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Hydrogen peroxide potentiates organophosphate toxicosis in chicks

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Abstract. Objective: The purpose of the present study was to examine the effect of hydrogen peroxide (H_2O_2) on the acute toxicity of organophosphate insecticides dichlorvos and diazinon and their inhibitory actions on plasma, brain and liver cholinesterase activities. Material and Methods: H_2O_2 was given in drinking water (0.5% v/v) for 2 weeks in unsexed day old chicks, a regimen known to induce oxidative stress in this species. A control group received drinking tap water. All experiments were conducted on the chicks at the age of 15 days after exposure to H_2O_2. The acute (24 h) oral LD50 values of dichlorvos and diazinon in the insecticidal preparations as determined by the up-and-down method in the control chicks were 9.4 and 15.6 mg/kg, respectively. Results: The poisoned chicks manifested signs of cholinergic toxicosis within one hour after the dosing including salivation, lacrimation, gasping, frequent defecation, drooping of wings, tremors, convulsions and recumbency. The acute (24 h) oral LD50 values of dichlorvos and diazinon in chicks provided with H_2O_2 were reduced to 3.5 and 6.5 mg/kg, by 63 and 58%, respectively when compared to respective control LD50 values. The intoxicated chicks also showed cholinergic signs of toxicosis as described above. Plasma, brain and liver cholinesterase activities of the chicks exposed to H_2O_2 were significantly lower than their respective control (H_2O) values by 25, 28 and 27%, respectively. Oral dosing of chicks with dichlorvos at 3 mg/kg significantly inhibited cholinesterase activities in the plasma, brain and liver of both control (42-67%) and H_2O_2-treated (15-59%) chicks. Diazinon at 5 mg/kg, orally also inhibited cholinesterase activities in the plasma, brain and liver of both control (36-66%) and H_2O_2-treated (15-30%) chicks. In the H_2O_2 groups, dichlorvos inhibition of liver cholinesterase activity and diazinon inhibition of liver and brain cholinesterase activities were significantly lesser than those of the respective values of the control group received tap water. Conclusion: The data suggest that H_2O_2 may potentiate the toxicity of organophosphate insecticides irrespective of the extent of cholinesterase inhibition, and further studies are needed to examine the role of oxidative stress in this potential toxicity outcome.
Key words: Cholinesterase, organophosphate, oxidative stress, H_2O_2, chicks, insecticide.

Introduction. Organophosphate (OP) pesticides are frequently used to control and eradicate parasitic insects in veterinary medicine and agriculture (Moffett 2006). They pose high threats to public health because of their intrinsic toxicity and injudicious uses (Jaga & Dharmani 2003). The toxic actions of OP in mammals are initiated by inhibiting the target enzyme cholinesterase (ChE) at the nerve endings and neuromuscular junctions, resulting in the accumulation of acetylcholine, and subsequently cause parasympathomimetic overstimulation manifested as muscarinic, nicotinic, and central nervous system effects (Wilson 2005; Lotti 2010). Reduction in blood and nervous tissue ChE activities is the most important diagnostic biomarker endpoint of OP exposure and toxicosis (Wilson et al 1999, 2005).

The toxicity of OP insecticides can be modulated by many therapeutic and toxic agents (Ecobichon 2001; Jaga & Dharmani 2003; Pope et al 2005). Some of these agents such as metals are known to produce oxidative stress in biological membranes characterized by metabolic alterations of body oxidative defence mechanisms, changes of blood or brain cholinesterase activities as well as behavioral alterations resulting from

