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Recognition and Assessment of Antidotal Effects of Diphenhydramine against Acute Carbaryl Insecticide Poisoning in a Chick Model

Ammar A. Mohammed[1] and Fouad K. Mohammad[2*]

[1]Department of Pharmacology, College of Pharmacy, University of Duhok, Duhok, Iraq

[2]Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Mosul, Mosul, Iraq; fouadmohammad@yahoo.com

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Abstract

Diphenhydramine antagonizes poisoning produced by cholinesterase (ChE) inhibiting insecticides. This study examines the effects of diphenhydramine against acute poisoning induced by the carbamate insecticide carbaryl in a chick model. The effects of diphenhydramine on the 24 h median Lethal Dose (LD50), and acute toxicity of carbaryl were assessed in chicks (7-15 days old). The plasma and whole brain ChE activities were measured electrometrically *in vitro* and *in vivo.* Diphenhydramine at 10mg/Kg Body wt. administered intramuscularly 15 min before carbaryl dosing increased the oral LD50 value of carbaryl (207 mg/Kg Body wt.) by 62%. Carbaryl at 250 mg/Kg Body wt. has orally produced toxidrome of cholinergic poisoning with 100% lethality in 24 h. Diphenhydramine (10mg/Kg Body wt.) used 15 min before carbaryl (250mg/Kg Body wt., orally) was the most effective dose (*vs* 5 and 20mg/Kg Body wt.) in delaying carbaryl-toxicity and increasing survivals in chicks. The intramuscular median effective dose (ED50) of diphenhydramine which prevented 24 h carbaryl-death in chicks was 8.6mg/Kg Body wt. The antidotal response to diphenhydramine was similar to that of the standard antidote atropine sulfate. Diphenhydramine at 10mg/Kg Body wt., given immediately after carbaryl (200mg/Kg Body wt.), reduced the percentages of plasma and whole brain ChE inhibitions *in vivo* by 12- and 13%, respectively. Carbaryl (10µmol/L) *in vitro* inhibited ChE activities in the plasma and brain by 53 and 77%, respectively; these inhibitions were reduced by 13- and 14%, respectively, when diphenhydramine (10µmol/L) was added to *in vitro* reactions. Diphenhydramine exerted antidotal action against a model of acute and lethal carbaryl intoxication in chicks.

**Author for correspondence*

1. Introduction

Carbaryl (1-naphthyl-N-methylcarbamate) is a commonly used carbamate insecticide in public health[1], agriculture[2] and veterinary clinical practice[3]. Carbaryl induces poisoning in mammals and birds by a reversible inhibitory carbamylation of cholinesterase (ChE) at the neuronal endings, causing accumulation of acetylcholine which leads to cholinergic overstimulation or crisis[4]. The clinical toxidrome of poisoning with carbamate pesticides is similar to that of organophosphates which is presented by muscarinic and nicotinic effects with stimulation of the central nervous system[4-6]. The toxicity of carbaryl has been described in the adult and young chicken[6-10], with limited information in younger ages[9,10]. Carbaryl reversibly inhibits chicken brain and plasma ChE activities both *in vivo* and *in vitro*[6-10].

Many studies have reported that the H1 antihistamine diphenhydramine antagonizes poisoning induced by organophosphate insecticides in rodents[11-13] and chickens[14] as well as those of carbamates in mice[15-17] and rats[18]. The action of diphenhydramine against anti ChE poisoning is thought to be through its antimuscarinic effects, similar to the standard antidote atropine[12,14,19,20]. There is also a possibility of the interaction of diphenhydramine with ChE inhibiting insecticides at the level of the ChE by reducing the extent of enzyme inhibition and at the nicotinic receptor sites by reducing the nicotinic signs of poisoning[14,18,20,21].

The purpose of the present study was to assess and recognize the potential antidotal action of diphenhydramine against carbaryl-induced acute poisoning in young chicks (7-15 days old). Such an animal model of acute and lethal ChE inhibitor poisoning has been previously well documented for organophosphate insecticides[14,22,23]. However, the effect of diphenhydramine on carbaryl-induced poisoning in chicks has not been reported yet. The standard antidote, atropine, is used for the treatment of poisoning induced by both organophosphates and carbamates due to its strong antimuscarinic effects[4,14,19,20]; and it has been used in the present study for comparison purposes with diphenhydramine.

2. Materials and Methods

2.1 Experimental Animals and Treatments

One day-old Cobb broiler chicks of both sexes were purchased from a local hatchery in Summeil, Duhok, Iraq. They were maintained in batches of 40-50 chicks at a time in an animal house facility containing wood shavings as floor litter, with constant lighting at a temperature of 25-30°C. Water and feed were available *ad libitum*. The age at which the chicks were subjected to the experiments ranged between 7-15 days (mean body weight + SD = 155 + 66 g). This is a chick model which could be used for the acute and lethal anti ChE poisoning that has been developed earlier[14,22,23]. The Committee of Postgraduate Studies at the College of Medicine, University of Duhok, Iraq has approved the present study according to the institutional regulations and ethics on the animal use and handling in biomedical research in compliance with ARRIVE (Animal Research: Reporting of *in vivo* Experiments; https://www.nc3rs.org.uk/arrive-guidelines) guidelines and the guide for the care and use of laboratory animals (https://www.ncbi.nlm.nih.gov/books/NBK54050/).

