Determination of Brain Cholinesterase Activity in Normal and Pesticide Exposed Wild Birds in Israel

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ABSTRACT

Whole brain cholinesterase activity was measured in 26 species of wild birds in Israel, and compared to depressed cholinesterase activities of birds known to having been exposed to certain organophosphorus and carbamate insecticides. The purpose of the study was to establish a reference range of normal whole brain cholinesterase activities, which will enable ecotoxicologists to evaluate suspected cases of organophosphorus and carbamate exposure in wild birds in Israel. Normal whole brain cholinesterase activity in birds of prey ranged from 7.4-12.5 μmol/min/g tissue, and the remaining avian species exhibited a wider range of 7.4-19.8 μmol/min/g. Birds' carcasses with residual pesticides in their proventriculus and gizzard, revealed a significant decrease of more than 50% of their normal whole brain cholinesterase activity, thereby confirming the cause of the death due to cholinesterase inhibition. The most prominent pesticides detected in the stomach content of wild birds were the carbamates, aldicarb and methomyl. The reference values presented in this study could facilitate routine diagnosis and monitoring of exposure to anticholinesterase agents in wild birds in Israel.

Key words: Brain cholinesterase, Carbamates, Organophosphorus, Insecticides

INTRODUCTION

Organophosphorus (OP) and carbamate (CA) insecticides, widely utilized in diverse wildlife habitats and farms, are potent cholinesterase inhibitors (1, 2, 3, 4, 5). Accidental exposure of wild birds has been shown to be responsible for the death of large numbers of wild birds of various species as a result of excess cholinergic transmission in the central and peripheral nervous system (1, 4, 6, 7, 8, 9, 10). Such exposure can precipitate toxicosis, manifested by signs similar to those observed in mammals including incoordination, weakness, ataxia, muscle tremor, diarrhea, convulsions, respiratory difficulty and bradycardia. Sudden death is usually due to respiratory failure from a high-dose exposure (2, 4). Exposure can also occur secondarily through predators and scavengers that eat their prey whole or ingest gastrointestinal contents of exposed animals.

Analysis of brain cholinesterase activity is used routinely as a diagnostic technique together with chemical detection of anticholinesterase residues in the birds, to determine exposure to OP and CA (2, 11, 12, 13, 14, 15, 16). In addition, brain cholinesterase activity of wild birds has been used as an indirect means of monitoring exposure to field applications of anticholinesterase insecticides (2, 4, 17). Diagnosis of insecticide poisoning as a cause of death in incident investigations relies heavily on the detection of insecticide residues in the proventriculus and gizzard contents, using a "multi-residue" gas chromatographic method with mass spectrometry and/or nitrogen-phosphorus de-

tection (2, 13). However, such pesticide detection methods utilizing gas chromatography is often limited, due to frequently enhanced autolysis of the birds' carcasses, unavailability of proventriculus and gizzard contents, insecticide concentrations below detection limit, or dermal exposure (18). Therefore, it is essential to measure whole brain cholinesterase activity as an additional diagnostic criterion for anticholinesterase poisoning, as brain cholinesterase activity is stable at ambient temperatures and little affected by post-mortem decomposition (11).

In order to diagnose lethal exposure to anticholinesterase insecticides based on whole brain cholinesterase activity, it is vital to know the normal values in each species (19). In the last two decades, numerous reference values of normal and pesticide-exposed brain cholinesterase activities under a standardized protocol for various wild bird species in the UK and North America have been published (2, 9, 12, 14, 19, 20). It has been widely accepted that inhibition in brain tissue to levels below two standard deviations of the mean normal activity for a species is considered evidence of exposure to one of the anticholinesterase pesticides. Furthermore, in birds found dead with brain cholinesterase activity inhibited greater than 50%, anticholinesterase, poisoning can be diagnosed as the cause of death (2, 11, 12, 19, 21, 22). However, a major confounding factor that might hamper diagnostic interpretation of whole brain cholinesterase activity measurement is the occasional occurrence of spontaneous post-mortem reactivation of carbamylated cholinesterases, which might partially mask a lethal exposure to CA pesticides (15). Moreover, reference values from different laboratories in different countries are not always closely matched and therefore it is advisable that in each country, the same laboratory using the same standardized protocol should be employed in analyzing whole brain cholinesterase activity and comparing the obtained values to their own established reference values, especially in cases where conclusions may be used to support prosecutions for violation of legislation covering pesticide use (11).