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insults to the central nervous system (Al-Baggou 2002; Gao et al 2009). The OP insecticides also induce oxidative stress in laboratory animals such as rats (Possamai et al 2007; Lukaszewicz-Hussain 2010) and many reports indicate changes in oxidative stress markers among patients exposed to OP pesticides with concurrent depression of blood ChE activities (Ranjbar et al 2005; Lukaszewicz-Hussain 2010; Hundekari et al 2011). Hydrogen peroxide (H_2O_2) has many therapeutic and industrial applications with potential toxic oxidative effects (Douglass 2003; Watt et al 2004) and it was reported to induce oxidative stress in laboratory animals and modify their responses to drugs such as sedatives and anaesthetics (Wohaeib et al 1994; Mohammad et al 1999) probably by causing lipid peroxidation in the central nervous system (Watt et al 2004). Information on the response of H_2O_2-stressed animals to OP is rather limited. One report indicates that H_2O_2 inhibits ChE of myometrium sarcolemma in vitro (Danylovych 2009). Another in vitro study showed that low concentrations of H_2O_2 (10^{-6} M) activate recombinant human acetylcholinesterase by increasing the Vmax > 2-fold, whereas high concentrations (10^{-3} M) inhibit the enzyme with a significant decrease in Vmax (Schallreuter et al 2004). This form of activation/deactivation of skin ChE by H_2O_2 was found to be a reversible oxidation process (Schallreuter & Elwary 2007). Furthermore, the implication of H_2O_2-induced oxidative stress has been suggested in Alzheimer's disease by inhibiting membrane bound erythrocyte ChE activity (Molochkina et al 2005).

Assessing ChE inhibition profiles in the plasma and brain of young chicks is a widely accepted standard for monitoring toxicosis and lethality induced by OP insecticides (Farage-Elawar & Francis 1988; Mohammad et al 2008). Chicks have already been used in experimental models of acute or subchronic OP poisoning (Farage-Elawar & Francis 1988; Wilson 1988; Al-Badrany & Mohammad 2007). Various reports indicate concurrent reductions in blood and brain ChE activities in birds acutely intoxicated by doses close to or higher than the median lethal doses (LD50) of OP insecticides (Farage-Elawar & Francis 1988; Wilson 1988; Burn & Leighton 1996; Mohammad & Al-Baggou 2005; Wilson et al 2005; Mohammad et al 2008). The aim of the present study was to examine the effect of H_2O_2 treatment (0.5% v/v in drinking water for 2 weeks) on acute OP toxicosis and ChE inhibition in chicks. In this context, the possible modulating effect of H_2O_2 on OP toxicosis can be assessed directly in vivo.

Material and Methods. Mixed breed day old unsexed chicks were used. They were maintained in a room with a temperature of 30-34°C, constant lighting and wood shavings as floor litter. The chicks had free access to water and feed. Chicks were either provided with plane tap water (control group) or H_2O_2 (Scharlau Chemie, Barcelona, Spain) prepared in tap water as 0.5% v/v drinking solution for two weeks in order to produce oxidative stress as reported before (Mohammad et al 1999; Ahmed 2010).The water sources were changed daily and freshly provided to chicks. Commercial insecticidal concentrate solutions of the OP insecticides dichlorvos (50% EC, Pacific Agrisience, Australia) and diazinon (60% Diazinon-60EC, VAPCO, Jordan) were further diluted in distilled water to obtain the desired concentrations of the individual insecticide for oral dosing of the chicks by a gavage needle in a volume of 5 mL/kg body weight (Mohammad et al 2008). The insecticidal solutions were freshly prepared before use, and all doses of the OP insecticide were based on their active ingredients. The selections of the doses of OP insecticides were based on our preliminary experiments in chicks and on the literature (Mohammad & Al-Baggou 2005; Mohammad et al 2008). All experiments were conducted on the chicks at the age of 15 days after exposure to H_2O_2.

Acute toxicity of OP insecticides. The acute (24 h) oral LD50 values of dichlorvos or diazinon were determined in chicks provided with tap water or with 0.5% H_2O_2 in the drinking water for two weeks by the up-and-down method (Dixon 1980). The chicks were individually observed for the appearance of signs of cholinergic toxicosis for

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1 h, and then the 24 h lethality was recorded (Wilson 1988; Al-Badrany & Mohammad 2007; Mohammad et al 2008). The LD50 experiments were concluded using only 5 or 6 chicks in each OP group over a period of 6 days. The LD50 of each OP in the insecticidal preparations was determined so that the relative changes in ChE activities at the doses of dichlorvos and diazinon in subsequent experiment could be compared to a bench mark dosage of acute toxicity.

