB is critical to several forms of use-dependent synaptic plasticity and transcription-dependent forms of memory, and evidence supports a major role for CREB in cell survival and differentiation during brain development. Since impairments of brain development and memory function are two primary neurological effects observed in laboratory studies with OPs, Schuh et al. have hypothesized that the mechanisms underlying these effects may include alterations in the expression or activational status of CREB. OPs caused similar effects in primary cultures of hippocampal neurons. Mechanism(s) by which OPs activate CREB is not known, but is probably not mediated by OP effects on adenylyl cyclase activity, which are predominantly inhibitory. There is documentation of OP effects on other transcription factors important in neurodevelopment and synaptic plasticity. Thus, OPs elevate levels and activation of c-fos cause developmental stage-specific changes in AP-1 and Sp-1 expression and DNA binding activity and stimulate phosphorylation of c-Jun. Recently Verma et al. have reported that dichlorvos at low dose exposure leads to reduction in the signal transduction cascade linked to muscarinic receptor subtypes and adenylyl cyclase-linked signaling pathway was impaired. This finally leads to significant reduction in the phosphorylation of CREB in both low dose and high dose group animals.
Neurobehavioral implication of dichlorvos exposure

Importance of neurobehavioral toxicity in risk assessment lies in the fact that behaviour can be regarded as the net output of the sensory, motor and cognitive functions occurring in the nervous system and can serve as potentially sensitive endpoints of chemical induced neurotoxicity. Neurobehavioral sequelae of acute and chronic organophosphate exposure have been described in the literature for decades\textsuperscript{60, 61}.

Due to the almost ubiquitous nature of cholinergic pathways and synaptic networks, organophosphate pesticide such as dichlorvos by virtue of their being anticholinergic agent may exert the widest range of behavioural effects. Schulz \textit{et al.}\textsuperscript{62} have reported dose related alterations in open field behaviour of animals along with impairment in cognitive functions following dichlorvos exposure during gestation. Dose and time related exposure to dichlorvos has been reported to alter the EEG pattern and the nerve conduction velocity in treated animals, with concomitant decrease in the AChE levels.

Significant motor deficit in terms of altered motor functions and coordination following a delayed neurotoxic insult by dichlorvos have been reported\textsuperscript{63}. While the effects of acute dichlorvos poisoning are well characterized, significant lacunae exist as regards the chronic effects, particularly those associated with behavioural aspects of dichlorvos exposure. We reported chronic dichlorvos administration caused a marked decrease in both the ambulatory and stereotypic components of spontaneous locomotor activity of rats. The muscle strength and coordination of the dichlorvos-treated animals was also significantly impaired. Besides, a marked deterioration in the memory function assessed in terms of the conditioned avoidance response was discernible at the end of the treatment schedule in the experimental animals. Verma \textit{et al.}\textsuperscript{59} also explained the molecular mechanism of neurobehavioral impairments, seen after chronic dichlorvos exposure, may be due to significant reduction of M\textsubscript{2} muscarinic receptor linked adenylyl cyclase signaling pathway and reduction in the phosphorylation of CREB (Fig. 3).

Recent advances in treatment of organophosphate poisoning

Therapeutic treatment against OP poisoning has been studied extensively since World War II. The characteristic signs and symptoms of acute OP intoxication are generally thought to arise from the inhibition of acetylcholinesterase and the ensuing cholinergic crisis. Nevertheless, the currently adopted treatments (such as those supplied by atropine-oxime autoinjectors) are not fully satisfactory under actual life threatening conditions\textsuperscript{64}. Atropine is suitable to counteract the muscarinic effects of excess acetylcholine, as bronchospasm, bronchorrhoea and

![Fig. 3—in-Chronic dichlorvos exposure, leads to significant reduction of M\textsubscript{2} muscarinic receptor linked adenylyl cyclase signaling pathway and reduction in the phosphorylation of CREB](image-url)
pulmonary oedema. But it is ineffective at nicotinic receptor sites, e.g. at respiratory muscles. To cope with the respiratory problems, antidotes reactivating inhibited AChE have been developed. Their clinical effectiveness, however, is still a matter of controversy. The reason for this uncertainty is caused by the difficulty in clearly assessing oxime effects due to the high complexity of the various microscopic reactions involved and problems in recording the distinct clinical changes in sedated and artificially ventilated patients during cholinergic crisis. In order to counteract the toxic effects of OP at striated muscles additional therapeutic measures are indispensable. Here reactivating oximes can be expected to act as specific antidotes. The search for oxime-based reactivators dates back to the early 1950s, starting with hydroxylamine and hydroxamic acids. Later on, ketoximes and aldoximes were investigated. Meanwhile, more than 1500 oximes have been tested, but only few have been studied for human use. One of the major drawbacks in the development of oximes as antidotes against poisoning is the inability to perform clinical studies in humans. Therefore, different in vitro and in vivo models have been used to evaluate the efficacy of oximes. One of the major problems in the assessment of oxime efficacy in humans is the difficulty to extrapolate animal data to humans.