Under the supervision of the second author, who is also a veterinarian, the chicks were daily monitored to ensure that they were healthy before using them in experiments between ages of 7-15 days. The desired concentrations of the active ingredient carbaryl were freshly prepared from a commercially available insecticide-carbaryl powder (85%, Ferasin 85 WP, Fertil, Turkey) as suspensions in distilled water for oral dosing by a gavage needle in chicks. The volume of administration was at 5mL/Kg Body wt.[14,22]. All diphenhydramine HCl (The State Company for Drug and Medical Appliance, Samarra, Iraq) doses ranging between 5 to 20mg/Kg Body wt. were prepared using physiological saline solution (normal saline) as a solvent, and the volume of administration was 2mL/Kg Body wt., given intramuscularly (i.m.)[14]. A solution of atropine sulfate (1%, Mallinckrodt Co., St. Louis, USA) was diluted with normal saline to obtain the desired antidotal dosage, which was given at 2mg/Kg Body wt., i.m. in a volume of 2mL/Kg Body wt.[14]. The selected doses of carbaryl, diphenhydramine and atropine were based

on preliminary experiments conducted in chicks, and on previous reports[8, 11, 13 - 15, 18, 24].

2.2 Experiment Design

Figure 1 illustrates the integrated experimental design of the study and allocation of chicks to experiments to assess effects of diphenhydramine on the acute toxidrome of carbaryl. The total number of chicks used in the present study was 189, excluding those birds used for preliminary experiments to identify suitable dosage forms of the drugs.

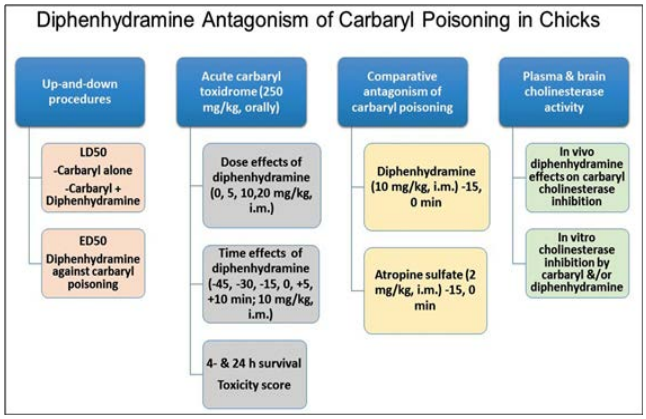


Figure 1. The integrated experimental design of the study to examine the effects of diphenhydramine on the acute toxidrome of carbaryl in 7-15 day-old chicks (total n= 189).

2.2.1 *Effect of Diphenhydramine on Acute Oral Median Lethal Dose (LD50) of Carbaryl in Chicks*

The up-and-down method[25] was used to determine acute (24h) oral LD50 values of carbaryl in chicks pretreated (-15min) with either normal saline at 2mL/KgBody wt., i.m. or with diphenhydramine at 10mg/Kg Body wt., i.m. The initial dose of carbaryl was 300 mg/Kg Body wt., orally. The increase or decrease in carbaryl doses was determined according to the survival or death of the chicks, respectively, and it was kept constant at 50mg/Kg Body wt., orally. The death of a chick was judged by the cessation of both heart beats and respiratory movements. Carbaryl LD50 value was computed according to the up-and-down method illustrated by Dixon[25]. The chicks were individually observed for the occurrence of signs of cholinergic poisoning, which included salivation, lacrimation, tremor, gasping, frequent defecation, convulsions and recumbency within 2 h, and thereafter, the 24h lethality was recorded[14, 22, 23]. The protection ratio of diphenhydramine against normal saline was calculated[14].

2.2.2 *Effects of Diphenhydramine Pretreatments on Carbaryl-Induced Acute Poisoning in Chicks*

Chicks (10 per dose group) were injected with diphenhydramine at doses of 0 (normal saline-control group), 5, 10 and 20mg/Kg Body wt., i.m. Fifteen min later, the chicks in all groups were dosed orally with carbaryl at 250mg/Kg Body wt. using a gavage needle. This dose of carbaryl was higher than its LD50 in chicks by 21%, and it was chosen in order to produce apparent signs of poisoning (toxidrome) in a manner that enabled us to choose the optimal dosage of diphenhydramine which prevented acute toxicity and lethality induced by carbaryl in chicks. After the carbaryl dosing, chicks were individually observed for the latencies to onset of signs of acute poisoning and death. Signs of acute poisoning within 4h and the 4h- and 24h lethality in chicks were recorded. The severity of carbaryl-induced poisoning in chicks was estimated by calculating the toxicity score (median, 25- and 75 percentile and total score) as described earlier[12, 14, 18].

2.2.3 *Effects of Time of Diphenhydramine Administrations on Carbaryl-Induced Acute Poisoning in Chicks*

To examine the effects of the time of diphenhydramine administration at 10mg/Kg Body wt., i.m. relative to the oral dosing of carbaryl at 250mg/Kg Body wt., seven groups of seven chicks each were treated as follows: chicks of the control group were treated with normal saline at 2mL/Kg Body wt., i.m., 15 min before the carbaryl dosing, where as chicks of the diphenhydramine groups were treated at –45, –30, –15, 0, + 5 and +10 min relative to the carbaryl dosing. After the carbaryl dosing, latencies to the onset of signs of poisoning and death were recorded; the chicks were also monitored individually for the occurrence of signs of carbaryl poisoning (4h) and

death (4h and 24h). The toxicity scores were estimated as described above.