The purpose of this paper is to establish such a reference range for whole brain cholinesterase activity of healthy wild birds in Israel, compare them to available whole brain cholinesterase activities following known exposure to anticholinesterase pesticides, and finally discuss the utility of the reference range for use in the field diagnosis of wild bird mortality from anticholinesterase poisoning.

MATERIALS AND METHODS

Specimen collections

Normal whole brain was collected over a three year period from 26 different bird species that died from health problems unrelated to cholinesterase inhibition, while whole brains from birds exposed to various anticholinesterase insecticides were obtained from the Israel Nature and Parks Authority. The dissected whole brains were immediately frozen at -20°C and evaluated within a few days. The presence of insecticides in the proventriculus and gizzard of each carcass was confirmed by gas chromatography and mass spectrometry/nitrogen-phosphorus detector (GC/MS).

Whole brain cholinesterase activity assay

Whole brain was homogenized in 0.1 M phosphate buffer at pH 8.0 using 1.25g brain tissue/10 mL buffer in a Heidolph SilentCrusher S (Heidolph Instruments GmbH Schwabach, Germany). The cholinesterase activity was measured immediately after sample preparation using a modification of Ellman's method (13, 23). In brief, 250 µl of brain homogenate was mixed in a 96 plate well with 25 µl solution of 5,5'-dithio-2-nitrobenzoic acid (0.82 mg/mL) followed by the addition of 25 µl of the enzyme substrate, acetylthiocholine (6.7 mg/mL) and the change in absorbance/min during a 5 min interval was measured at 405 nm using a Sunrise Microplate reader (Tecan Group Ltd., Männedorf, Switzerland). The change in absorbance/min was eventually converted into enzyme activity by utilizing a previously derived formula (23). Enzyme activity was expressed in µmoles of acetylthiocholine hydrolyzed/ min/ gram of wet tissue. All measurements were made in triplicates at 22°C.

Determination of insecticides residues in bird's gut content by qualitative GCMS

To identify the presence of insecticides, the stomach content (up to 5 g) was extracted with 10 mL acetonitrile, dried over $MgSO_4$ and NaCl and subsequently 2 mL of the acetonitrile fraction was transferred into a new 15 mL Falcon tube and dried over $MgSO_4$ and Bondesil DEA bulk sorbent. The dried organic solvent was transferred into a new tube, of which 1 mL was evaporated using a stream of N_2 and the dried residual reconstituted with 1 mL ethylacetate and subjected to GCMS analysis. The qualitative analysis was made on a model 7890A gas chromatograph (Agilent

Technologies, Santa Clara, USA) equipped with a single quadrupole 5975C VL-MSD, nitrogen phosphorus detector and a J&W Megabore 5% phenyl-95% methyl silicone capillary column (0.25 µm · 15 m · 0.25 mm; Agilent Technologies, Santa Clara, USA). The temperature program for identifying organophosphorus pesticides was as follows: injector temperature, 220^{0}C; initial temperature, 80^{0}C for 0 min; gradient of 17^{0}C/min until 180^{0}C; gradient of 10^{0}C until 250^{0}C; gradient of 20^{0}C until 300^{0}C. The MS parameters were set as follows: source temperature, 230^{0}C; transfer line, 230^{0}C; positive ion monitoring; EI-MS (70 eV). For the identification of carbamate pesticides, the following temperature program was employed: injector temperature, 150^{0}C; initial temperature, 40^{0}C for 1 min; gradient of 15^{0}C/min until 150^{0}C; gradient of 20^{0}C until 280^{0}C; hold time, 5 min. The MS parameters were set as follows: source temperature, 250^{0}C; transfer line, 200^{0}C; positive ion monitoring; EI-MS (70 eV). Pesticide identification was accomplished by comparing the pure mass spectrum and retention time of each eluting compound with those in the NIST 05 mass spectral library.