Presently, the main focus in oxime research is on the development of broad spectrum reactivators being able to counteract the effects of structurally different OPs. While 2-PAM is a generally weak reactivator of OP-inhibited AChE, obidoxime effectively reactivates human AChE inhibited by different OP pesticides. HI 6 has been shown to be effective with most nerve agents, including cyclosarin, but is a weak reactivator of pesticide-inhibited AChE and fails to reactivate tabun-inhibited enzyme. MMB-4 is superior to 2-PAM, but mostly less potent than obidoxime and HI 6. Presently, Hagedorn oxime HLö 7 may be considered as a broad spectrum reactivator active against organophosphonates, phosphates and phosphoramidates, but this oxime is difficult to synthesize and rather unstable in solution. Data with newer oximes are scarce and are mostly generated using animal AChE.

Organophosphate poisoning can result in seizures and subsequent neuropathology. One possible therapeutic approach would be to employ adenosine A(1) receptor agonists, which have already been shown to have protective effects against organophosphate poisoning. Harrison et al. have investigated the ability of several adenosine A(1) receptor agonists to inhibit epileptiform activity induced by the organophosphate sarin, in CA1 stratum pyramidale of guinea pig hippocampal slice. Efforts have now been expanded to identify a catalytic protein capable of not only binding, but also rapidly hydrolyzing the standard threat nerve agents. Recent work has focused on paraoxonase-1(PON1). The specific hydrolytic activity of PON1 paraoxonase/arylesterase enzymes in liver and blood provide a natural barrier against the entry of organophosphate toxins into the central and peripheral nervous systems. Cowan et al. have reported that boosting serum levels of PON1 enzymes by a gene delivery vector raises the threshold for organophosphate toxicity by hydrolytic destruction before the chemical can enter the brain. Moreover, recently Stevens et al. have reported that engineered recombinant human PON1 purified from Escherichia coli protects against organophosphate poisoning.

Conclusion

As dichlorvos displays relatively limited selectivity between insects and non target species, including humans, concerns on their potential adverse effects in human populations will continue. Further investigation of the low level long-term exposure leading to dichlorvos neurotoxicity should be undertaken. More information is required to properly evaluate the chronic exposure to humans leading to neuropsychological manifestations. Neurotoxicity studies at low concentrations with large groups of rodents must be undertaken to settle the present dilemma of acetylcholine independent neurotoxicity vs. acetylcholine independent neurotoxicity. Moreover, an epidemiological study of long-term exposed workers might reveal information concerning the toxicology of dichlorvos as well as its possible neurotoxicity to humans. The issues discussed in this review represent real-life problems, with clinical, societal and legal ramifications. Continuing research in all these areas, and others not mentioned, is warranted. The extreme toxicity of dichlorvos highlights the need for a more complete understanding of it’s mechanisms of toxic actions. This information also provides new insights into neurotoxicity and opens new vistas for research to explore mechanisms of OP toxicity. Even though, the
overall toxicity and the general mechanisms of dichlorvos toxic actions have been rather well clarified over the years, a more thorough understanding of the cascades of cellular and subcellular toxic events of dichlorvos in the nervous systems is needed for effective prevention and treatment of dichlorvos induced poisoning.

References
11 Choudhary S, Joshi K & Gill K D, Possible role of enhanced microtubule phosphorylation in dichlorvos induced delayed neurotoxicity in rat, *Brain Res*, 897 (2002a) 60.
38 Kobayashi H, Yuyama A & Chiba K I, Cholinergic system of brain tissue in rats poisoned with the organophosphate 0,0-dimethyl-(2,2-dichlorovinyl) phosphate, *Toxicol appl Pharmacol*, 82 (1986) 32.
dimethanesulfonate, a potent bispyridinium-dioxime against anticholinesterases, *Arch Toxicol*, 66 (1992) 603