2.2.4 Determination of the Median Effective Dose (ED50) of Diphenhydramine to Prevent Death within 24h in Chicks Intoxicated with Carbaryl (250 mg/Kg Body wt., Orally)

The up-and-down method[25] was used to determine the ED50 of diphenhydramine in preventing the 24h lethality in carbaryl (250mg/Kg Body wt., orally) -intoxicated chicks. The initial dose of diphenhydramine was 10mg/Kg Body wt., i.m., given immediately after the carbaryl dosing. The increase or decrease in diphenhydramine doses was determined according to the death or survival of the chicks, respectively, and it was kept constant at 2mg/Kg Body wt., i.m.

2.2.5 Comparison of the Efficacy of Diphenhydramine (10 mg/kg Body wt., i.m.) with that of the Standard Antidote Atropine Sulfate (2 mg/kg Body wt., i.m.) against Carbaryl-Induced Acute Poisoning in Chicks

Chicks (eight per group) were subjected to the following treatments: Chicks of group1 (control) were injected with normal saline at 2 mL/Kg Body wt., i.m., 15 min before the carbaryl dosing (250mg/Kg Body wt., orally). Group 2 and 3 chicks were injected with diphenhydramine and atropine at 10 and 2 mg/Kg Body wt., i.m., respectively, 15 min before the carbaryl dosing (250mg/Kg Body wt., orally), whereas those of groups 4 and 5 were injected with diphenhydramine and atropine at 10 and 2 mg/Kg Body wt., i.m., respectively, immediately after the carbaryl dosing (250mg/Kg Body wt., orally). The chicks were individually observed for the latencies to the onset of poisoning and death; the occurrence of acute signs of poisoning, 4 h- and 24 h lethality. Toxicity scores were recorded as mentioned above.

2.2.6 In Vivo Effects of Diphenhydramine on Plasma and whole Brain ChE Activities in Chicks Dosed Orally with Carbaryl

Chicks (six per group) were treated as follows: normal saline at 2mL/Kg Body wt., i.m., immediately after dosing them with distilled water at 5mL/Kg Body wt., orally (Group 1), diphenhydramine at 10mg/Kg Body wt., i.m., immediately after dosing them with distilled water at 5mL/Kg Body wt., orally (Group 2), normal saline at 2mL/Kg Body wt., i.m., immediately after dosing them with carbaryl at 200mg/Kg Body wt., orally (Group 3) and diphenhydramine at 10mg/Kg Body wt., i.m., immediately after dosing them with carbaryl at 200mg/Kg Body wt., orally (Group 4). Blood samples(0.5-1.0 mL) were collected from chicks by jugular vein bleeding into heparinized test tubes 30 min after the distilled water or carbaryl dosing[14,26]. Thereafter, the chicks were euthanized by cervical dislocation to excise the whole brain[14,27]. Blood samples were centrifuged at 3000 rpm for 15 minutes to obtain the plasma. Plasma and whole brain samples were kept at –20 °C pending for ChE determination within one week.

A glass homogenizer was used to homogenize the whole brain in an ice bath using barbital-phosphate buffer solution (pH 8.1) at 3mL/100 mg wet weight[14,22,24,28]. An electrometric method was used to determine ChE activity in the plasma and whole brain homogenate as described before[14,22,24,28,29]. The pH of reaction mixtures were measured with the electrode of a pH meter (CamLab Co., Cambridge, U.K.) to calculate ChE activity and % ChE inhibition.

2.2.7 In Vitro Effects of Diphenhydramine and Carbaryl on Plasma and Whole Brain ChE Activities in Chicks

As described above, the blood plasma samples and whole brains were obtained from each of 10 healthy chicks per group. Separate aliquots of plasma and whole brain homogenate were used for the ChE inhibition assay by applying the method of incubating the ChE inhibitor with the enzyme source *in vitro*[30,31]. The residual ChE activity and % ChE inhibition in the plasma or whole brain samples was calculated as mentioned above.

In all experiments that involved monitoring of the treated chicks for 24h survival, after recording the outcome according to experiment protocols mentioned above, all survived chicks were euthanized 24h post-treatment by cervical dislocation regardless of their health status[14,27]. This is to prevent unnecessary suffering that might have been carried over because of insecticide/drug treatments.

2.3 Statistics

Parametric data presented as multiple means were statistically analyzed by the one way analysis of variance

followed by Tukey's test[32]. The frequency data were analyzed by the Fisher's exact probability test[33], and scores of the severity of poisoning were analyzed by the Kruskal-Wallis test followed by the Dunn's test[32,33]. The statistics software package PAST 4.08 (https://www.nhm.uio.no/english/research/infrastructure/past/) was used to analyze the data. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1 Effect of Diphenhydramine on the LD50 Value of Carbaryl in Chicks

The acute (24h) LD50 value of carbaryl in chicks pretreated with normal saline solution was 207mg/Kg Body wt., orally (Table 1). The signs of cholinergic poisoning which appeared on the chicks within 2-10 min after the carbaryl dosing included salivation, lacrimation, lethargy, gasping, frequent and watery defecation, extending feet, loss of equilibrium, tremor, drooping of the neck and wings, ruffled feathers, muscle stiffness, recumbency, paralysis or convulsions followed by death. Diphenhydramine at 10mg/Kg Body wt., i.m., 15 min before carbaryl dosing increased the oral LD50 value of carbaryl by 62% to 335mg/Kg Body wt., with a protection ratio of 1.62 (Table 1). Also, diphenhydramine prolonged the latency to the onset of poisoning to 10-18 min, but did not prevent the signs of carbaryl-induced poisoning in chicks succumbed to death (Table 1).

