Mitochondrial complex I inhibition as a possible mechanism of chlorpyrifos induced neurotoxicity

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ABSTRACT

Background: Organophosphates (OPs) represent the most widely used class of pesticides. Although perceived as low toxicity compounds compared to the previous organochlorines, they still possess neurotoxic effects both on acute and delayed levels. Delayed neurotoxic effects of OPs include OPIDN and OPICN. The mechanisms of these delayed effects have not been totally unraveled yet. One possible contributor for neurotoxicity is mitochondrial complex I (CI) inhibition. **Purpose:** in the present study we evaluated the contributing role of (CI) inhibition in chlorpyrifos (CPF) induced delayed neuropathy in hens. **Methods:** Experimented birds received 150 mg/kg of CPF, and evaluated behaviorally and biochemically. **Results:** CPF treated hens received 150 mg/kg and developed signs of delayed neurotoxicity, which were verified by NTE inhibition. These effects were paralleled by CI inhibition and decrease in ATP level. **Conclusions:** The data confirms the possible role of CI inhibition in CPF induced delayed neuropathy.

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Introduction

Pesticides are a group of chemicals that are "used to prevent, destroy, repel or mitigate any pest ranging from insects, animals and weeds to microorganisms.[1] They are widely used in various settings including agriculture, public health and households. Owing to their different origins, pesticides have different classifications: commonly referred to by the organisms that they are designed to control (e.g., herbicides, insecticides, or fungicides). They may also be grouped by their chemical class e.g., organophosphate insecticides or triazine herbicides.[2]

Organophosphorus pesticides (OPs) are currently the most commonly utilized pesticides in the world. They combine almost 40 different chemical members registered by the US-EPA (www.epa.gov). About 70% of all insecticides used in USA are OPs, constituting about 73 million pounds in 2001.[3] Moreover, OPs problem involves both developed and developing countries.[4] The cholinesterase inhibiting effect of OPs represents the main mechanism underlying their acute toxicity. However, two other neurotoxic syndromes have been linked to OPs. The first is organophosphate-induced chronic neurotoxicity (OPICN). This entails neurobehavioral alterations with possible neurodegenerative effects that have been characterized.[5] The second syndrome is organophosphates-induced delayed neurotoxicity (OPIDN).[6–8] This is characterized by a primary wallerian degeneration of the axons ending with possible paralysis. Although, OPIDN has been attributed to inhibition of brain Neurotoxicity Esterase Enzyme (NTE),[9] such mechanism has not been linked to OPICN. Chlorpyrifos (CPF), an organophosphorothioate insecticide, (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate)[10] that has been shown to induce OPIDN and OPICN. Subclinical exposure to CPF has led to persistent long-term cognitive dysfunction and defects in concentration.[11] These neurobehavioral effects have been noticed following exposure to low-level of CPF in 22 patients. Moreover, daily dermal application of 1.0 mg/kg of chlorpyrifos caused sensorimotor disturbance in rats.[12]

Affection of mitochondrial function plays an important role in the development of many neurodegenerative disorders.[13] These eukaryotic intracellular organelles are responsible for utilizing the energy by cells. Inside the mitochondria, oxidative phosphorylation (OXPHOS) pathway harvests the energy in nutrients and transforms it into adenosine triphosphate (ATP).[14] Mitochondrial dysfunction leads to impairment in energy metabolism and oxidative stress, effects that are linked to many neurodegenerative conditions. The OXPHOS system, which is embedded in the lipid bilayer of the mitochondrial inner membrane, is the final biochemical pathway in the energy production of the cell.[15] This system consists of five enzyme complexes with two mobile electron carriers. NADH: Ubiquinone oxidoreductase (complex I) the first and the largest of the five complexes is one of the two entry points of the OXPHOS system; complex II being the other. It initiates electron transfer by oxidizing NADH and using the lipid soluble ubiquinone as the electron acceptor.[16] Complex I (CI) inhibition plays a critical role in many nervous disorders e.g. Parkinson's disease (PD), tauopathy, Alzheimer's disease (AD) and many others. Many toxicants have shown CI inhibition effects e.g. rotenone, annonacine and MPP+. Recently, OPs related neurobehavioral complications have been linked to CI inhibition. Although some have studied this hypothesis *in-vitro,* the *in-vivo* study on hens (which are the most sensitive species for OPIDN) has not been carried out yet. In the present study we tested the hypothesis that affection of mitochondrial complex I by CPF can contribute to development of chloropyrifos induced neurological complications inhens.