Data analysis

Descriptive statistics were used to determine mean and standard deviation in each bird species. Statistical comparisons of whole brain cholinesterase activity between insecticide-exposed and normal birds were made by one-way analysis of variance. Statistical significance was set at $p \leq 0.05$. Normal range of whole brain cholinesterase activities were defined as two standard deviations above and below the arithmetic mean.

RESULTS

The pesticides detected most frequently in the proventriculus and gizzard of wild bird carcasses are listed in Table 1, which includes four OP and two CA compounds presented together with their corresponding LD_{50}'s in mallards (24). The most frequently encountered insec-

Table 1. Pesticides detected most frequently in wild birds in Israel in the years 2009-11

Species	Number of poisoned wildlife birds in Israel	Acute oral LD_{50} Range in mg/kg in mallards (95% CI)[a]
Organophosphorus compounds		
Chlorpyrifos	4	75 (35.4-161)
Diazinon	5	3.5 (2.4-5.3)
Methamidophos	7	8.5 (6.7-10.7)
Monocrotophos	2	4.7 (3.4-6.6)
Parathion	8	2.3 (1.8-2.9)
Carbamate compounds		
Aldicarb	23	3.4 (2.7-4.3)
Methomyl	15	15.9 (11.4-22)
Carbofuran[b]	3	0.5 (0.3-0.6)

[a]Reference 4
[b]Not commercially available in Israel

Table 2. Whole brain cholinesterase activity in insecticide-exposed compared with unexposed, birds of prey in Israel

Species	Unexposed whole brain acetylcholinesterase activity Mean ± std (n)[a] (µmol acetylthiocholine/ min/g tissue)	Pesticides exposed whole brain acetylcholinesterase activity Mean ± std (n) (µmol acetylthiocholine/ min/g tissue)
Long eared owl (*Asio otus*)	9.9 ± 1.2 (6)	ND[b]
Eagle owl (*Bubo bubo*)	8.7 ± 1.2 (7)	ND
Tawny owl (*Strix aluco*)	9.9 ± 1.2 (3)	ND
Barn owl (*Tyto alba*)	7.4 ± 1.2 (20)	ND
European honey-buzzard (*Pernis apivorus*)	9.9 ± 2.5 (5)	ND
Common buzzard (*Buteo buteo*)	11 ± 2.5 (13)	4.7 (1, methomyl)
Kestrel (*Falco tinnunculus*)	12.4 ± 2.5 (21)	ND
Griffon vulture (*Gyps fulvus*)	11 ± 2.4 (9)	3 ± 0.1 (2, aldicarb)
Short-toed eagle (*Circaetus gallicus*)	12.4 ± 2.4 (4)	ND
Bonellis eagle (*Hieraetus fasciatus*)	12.4 ± 1.2 (8)	4.9 ± 0.6 (3, aldicarb)
Black kite (*Milvus migrans*)	12.5 ± 1.8 (12)	0.4 ± 0.01 (2, monocrotophos) 4.9 (1, aldicarb)

[a]Number of birds. [b]Not determined

Table 3. Whole brain cholinesterase activity in insecticide-exposed compared with. unexposed wild birds in Israel