3.2 Effects of Diphenhydramine Pretreatments on Acute Carbaryl-Induced Poisoning in Chicks

Dosing of chicks with carbaryl (pretreated with normal saline) at 250mg/Kg Body wt., orally produced signs of acute poisoning, which included salivation, lacrimation, tremor, gasping, frequent defecation, convulsions and recumbency within 8.4 $\pm$ 2.16 min (Table 2). The percentage of death was 90% in the carbaryl-treated chicks, which occurred within 62.2 $\pm$ 8.02 min after the insecticide dosing, and all chicks died within 24h

Table 1. The 24h oral median lethal dose (LD50) of carbaryl in chicks determined by the up-and-down method after pretreatment with normal saline solution (2mL/Kg Body wt., i.m.) or diphenhydramine (10mg/Kg Body wt., i.m.)

Variables	Results
Carbaryl + normal saline	
LD50 of carbaryl	207mg/Kg Body wt., orally
Range of the doses used	300–200 = 100mg/Kg Body wt., orally
Initial dose	300mg/Kg Body wt., orally
Last dose	200mg/Kg Body wt., orally
Increase or decrease in dose	50mg/Kg Body wt., orally
Number of chicks used	7 (XXXOOXO)
Range of latency to the onset of poisoning	10–2= 8 minutes
Carbaryl + diphenhydramine	
LD50 of carbaryl with diphenhydramine	335mg/Kg Body wt., orally
Range of the doses used	350–300 = 50mg/Kg Body wt., orally
Initial dose	300mg/Kg Body wt., orally
Last dose	300mg/Kg Body wt., orally
Increase or decrease in dose	50mg/Kg Body wt., orally
Number of chicks used	5 (OXOXO)
Range of latency to the onset of poisoning	18–10= 8 minutes
Protection ratio	1.62

Diphenhydramine and normal saline were injected intramuscularly (i.m.) 15 min before the carbaryl dosing. X= 24h death; O= 24h survival.

Table 2. Effects of diphenhydramine treatments on carbaryl (250mg/Kg Body wt., orally)-induced poisoning in chicks

Variables	Diphenhydramine (mg/Kg Body wt., i.m.)			
	0 (saline control)	5	10	20
Latency to onset of signs of poisoning (min)	8.4 + 2.16	13.7 + 2.45	13.5 + 0.93*	27.3 + 5.95*,a,b
Latency to onset of death (min)	62.2 + 8.02	92.0 + 16.0	144.7 + 18.28*	114.4 + 7.22*
% Occurrence of signs of poisoning				
Salivation	100	50*	70*	60*
Lacrimation	100	90	90	80
Gasping	100	100	100	100
Tremor	100	100	80	80
Frequent defecation	100	100	100	100
Convulsions	90	40*	20*	60
Recumbency	90	40*	30*	70
4 h lethality (%)	90	20*	20*	50*
24 h lethality (%)	100	50*	40*	60*
Median toxicity score	4	2*	3*	3
25 percentile	4	2	1.5	3
75 percentile	4	4	4	4
Total toxicity score	36	25	25	30

Diphenhydramine was injected intramuscularly (i.m.) 15 min before the carbaryl dosing. Latency values are mean + SE. n= ten chicks per group.

* Significantly different from the respective control value, $p < 0.05$.

[a] Significantly different from the respective 5mg/Kg Body wt., i.m. dose group of diphenhydramine, $p < 0.05$.

[b] Significantly different from the respective 10mg/Kg Body wt., i.m. dose group of diphenhydramine, $p < 0.05$.

thereafter; the total toxicity score in this group was 36 (Table 2).

Diphenhydramine at 5mg/Kg Body wt., i.m. given 15 min before carbaryl (250mg/Kg Body wt., orally) non-significantly increased the latencies to the onset of toxic signs and death induced by the insecticide (Table 2). However, there was a significant decrease in the occurrence of signs of carbaryl poisoning (salivation, convulsions and recumbency) and a significant increase in the 4h- and 24h survival up to 80 and 50%, respectively, in comparison with the control values (Table 2). Correspondingly, the total toxicity score in the 5mg/Kg Body wt., i.m. dose group of diphenhydramine was reduced by 31% to 25 (Table 2). Diphenhydramine pretreatment at 10mg/Kg Body wt., i.m. significantly increased both of the latencies to the onset of poisoning and death, significantly decreased the occurrence of salivation, convulsions and recumbency, and significantly increased the percentages of survival 4 h- and 24 h after the carbaryl dosing to 80 and 60%, respectively, when compared to control values (Table 2). Furthermore, this dose of diphenhydramine decreased the total toxicity score by 31% to 25 (Table 2). However, the highest dose of diphenhydramine pretreatment at 20mg/Kg Body wt., i.m. was not as effective as the 10mg/Kg Body wt., i.m. dose. Diphenhydramine at 20mg/Kg Body wt., i.m. significantly increased the latencies to the onset of poisoning and death, significantly decreased only the occurrence of salivation and significantly increased the percentages of survival within 4h- and 24h of carbaryl administration, without significantly affecting the toxicity score (Table 2). It seemed from this experiment that the most effective and protective dose of diphenhydramine pretreatment against carbaryl-induced acute poisoning in chicks was at 10 mg/Kg Body wt., i.m.