In vivo effects of OP insecticides on plasma, brain and liver ChE activities. Chicks from the control group (tap water) and the H_2O_2 group (0.5% in the drinking water) were dosed orally using a gavage needle with either distilled water at 5 mL/kg (control) or with dichlorvos at 3 mg/kg body weight and diazinon at 5 mg/kg body weight (6-9 chicks /each group). The doses of the OP were chosen so that they comprised about 30% of their respective LD50 values, and other than salivation or slight depression, they did not cause overt acute signs of cholinergic toxicosis or death in chicks within two hours after the dosing. Two hours after each OP dosing, chicks were euthanized to obtain the plasma, whole brain and liver for determination of ChE activity by an electrometric method described previously in chickens (Mohammad 2007; Al-Badrany & Mohammad 2007; Mohammad et al 2008). All samples were kept at −20°C pending ChE analysis within one week.

The whole brain and liver were homogenized on an ice bath by a glass homogenizer in a pH 8.1 barbital-phosphate buffer solution (1.237 g sodium barbital 0.163 g potassium dihydrogen phosphate and 35.07 g sodium chloride/L of distilled water) at 3 mL/100 mg wet weight (Mohammad & Al-Baggou 2005; Mohammad 2007; Al-Badrany & Mohammad 2007). We determined ChE activity in the plasma, brain and liver samples by the electrometric method as follows: the reaction mixture contained 3 mL distilled water, 0.2 mL plasma or whole brain homogenate and 3 mL of pH 8.1 buffer described above. Initial pH of the mixture (pH1) was measured with a glass electrode using a pH meter (Hanna, Romania), and then 0.10 mL of the substrate 7.5% acetylthiocholine iodide was added to the mixture which was incubated at 37 °C for 30 min. At the end of the incubation period, the pH of the reaction mixture (pH2) was measured. The enzyme activity (expressed as ΔpH/30 min) was calculated as follows:
ChE activity (ΔpH/30 min) = (pH1 − pH2) - Δ pH of blank

The blank was without the plasma or brain homogenate sample. The % of ChE inhibition was calculated as follows:
% ChE inhibition = [ChE activity (without OP)-ChE activity (with OP)/ ChE activity (without OP)] X 100

Data as multiple means were subjected to the analysis of variance followed by least significant difference test (Petrie & Watson 1999). The statistical difference between two groups was measured by Student's-t-test. The level of statistical significance was at $p < 0.05$.

Results. The acute (24 h) oral LD50 values of dichlorvos and diazinon in the insecticidal preparations as determined by the up-and-down method in the control chicks provided with tap water were 9.4 and 15.6 mg/kg, respectively (Table 1). The intoxicated chicks manifested signs of cholinergic toxicosis within one hour after the dosing including salivation, lacrimation, gasping, frequent defecation, drooping of wings, tremors, convulsions and recumbency. The acute (24 h) oral LD50 values of dichlorvos and diazinon in chicks provided with H_2O_2 (0.5%) in tap water were reduced to 3.5 and 6.5 mg/kg, by 63 and 58%, respectively when compared to respective control LD50 values (Table 1). The intoxicated chicks also showed cholinergic signs of poisoning as described above.