Methods

Chemicals

Chlorpyrifos (99%) (*O*,*O*-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate Phenyl Valerate) was prepared following the method of Johnson.[17] Mipafox (*N*,*N*-di-isopropyl diamidophosphorofluoridate), Tri-ortho-Cresyl Phosphate (TOCP, 99%), Bicinchonic acid (BCA), Paraoxon (O,O-diethyl-4-nitrophenyl phosphate), acetylcholine iodide, and S-butyrylthiocholine iodide were obtained from Sigma Labs, St. Louis, MO.

Animals

Test Animals

Healthy, young adult laying hens, 18 months old, and weighing 1.5 ± 0.03 kg were purchased from local breeders. These hens were vaccinated against common chicken diseases and were considered specific pathogen and medication free and without abnormalities of gait. Groups of five hens were placed in 3 × 3 × 3 ft stainless- steel cages in rooms with a temperature of 22–24°C and with a 12-hour chicken feed and free water supply.

Determination of LD_{50}

The acute oral LD_{50} values for chlorpyrifos were determined in hens unprotected from cholinergic toxicity. Groups of 3–5 hens were given single oral doses ranging from 10 to 100 mg/kg of either test compounds. The LD_{50} values were determined using the method of Litchfield and Wilcoxon.[18]

Treatments

In this study two experiments were carried out: a 96-hour study to determine brain and blood enzymes and a 16-day study to assess clinical changes. In both studies, 30 minutes prior to administration of test compounds, each hen received an intramuscular injection of 20 mg/kg (0.1 ml/kg atropine sulfate of 200 mg atropine sulfate/ml saline). Glycerol was used as a vehicle for CPF, at 1 ml/kg. One group of five hens was treated with a single oral dose of 150 mg/kg of CPF. Birds were monitored for signs of acute toxicity of OPs and given atropine sulfate and 2-PAM as needed during the first 96 hours. Positive control hens were given 750 mg/kg TOCP without protection from acute toxicity. Vehicle control hens received 1 ml/kg of the vehicle.

In the second experiment, five hens were treated with CPF (150 mg/kg), or the glycerol/oil vehicle. Animals were supported with 2-PAM and atropine sulfate as needed over the first five days following treatment. During the 16-day experiment, hens from treatment group and controls were carefully observed daily in and outside the cage for signs of behavioral abnormalities, disturbance in locomotion, ataxia and paralysis. Neurologic deficits characteristic of OPIDN were categorized into four stages of ataxia before the onset of paralysis: T_1: mild ataxia, T_2: moderate ataxia, T_3: severe ataxia and T_4: ataxia with near paralysis as previously described.[19]

Enzymatic Studies

Preparation of Tissues

At 96 hours after dosing test compound treated and control hens were sacrificed by decapitation. Brains were removed and homogenized (1% x/v) in 50 mM Tris-HCl buffer, pH 8.0 with 0.1 mM EDTA and Neuropathy Target Esterase (NTE) assay was performed immediately.

Blood samples were collected into heparinized tubes and spun in a centrifuge (Beckman Model J2-21 Centrifuge; Beckman Instruments Corporation; CA) at 5000 g for 30 minutes at 40°C using a JA-20 rotor to separate plasma and red blood cells. All samples were stored at –70°C until use.

Determination of Enzyme activity

Neurotoxicity Target Esterase assay (*NTE*) Brain NTE activity was determined following the method of Johnson[17] in the following steps: 50 ul of brain homogenate was added into each of the paired 1.95 mL of 40 mM paraoxon. Paired tubes containing the blank solution composed of paraoxon plus 50 mM mipafox were run in parallel with each ample tested. Tubes were incubated in a 37°C water bath for 20 minutes. Afterwards, 2 mL of the dispersion solution made of 500 mM phenylvalerate and 0.03% Triton X-100 was added into each tube, and the tubes were incubated for 15 minutes. Lastly, 2 mL of 1% (w/v) sodium dodecyl sulfate and 0.025% 4-aminoantipyrine were added into each tube to stop the reaction and 0.5 mL of 0.8% (w/v) potassium ferricyanide was added to develop the color. The solution was then vortexed and the optical density reading was taken from a spectrophotometer at 512 nm.

Protein Level Determination: Protein concentration for brain and plasma were determined using the bicinchonic acid method (BCA).[20]

Brain Cholinesterase Assay: Cholinesterase activity for brain was determined following the method of Ellman et al.[21] The assay was carried in the following steps: for each sample, two tubes containing 4 ml substrate solution [20 mN Tris buffer at pH 8.2 with 20 mM magnesium chloride ($MgCl_2$) and 100 mM NaCl, 0.001 M Dithio-2-nitrobenzoic acid (DTNB) as reagent], and 0.001 M acetylcholine iodide (as substrate) were pre-incubated at 37°C in a water bath for ten minutes. 20 μl of tissue homogenate was added into each tube. The sample was vortexed and transferred into a cuvette for absorption rating. Absorption reading was taken at one minute time interval for 3 minutes using a spectrophotometer (Shimadzu UV-3000; Shimadzu Corporation; Kyoto, Japan) at 412 nm. A substrate free blank was run in parallel with each sample tested.