3.3 Effects of Time of Diphenhydramine Administrations on Carbaryl-Induced

Acute Poisoning in Chicks

Diphenhydramine (10mg/Kg Body wt., i.m.) treatments at times of –45, –30, –15, 0, +5 and +10 min relative to the carbaryl (250mg/Kg Body wt., orally) dosing, generally though, variably alleviated the carbaryl-induced acute poisoning in chicks (Table 3). Alleviation of carbaryl-induced poisoning by diphenhydramine treatments was mostly evident by statistically significant increases in the latency to onset of poisoning (–45, –30, –15 and 0 min), decreases in the occurrence of some signs of poisoning (0 and +5 min), increases of 4 h survival (–15 min) and a decrease in the toxicity score (0 time) in comparison with respective control values (Table 3). Based on the total toxicity score, the best protection obtained by diphenhydramine treatment against the carbaryl poisoning was at the 0 time; i.e. immediately after the carbaryl dosing (Table 3).

3.4 Determination of the Median Effective Dose (ED50) of Diphenhydramine to Prevent Death within 24h in Chicks Intoxicated with Carbaryl (250mg/Kg Body wt., Orally)

The ED50 of diphenhydramine which prevented death within 24h in chicks intoxicated with carbaryl (250mg/

Table 3. Cholinergic poisoning in chicks treated with diphenhydramine at 10mg/Kg Body wt., intramuscularly (i.m.) relative to the time of carbaryl dosing (250mg/Kg Body wt., orally)

Variables	Saline control	Diphenhydramine treatment time (min)					
		–45	–30	–15	0	+5	+10
Latency to onset of signs of poisoning (min)	2.0 + 0.31	6.4 + 1.09*	5.8 + 0.57*	8.0 + 1.07*	22.6 + 1.59*,a,b,c	2.0 + 0.22a,b,c,d	1.7 + 2.29a,b,c,d
Latency to onset of death (min)	144.0 + 28.7	240.0†	183.3 + 40.2	None	165.0†	164.0 + 60.0	213.0 + 26.0
% occurrence of signs of poisoning							
Salivation	100	86	86	86	71	57	71
Lacrimation	100	86	100	100	86	57	86
Gasping	100	100	100	100	100	100	100
Tremor	100	100	100	86	71	86	100
Frequent defecation	100	100	100	100	100	100	100
Convulsions	57	57	29	14	0*	29	29
Recumbency	85	43	43	57	0*	29*	43
4 h lethality (%)	43	14	29	0*	14	29	43
24 h lethality (%)	86	86	71	71	57	57	71
Median toxicity score	4	4	4	4	3*	3	3
25 percentile	3.5	2.5	2	2	0.5	2	2
75 percentile	4	4	4	4	4	4	4
Total toxicity score	33	30	29	27	22	27	28

Latencies are mean + SE (except at †, one chick died in 4 h). n= seven chicks per group.
* Significantly different from the respective control value, $p < 0.05$.
[a] Significantly different from the respective value of the –45 min diphenhydramine group, $p < 0.05$.
[b] Significantly different from the respective value of the –30 min diphenhydramine group, $p < 0.05$.
[c] Significantly different from the respective value of the –15 min diphenhydramine group, $p < 0.05$.
[d] Significantly different from the respective value of the 0 min diphenhydramine group, $p < 0.05$.

Table 4. Median Effective Dose (ED50) of diphenhydramine in preventing death within 24h in chicks intoxicated with carbaryl (250mg/Kg Body wt., orally) using the up-and-down method

Variables	Results
ED50 of diphenhydramine	8.6mg/Kg Body wt., i.m.
Range of the doses used	10–8= 2mg/Kg Body wt., i.m.
Initial dose	10mg/Kg Body wt., i.m.
Last dose	10mg/Kg Body wt., i.m.
Increase or decrease in dose	2mg/Kg Body wt., i.m.
Number of chicks used	5 (XOXOX)
Range of latency to the onset of carbaryl poisoning	29–9= 20 min

Diphenhydramine was injected intramuscularly (i.m.) immediately after the carbaryl dosing.
X: 24 h survival (effect), O: 24 h death (no effect).

Kg Body wt., orally) was 8.6mg/Kg Body wt., i.m. (Table 4). The signs of acute carbaryl poisoning in the chicks appeared within 9-29 min which included salivation, lacrimation, gasping, tremor, frequent defecation and then death (Table 4).

3.5 Comparison of the Efficacy of Diphenhydramine (10mg/Kg Body wt., i.m.) with that of the Standard Antidote Atropine Sulfate (2mg/Kg Body wt., i.m.) Against Carbaryl-Induced Acute Poisoning in Chicks

As in previous experiments, dosing of chicks with carbaryl at 250mg/Kg Body wt., orally (normal saline-control) produced signs of acute cholinergic poisoning within 2.4+0.32min, which included salivation, lacrimation, tremor, gasping, frequent defecation, convulsions and recumbency (Table 5). In this group, death occurred in 87 % of the chicks within 171.9+ 19.56 min, and all birds died within 24 h after the carbaryl dosing, with a toxicity score of 35 (Table 5). Diphenhydramine at 10mg/Kg Body wt., i.m. given 15 min before-or immediately after the carbaryl dosing (250mg/Kg Body wt., orally) significantly reduced cholinergic poisoning in chicks in a manner comparable to the standard antidote atropine treatment at 2mg/Kg Body wt., i.m. (Table 5). Both diphenhydramine and atropine treatments (before or immediately after the carbaryl dosing) significantly increased latency to the onset of acute poisoning and survival rate along with significant decrease in occurrence of cholinergic signs of poisoning and total toxicity scores by 29-63% (Table 5). Diphenhydramine treatment, immediately after the carbaryl dosing of chicks, appeared to produce an antidotal response (based on the 24h survivability and toxicity score) almost similar to that of the atropine (Table 5).