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Table 1

The 24-hour median lethal doses (LD50) of the organophosphate insecticides dichlorvos and diazinon in chicks treated with hydrogen peroxide (H_2O_2) at 0.5% in the drinking water for two weeks

Variable	Result	
	H_2O (control)	H_2O_2
Dichlorvos		
LD50 (mg/ kg, orally)	9.4	3.5
Range of the doses used (mg/ kg, orally)	15-5=10	5-2=3
Initial dose (mg/ kg, orally)	15	5
Last dose (mg/ kg, orally)	5	5
Number of chicks used	5 XOXXO	7 XXXOOOX
Increase or decrease in dose (mg/ kg, orally)	5	1
Diazinon		
LD50 (mg/ kg, orally)	15.6	6.5
Range of the doses used (mg/ kg, orally)	20-10=10	10-5=5
Initial dose (mg/ kg, orally)	10	10
Last dose (mg/ kg, orally)	20	10
Number of chicks used	5 OXOOX	5 XOXOX
Increase or decrease in dose (mg/ kg, orally)	5	5

*X=death; O=survival The LD50 was determined by the up-and-down method (Dixon 1980).

Plasma, brain and liver ChE activities of the chicks exposed to H_2O_2 (0.5%) were significantly lower than their respective counter parts of the control (H_2O) group by 25, 28 and 27%, respectively (Figure 1). Oral dosing of chicks with dichlorvos at 3 mg/kg significantly inhibited ChE activities in the plasma, brain and liver of both control (42-67%) and H_2O_2-treated (15-59%) chicks (Table 2). Diazinon at 5 mg/kg, orally also inhibited ChE activities in the plasma, brain and liver of both control (36-66%) and H_2O_2-treated (15-30%) chicks (Table 2). In the H_2O_2 groups, dichlorvos inhibition of liver ChE activity and diazinon inhibition of liver and brain ChE activities were significantly lesser than those of the respective values of the control group that received tap water (Table 2).

Table 2

Percentages of inhibition of plasma, whole brain and liver cholinesterase activities in chicks exposed to H_2O_2 (0.5% in drinking water for 2 weeks) and subsequently dosed orally with organophosphate insecticides dichlorvos (3 mg/kg) and diazinon (5 mg/kg)

Cholinesterase	Dichlorvos		Diazinon	
	H_2O	H_2O_2	H_2O	H_2O_2
Plasma	67 ± 6	59 ± 5	$36\pm5^{\dagger}$	$30\pm9^{\dagger}$
Brain	55 ± 5	48 ± 7	66 ± 5	$19\pm9^{*\dagger}$
Liver	42 ± 5	$15\pm5^{*}$	41 ± 4	$15\pm5^{*}$

Values are mean$\pm$SE of 6-9 chick/group.
*Significantly different from the respective control (H_2O) value, $p < 0.05$.
†Significantly different from the respective dichlorvos value, $p < 0.05$.
Cholinesterase activity was measured 2 hour after the organophosphate dosing.
Mean baseline cholinesterase activities (ΔpH/30 min) of the plasma, brain and liver in the control group were 1.1, 0.66 and 0.265, respectively, and in the H_2O_2 they were 0.83, 0.41 and 0.193, respectively. These values were used to calculate the % of cholinesterase inhibition.
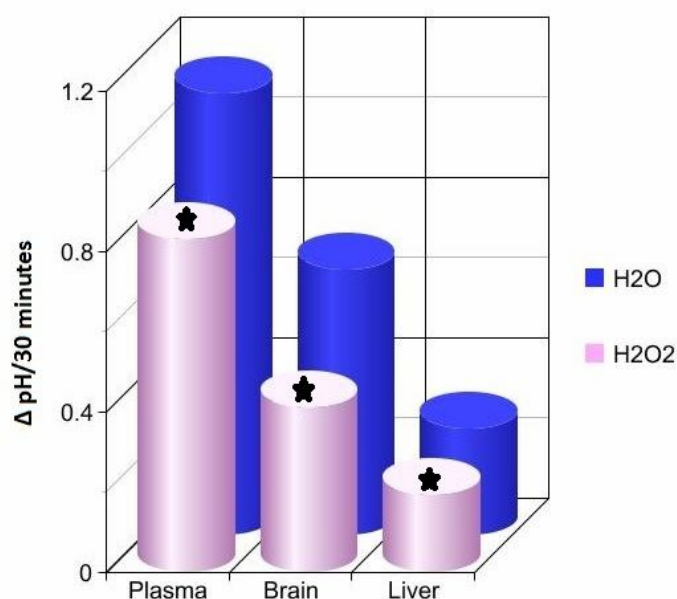
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Figure 1. Cholinesterase activities (Δ pH/30 minutes) of the plasma, whole brain and liver of chicks exposed to H_2O_2 (0.5%) in the drinking water for 2 weeks. The barrels represent the mean values of 6-8 chicks/tissue measurement. The standard errors of the mean values in the control (H_2O) group ranged between 4.9-15.2%, whereas those of the H_2O_2 group were 7.8-12.0%. *Significantly different from the respective control value, p < 0.05.