Plasma Cholinesterase Activity: Chloinesterase activity in plasma was also determined by the method of Ellman et al.,[21] except the substrate is made with 4 mM tris buffer at pH of 7.4 with 40 mM $MgCl_2$, 0.1 mM $\times 10^{-4}$ M DTNB, and 0.2 mM $\times 10^{-4}$ M of S-butyrylthiocholine iodide as substrate.

Quantification of mitochondrial CI activity: As described previously[22] cells were homogenized mechanically to create submitochondrial particles with the inner side of the inner mitochondrial membrane turned out. These were centrifuged at 600×g (4°C, 20 min). The post-nuclear supernatants was solved in 25 mM phosphate buffer pH 7.4 at a concentration of 40 μg protein per ml. CI activity was determined spectrophotometrically at 37°C during the following 4 min at 340 nm as the amount of NADH oxidized per minute per milligram protein in post-nuclear extracts of the homogenates.

Measurement of ATP levels: ATP levels in 10 μL of the lysates was quantified in a luminometer using the Vialight HS Kit (Bio Whittaker, Verviers, Belgium), which utilizes luciferase to catalyze the formation of light from ATP and luciferin, as described previously.[23] The results obtained in arbitrary units were normalized with respect to the protein content of the extracts and are represented as the percentage of values obtained in controls.

Results

Clinical evaluation

Acute Toxicity

Animals treated with a single 150 mg/kg dose of CPF displayed signs of acute OP toxicity, the onset of signs varied from

Table 1: Clinical evaluation of hens following treatment with CPF

Bird	Treatment	Dose	Clinical signs@		Hen weight (kg)				
			12 d	16 d	initial	10 d	% initial	16 d	% initial
1	Vehicle	1 ml	Normal	Normal	1.68	1.67	96.9	1.69	100.6
2	Vehicle	1 ml	Normal	Normal	1.66	1.66	100	1.62	98
3	Vehicle	1 ml	Normal	Normal	1.59	1.60	101.2	1.61	102.5
4	Vehicle	1 ml	Normal	Normal	1.70	1.69	98	1.72	100.1
5	Vehicle	1 ml	Normal	Normal	1.64	1.60	97.4	1.60	97.4
6	CPF	150	T1-2	T2	1.53	1.42	92	1.44	95.8
7	CPF	150	T2	T3	1.47	1.32	89.8	1.38	93.9
8	CPF	150	T1-2	T1-2	1.55	1.56	100.3	1.51	95.8
9	CPF	150	T1-2	T2	1.62	1.58	96.4	1.57	95.3
10	CPF	150	T1-2	T2-3	1.61	1.61	100	1.51	91

@Hens were evaluated for clinical signs daily (through day 12 and 16) following administration of test compounds.

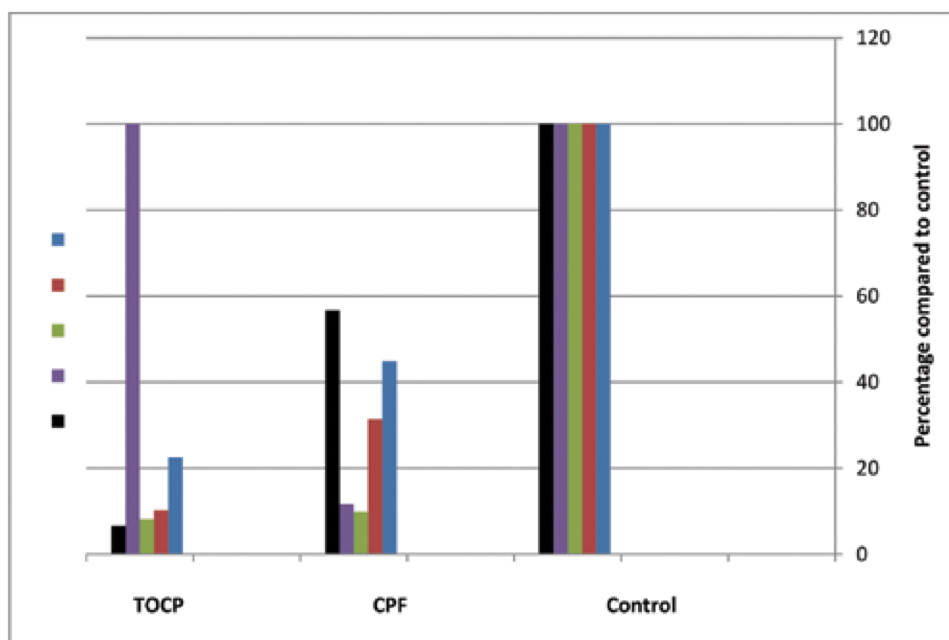

Fig. 1: Different enzyme assays in the three tested groups. Control, CPF (Chloropyrifos treated group) and TOCP treated group. The values are for: Blue for the ATP, red for CI assay, green for Bu ChE, violet for A chE and black for NTE.