3.6 *In vivo* Effects of Diphenhydramine on Plasma and whole Brain ChE Activities in Chicks Dosed Orally with Carbaryl

Injecting chicks with diphenhydramine alone at 10mg/Kg Body wt., i.m. did not significantly affect the plasma or whole brain ChE activities in comparison with the respective control values (Table 6). Carbaryl at 200mg/Kg Body wt., orally significantly inhibited ChE activities in the plasma and the whole brain by 35 and 48%, respectively, in comparison with the respective control values (Table 6). Diphenhydramine at 10mg/Kg Body wt., i.m. given immediately after the carbaryl dosing (200mg/Kg Body wt., orally) decreased the percentages of carbaryl-induced inhibitions of plasma and whole brain ChE activities by 12- and 13%, respectively, compared with those of the control group, although both ChE activities remained significantly below control and diphenhydramine values (Table 6).

Table 5. Comparison of the antidotal efficacy of diphenhydramine (10mg/Kg Body wt.) with that of the standard antidote atropine sulfate (2mg/Kg Body wt.) given intramuscularly against carbaryl (250mg/Kg Body wt., orally)-induced acute poisoning in chicks

Variables	Treatment groups				
	Saline control	Diphenhydramine		Atropine sulfate	
		–15 min	0	–15 min	0
Latency to onset of signs of poisoning (min)	2.4 + 0.32	7.4 + 0.71*	19.0 + 2.1*	7.9 + 0.64*	12.1 + 1.03*
Latency to onset of death (min)	171.9 + 19.56	233†	None	None	None
% occurrence of signs of poisoning					
Salivation	88	63	25*	13*	0*
Lacrimation	100	100	75	88	88
Gasping	100	100	63	100	100
Tremor	100	100	50*	50*	0*
Frequent defecation	100	100	100	100	88
Convulsions	63	0*	13*	0*	0*
Recumbency	88	25*	0*	0*	0*
4 h lethality (%)	87	12*	0*	0*	0*
24 h lethality (%)	100	87	12*	0*	25*
Median toxicity score	4	4	1*	1*	0*
25 percentile	4	1	0.5	0	0
75 percentile	4	4	3	4	4
Total toxicity score	35	25	15	15	13

Latencies are mean + SE (except at †, one chick died in 4h).
n= eight chicks per group. Saline was injected 15min before the carbaryl dosing; 0 time treatment= given immediately after the carbaryl dosing.
* Significantly different from the respective control value, $p < 0.05$.

Table 6. *In vivo* inhibition of plasma and whole brain cholinesterase (ChE) activities in chicks dosed with either carbaryl (200mg/Kg Body wt., orally) and/or diphenhydramine (10mg/Kg Body wt., i.m.)

Treatment groups	Plasma		Whole brain	
	ChE activity (Δ pH/30 min)	% inhibition	ChE activity (Δ pH/30 min)	% inhibition
Control (vehicle)	0.315 + 0.013		0.155 + 0.006	
Diphenhydramine	0.318 + 0.003	0%	0.147 + 0.006	5%
Carbaryl	0.205 + 0.006*	35%	0.08 + 0.009 *	48%
Carbaryl + Diphenhydramine	0.242 + 0.005*	23%	0.10 + 0.008 *	35%

Values of ChE activities are mean + SE of six chicks per group.
Diphenhydramine was injected intramuscularly (i.m.) immediately after the carbaryl dosing. The enzyme activity was determined 30 min after the carbaryl dosing.
* Significantly different from values of the respective control and diphenhydramine groups, $p < 0.05$.

3. *In vitro* Effects of Diphenhydramine and Carbaryl on Plasma and whole Brain ChE Activities in Chicks

Diphenhydramine (10µmol/L, *in vitro*) did not significantly inhibit plasma and whole brain ChE activities of chicks in comparison with the respective control values (Table 7). In contrast, carbaryl (10µmol/L) significantly

Table 7. Inhibition of chick plasma and whole brain cholinesterase (ChE) activities after *in vitro* incubation at 37 °C for 10 minutes with carbaryl (10 µmol/L) and/or diphenhydramine (10 µmol/L)

Group	Plasma		Whole brain	
	ChE activity (Δ pH/30 min)	% ChE inhibition	ChE activity (Δ pH/30 min)	% ChE inhibition
Control (vehicle)	0.501 + 0.011	-	0.388 + 0.009	-
Diphenhydramine	0.567 + 0.015	-	0.544 + 0.010	-
Carbaryl	0.233 + 0.008 *	53%	0.09 + 0.013 *	77%
Diphenhydramine + Carbaryl	0.301 + 0.005 *	40%	0.144 + 0.004 *	63%

Values of ChE activities are mean + SE of ten chicks per group. * Significantly different from values of the respective control and diphenhydramine groups, $p < 0.05$.

inhibited both plasma and whole brain ChE activities of chicks by 53 and 77%, respectively when compared with the respective control values (Table 7). When the combination of diphenhydramine and carbaryl was used *in vitro*, inhibitions of ChE activities were reduced in both the plasma and whole brain by 13- and 14%, respectively, in comparison with those of the carbaryl alone (Table 7).