Discussion and Conclusions. The findings of the present study showed that chicks exposed orally to H_2O_2 became susceptible to OP toxicosis more than the control (tap water) group as was evident by the decreases of the LD50 values of dichlorvos and diazinon by 63 and 58%, respectively. H_2O_2 treatment in the drinking water rendered rats and rabbits sensitive to sedatives and anesthetics (Wohaeib et al 1994; Mohammad et al 1999). Treatment of laboratory animals including the chicken with H_2O_2 in the drinking water for periods ranging between 2-8 weeks was reported to induce tissue oxidative stress (Imre & Juhásza 1987; Mohammad et al 1999; Ahmed 2010). Oxidative stress was reported to severely damage muscarinic receptors signaling system which might in turn add an additional burden on the toxic insult of OP insecticides (de Jongh et al 2007). H_2O_2 was found to modulate (activation/deactivation) ChE activity in a concentration dependent manner (Schallreuter et al 2004). It is possible that the H_2O_2-induced oxidative stress has potentiated the OP poisoning in the chicks. Oxidative stress has been also implicated in the adverse effects of OP insecticides (Possamai et al 2007; Shadnia et al 2007; Lukaszewicz-Hussain 2010). The role of oxidative stress in ChE inhibition is not clear yet. It may activate or even deactivate the enzyme (Schallreuter et al 2004) independently from the OP and its binding site. In support of this notion, the baseline ChE activities in the plasma, brain and liver of H_2O_2-treated chicks were lower than those of the control values by 25-28%, suggesting a deactivation process of the ChE. However, the acute LD50 experiment indicated increased toxic interaction (potentiation) between the OP and H_2O_2.

Dichlorvos and diazinon variably inhibited plasma, brain and liver ChE activities in both control and H_2O_2-exposed chicks by 15-67%. Organophosphates are known to differentially inhibit blood and tissue ChE activities (Pope et al 2005; Wilson 2005; Lotti 2010). However, in the H_2O_2-treated chick, dichlorvos inhibited liver ChE to a lesser extent than that of the respective value of the control group supplied with tap water. Similarly, diazinon inhibited liver and brain ChE activities to a lesser extent when compared with the control (Table 2). This effect could be attributed to H_2O_2 which modulates ChE activity and modifies its susceptibility to further inhibition by other agents

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(Schallreuter et al 2004; Molochkina et al 2005). H_2O_2 was reported to modify erythrocyte membrane structure and activity of acetylcholinesterase (Molochkina et al 2005). H_2O_2 may deactivate ChE active site via oxidation of the Trp432, Trp435, and Met[436] residues inducing conformational changes and loss of the physiological function (Schallreuter et al 2004). Several studies also suggested that oxidative stress and subsequent deleterious effects of OP may be an alternative mechanism of cytotoxic effects of these pesticides (Poovala et al 1999; Shadnia et al 2007; Lukaszewicz-Hussain 2010). Recent reviews suggested that many non-ChE mechanisms could be involved in the acute toxicity of OP (Pope et al 2005; Lukaszewicz-Hussain 2010; Lotti 2010). In this context the current data suggest that H_2O_2 may potentiate the toxicity of OP irrespective of the extent of ChE inhibition, and further studies are needed to examine the role of oxidative stress in this potential toxicity outcome of the OP insecticides.

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