0.5–4 hours following dosing in individual birds. Over the first 48 hours following dosing all chlorpyrifos treated birds showed signs of severe OP poisoning. At 96 hours, birds treated with 150 mg/kg were severely affected with persistent diarrhea, unsteadiness and slight leg splay.

Delayed Neurotoxicity

Results presented in Table 1 show that hens receiving a single dose of 150 mg/kg CPF displayed signs of ataxia and gait disturbance at 12 and 16 days.

Body Weight

Body weight that was determined initially and weekly after dosing, showed that by day 16, hens treated with CPF lost 5% that was significant compared to the control group.

Effects on Esterase Activities

Brain NTE

Brain NTE activity was 56.6 $\pm$ 6.0% of control after 150 mg/kg CPF. TOCP resulted in brain NTE activity of 6.64 $\pm$ 0.3% of the control. Effects on Esterase Activities (Fig-1).

Brain AChE

CPF resulted in AChE activity of 11.7 $\pm$ 8% of the control values after treatment with 150 mg/kg. TOCP did not inhibit brain AChE. Effects on Esterase Activities (Fig-1).

Plasma BChE

CPF treatment at 150 mg/kg resulted in BChE activity of 9.9 $\pm$ 6.5% of the control value. TOCP inhibition of plasma BChE was

similar to that of CPF with activity of 8.2 ± 3.24% of the control value. Effects on Esterase Activities (Fig-1).

Effects on complex I activity

Complex I

CPF treatment at 150 mg/kg resulted in complex I activity of 31.5 ± 7.5 % of the control value. TOCP resulted in CI activity of 10.3 + 2.2% of control value.

ATP level

CPF treatment at 150 mg/kg resulted in ATP level of 44.9 ± 6.5% of the control value. TOCP resulted in ATP levels of 22.6 + 4.32% of control value.

Discussion

Chlorpyrifos (CPF) is an organophosphate with known neurotoxic effects. The effects include: acute cholinergic crisis followed by possible intermediate syndrome and finally OPIDN. The delayed neuropathy imparted by CPF (as in many other OPs) is attributed to the inhibitory effects of such substances on Neuropathy Target Esterase (NTE) enzyme. Although this mechanism of action is well established, it still cannot justify the whole sequence of events in case of OPIDN.

One possible target that can be affected by OPs and lead to neuropathy is the mitochondrial complex I (CI). Mitochondria play an important role in neurotoxicity. This may be related to their relation to cellular energy. Previous studies have revealed possible contribution of CI inhibition to many neurological disorders e.g. Parkinson's disease, Alzheimer's disease, tauopathy and peripheral neuropathy.[13]

Most interestingly, CI inhibition was noticed in neuroblastoma cell cultures exposed to OPs with OPIDN effects; phenyl saligenin phosphate (PSP) and mipafox on the other-hand non-OPIDN inducing OPs (Parathione) was not associated with CI inhibition in the same study.[24] Moreover, CI inhibitory effects were verified in *rats* exposed to neurotoxic OPs e.g. dichlorvos (DDVP),[25] and monocrotophos (MCP).[26]

In the present study we evaluated the role of CI inhibition in CPF induced delayed neuropathy in hens. Since hens represent the ideal model to study OPIDN effects in a similar pattern to humans, studying the effect of CPF on this model would give an insight into OPIDN pathogenesis. Animals treated with CPF were evaluated clinically, then biochemically to verify the effects on different esterases, mitochondrial CI and ATP levels. Clinically, animals treated with CPF displayed features of delayed neuropathy as shown by signs of ataxia and locomotor disturbances. Biochemical studies revealed inhibition of various esterases (Brain AchE, Plasma AchE and NTE) by CPF treatment. Most important was the concomitant inhibition of CI activity. The inhibition of CI was reflected on similar decrease in ATP level on the CPF treated animals. A striking finding was that the decline in CI activity and resultant ATP level was analysed to the inhibition rate in NTE and clinical deterioration. This was noticed in TOCP treated hens showing more CI inhibitionthan CPF treated birds in a similar pattern to their stronger inhibition of NTE.

In the present study CPF treated hens received 150 mg/kg and developed signs of delayed neurotoxicity, which were verified by NTE inhibition. These effects were paralleled by CI inhibition and decrease in ATP level.

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