4. Discussion

In the experiments of the present study, dosing of chicks orally with toxic doses of carbaryl has produced mixed muscarinic and nicotinic signs of poisoning, in addition to stimulation of the central nervous system. These are the toxidrome, collectively and typically, seen in cases of organophosphate and carbamate intoxications in mammals and avian species[3-6,14,17,20,22,34]. Carbaryl reversibly inhibits ChE in different neuronal systems resulting in an accumulation of acetylcholine at the nerve endings and subsequently causing parasympathetic hyperstimulation, which in turn produces the toxidrome of muscarinic, nicotinic, and central nervous system effects[4,6,20,34,35]. Death is usually due to asphyxia as a result of inhibition of the respiratory center in the medulla oblongata in the brain accompanied with bronchoconstriction, increased bronchial secretions and bronchial muscle paralysis[6,20,22,34]. The results of the present study in a chick model of acute and lethal anti ChE (carbaryl) poisoning are in accordance with previous findings on the toxic signs of this insecticide in the avian species[6,7,10].

The LD50 value of carbaryl in chicks (pretreated with normal saline) was 207 mg/Kg Body wt., orally as determined by the up-and-down method[25]. This value is a new finding in young chicks which is currently used as a model of acute and lethal poisoning with the insecticidal formulation of carbaryl. Based on the recommendations of Australian Pesticides and Veterinary Medicines Authority[36] as well as those of others[6,34,35], such a low LD50 value of carbaryl in chicks ranks the insecticide at the level of compounds with a toxicological risk. For comparison purposes, the LD50 value of carbaryl in adult chickens was reported to be < 2000mg/Kg Body wt., orally[35]. In rats, it was variably reported to be (in mg/Kg Body wt., orally) 246[36], 307[5] and with a range of 250-850 depending on the studies[35]. In mice, the LD50 of carbaryl ranged between 100-650mg/Kg Body wt., orally, depending on the studies too[35]. Interspecies differences and age factors were expected in acute toxicity of ChE inhibiting pesticides[5,31,34-36]. Overall, the LD50 experimental findings of the present study, as well as the subsequent experiments of carbaryl intoxication in chicks, suggest the sensitivity of this avian species at the specified age range (7-15 days) for anti ChE toxicity evaluation. Such a notion further supports similar reports on the acute toxicological studies of organophosphates in a chick model[14,22,23].

The increase in the LD50 value of carbaryl in chicks by diphenhydramine pretreatment was 62%, and this antihistamine reduced the toxic signs and prolonged their

onset latency. Further, diphenhydramine was effective in preventing the carbaryl-induced acute poisoning in chicks (Table 2). The toxic dose of carbaryl (250mg/Kg Body wt., orally) used in the present experiment, being higher than its oral LD50 by 21%, caused acute poisoning in chicks within 4h and 100% death within 24h. The most effective dose of diphenhydramine in ameliorating the carbaryl-induced acute poisoning was 10mg/Kg Body wt., i.m. (Table 2), as it reduced the percentages of occurrence of acute toxic signs and increased the survival by 80 and 60% within 4h and 24h, respectively. Furthermore, diphenhydramine efficacy in reducing the carbaryl poisoning was best at the time of 0 min; i.e. immediately after the carbaryl dosing (Table 3). This is based on measurements of the 24h survivals and total toxicity score. The finding of the best time of diphenhydramine administration relative to the carbaryl dosing does not negate the beneficial effects of this antihistamine when given at other times to antagonize anti ChE poisoning. However, clinical trials would possibly clarify this point. These results together with those of the LD50 experiment in a chick model of acute and lethal carbaryl poisoning also confirm earlier suggestions concerning the protective benefits of diphenhydramine against acute poisoning induced by the carbamate insecticides, carbaryl in mice[17], methomyl in rats[18] and the non-insecticidal carbamates physostigmine and neostigmine in mice[15]. In addition, the results of the present study-reported for the first time in chicks expand and ascertain previous reports concerning the protective benefits of diphenhydramine against acute poisoning induced by organophosphates[11, 12 - 14, 19 - 21].

An additional new finding in this study reports the effectiveness of diphenhydramine when given immediately after the carbaryl dosing in chicks with an ED50 value of 8.60mg/Kg Body wt., i.m. (Table 4). The most effective dose of diphenhydramine applied in this study was 10mg/Kg Body wt., i.m. and it exceeded the ED50 by just 16.3%. The reported LD50 of diphenhydramine in chicks is 49.3mg/Kg Body wt., i.m.[24], and in this study, the antidotal dose of diphenhydramine in chicks dosed with carbaryl was not increased to more than 10mg/Kg Body wt., i.m. (Table 5). Furthermore, doses of diphenhydramine more than 30mg/Kg Body wt., i.m. were reported to produce tremors in chicks[24]. A similar conclusion has been drawn on the antidotal therapy with diphenhydramine (10mg/Kg Body wt., i.m.) in chicks intoxicated with dichlorvos[14].

In all the experiments of the present study, diphenhydramine effectively reduced the toxidrome of acute carbaryl poisoning and reduced death in chicks (Tables 1-5). These effects of diphenhydramine were similar to those of the standard antidote atropine (Table 5), a finding which is in accordance with those of other studies in rats[12] or chicks[14] intoxicated by organophosphates. The mechanism of such protective and ameliorative effects of diphenhydramine in cases of carbamate or organophosphate poisoning could be due the peripheral and/or central antimuscarinic and possibly antinicotinic effects of this antihistamine[12, 14, 18 - 21]. The nicotinic effects of carbaryl in the chicks of the present study might have contributed to the occurrence of tremor, recumbency and death as a result of muscle fasciculation, paralysis and weakness as well as central effects[4 - 6, 19, 20]. Diphenhydramine treatments variably but significantly reduced these toxic signs in addition to others in carbaryl-intoxicated chicks (Tables 2,3,5). These effects of diphenhydramine could be an added benefit to its antidotal action (mainly antimuscarinic) against anti ChE insecticides[19 - 21]. It is worth mentioning that the standard antidote atropine antagonizes muscarinic toxic effects in cases of organophosphate and carbamate poisoning[3 - 5, 19, 20, 34]. In this context, the present study further suggests and confirms that diphenhydramine could be a useful alternative antidote for protection and treatment of acute carbamate insecticides poisoning. Additional antidotes against poisons are usually beneficial to broaden the choice of therapies available for veterinarians. For example, in cases of organophosphate poisoning, oximes can be used to supplement the antidotal action of atropine[4, 20], whereas such an oxime therapy is controversial and contraindicated in cases of carbamate poisoning because of potentiation of the toxicity of the latter[37]. Therefore, the antihistamine diphenhydramine could be repurposed for use against carbaryl poisoning, albeit, clinical trials are still needed.

The measurement of blood ChE activity is a useful diagnostic tool for monitoring anti ChE poisoning or exposure in man and animals[4, 20, 28, 34]. Birds have no ChE activity in erythrocytes; therefore, their exposure to carbamates and organophosphates could be assessed by monitoring the ChE activity in the plasma (or serum) as well as in the nervous tissue[34, 38]. The present study has applied both *in vivo* and *in vitro* experiments to assess the inhibition of plasma and whole brain ChE activities in chicks, and whether there is any possible interaction of diphenhydramine and carbaryl at the enzyme level (Tables 6 and 7).

In vitro inhibition of blood or brain ChE activity is an assessment tool to evaluate the enzyme inhibitory actions of organophosphates and carbamates, and hence, their potential toxicity in mammals and avian species[29,30,31,39]. In the present study, the *in vitro* ChE inhibition experiment (Table 7) shows that diphenhydramine might be, apart from its antimuscarinic action, effective in reducing the carbaryl toxicity at the level of ChE, by protecting the enzyme from further inhibition by this insecticide. Such a notion has been reported earlier with weak ChE inhibitors in preventing organophosphate poisoning[23,40]. In this context, a previous study in chicks treated with diphenhydramine at 2.5 and 5mg/Kg Body wt., i.m. reported a weak inhibition of plasma and brain ChE activities[24]. This is also in support of earlier reports regarding the possibility of weak and reversible inhibitory actions of antihistamines on blood ChE activities[41]. Such a weak inhibitory action of diphenhydramine would possibly be effective in preventing additional toxic ChE inhibition by carbaryl.

In accordance with the *in vitro* ChE inhibition results, diphenhydramine treatment given immediately after the carbaryl dosing decreased (*in vivo)*, the percentages of ChE inhibition induced by carbaryl in both the plasma and brain of the chicks by 12- and 13%, respectively (Table 6). The reduction in both plasma and whole brain ChE activities in chicks dosed orally with carbaryl at 200mg/Kg Body wt. (Table 6) is in agreement with the toxic action of this insecticide that produces muscarinic, nicotinic and central nervous system stimulation[3-6,35]. A report indicated that ChE inhibition in the plasma and brain of rats dosed with carbaryl at 30mg/Kg Body wt., orally was related to tissue concentration of the insecticide within 40 min after the dosing[42]. In the adult chicken dosed orally with carbaryl at 900mg/Kg Body wt., brain ChE inhibition occurred 60 min after the administration[7], which also correlated with the appearance of signs of cholinergic poisoning. The total clearance of carbaryl in the adult chicken after the intravenous administration of carbaryl at 5mg/Kg Body wt. was 0.26L/kg/min regardless of the esterase inhibition in the liver[7]. Furthermore, in the present study, the *in vitro* and *in vivo* inhibitions of both plasma and whole brain ChE activities by carbaryl with or without diphenhydramine (Tables 6 and 7) are consistent with previous suggestions on the effects of a combination treatment with diphenhydramine and the carbamate insecticide methomyl on brain ChE activity in rats[18]. However, such a reduction of the ChE inhibitory effect of carbaryl by diphenhydramine might not be the main factor for the proposed antidotal action of diphenhydramine against ChE inhibitors poisoning, as this antihistamine possesses, most importantly, a strong antimuscarinic effect[14,19,20]. In addition, no toxic interactions occurred between diphenhydramine and the organophosphate insecticide, dichlorvos regarding the inhibition of blood or brain ChE activities in rats[12].

5. Conclusions

This study has described a model of acute and lethal poisoning with the commercially used insecticide carbaryl in young (7-15 days old) chicks which showed the characteristic toxidrome of muscarinic, nicotinic and central nervous system effects together with ChE inhibition in the plasma and whole brain. Diphenhydramine, similar to the standard antidote atropine has effectively produced protective and alleviating antidotal actions against this model of acute carbaryl intoxication in chicks, with a potential of partial protection of the ChE activity from further inhibition.

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7. Conflict of Interests Statement

The authors declare that they have no conflicts of interest.

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