Species	Unexposed whole brain acetylcholinesterase activity Mean ± SD (n)[a] (µmol acetylthiocholine/min/g tissue)	Pesticides exposed whole brain acetylcholinesterase activity Mean ± SD (n, pesticide) (µmol acetylthiocholine/min/g tissue)
Mallard (*Anas platyrynchos*)	8.7 ± 1.2 (5)	ND[b]
Cattle egret (*Bubulcus ibis*)	10.5 ± 1.8 (9)	2.5 (1, methomyl)
White stork (*Ciconia ciconia*)	11 ± 2.5 (15)	ND
Marsh harrier (*Circus aeruginosus*)	16 ± 2.5 (6)	ND
Rock pigeon (*Columba livia*)	16.1 ± 1.5 (7)	ND
Hooded crow (*Corvus corone*)	12.4 ± 2.5 (3)	3.7 ± 1.2 (4, methomyl) 4.9 ± 1.8 (2, aldicarb)
Jackdaw (*Corvus monedula*)	9.9 ± 2.5 (3)	1.8 ± 0.2 (2, methomyl)
Mute swan (*Cygnus olor*)	7.4 ± 1.2 (4)	ND
Domestic chicken (*Gallus domesticus*)	13.6 ± 1.2 (5)	3 ± 2.7 (2, diazinone)
Black headed gull (*Larus ridibundus*)	19.8 ± 4.9 (16)	ND
House sparrow (*Passer domesticus*)	15 ± 2.5(6)	ND
White pelican (*Pelecanus onocrotalus*)	9.9 ± 2.5 (3)	ND
Pygmy cormorant (*Phalacrocorax pygmeus*)	16.1 ± 3.7 (6)	ND
Glossy ibis (*Plegadis falcinella*)	13.6 ± 1.2 (4)	ND
Chukar (*Alectoris chukar*)	9.9 ± 1.2 (3)	0.7 ± 0.3 (4, methamidophos and chlorpyrifos)
Common coot (*Fulica atra*)	ND	6.0 ± 0.3 (3, carbofuran)

[a]Number of birds. [b]Not determined

ticides in the wild birds were the highly toxic CA's, aldicarb and methomyl with LD_{50}'s of 3.4 and 15.9 mg/kg respectively (Table 1).

Normal whole brain cholinesterase activity in diurnal and nocturnal birds of prey showed small differences between species, ranging from 7.4 – 12.5 µmol/min/g tissue (Table 2). Only in three species, namely the Griffon vulture (*Gyps fulvus*), Bonellis eagle (*Hieraetus fasciatus*) and Black kite (*Milvus migrans*), could residual insecticides be detected unequivocally (Table 2) and their corresponding whole brain cholinesterase activities were determined. All birds that were found dead and in which insecticides were detected, revealed a significant decrease of more than 50% of their normal whole brain cholinesterase activity, thereby confirming the cause of death due to cholinesterase inhibition. Birds exposed to the OP insecticide monocrotophos, showed a pronounced inhibition of whole brain cholinesterase activity as compared to birds exposed to the CA, aldicarb (97% vs. 65% depression, respectively; Table 2). Aldicarb was the most prevalent detected insecticide in birds of prey, which

is not surprising since most of the birds were found near aldicarb impregnated meat baits that were in some cases were scattered by local farmers intending to illegally kill animal mammalian pests.

Table 3 lists normal whole brain cholinesterase activity values of several wild birds in Israel, revealing a wider range of cholinesterase activity (7.4-19.8 µmol/min/g tissue) as compared to the cholinesterase activity range in birds of prey (7.4-12.5 µmol/min/g tissue; Table 2 and 3). In 4 different wild bird species, namely hooded crow (*Corvus corone*), jackdaw (*Corvus monedula*), cattle egret (*Bubulcus ibis*) and chukar (*Alectoris chukar*), anticholinergic insecticides were detected in their proventriculus and gizzard and their corresponding whole brain cholinesterase activities were established (Table 3). All of the depressed cholinesterase activity values were below 50% of the normal values, thereby confirming poisoning due to anticholinergic insecticides. The most prominent insecticides detected in stomach contents, were the CA, methomyl and aldicarb, in accordance to the most abundant insecticides found in birds of prey. The most pronounced depression of cholinesterase activity was found in 4 chukars poisoned with a mixture of methamidophos and chlorpyrifos, two highly toxic OP compounds with an oral LD_{50} in mallards of 8.5 and 83 mg/kg, respectively.

DISCUSSION

The frequent poisoning of wild birds in Israel as a result of exposure to anticholinergic pesticides each year necessitates rapid and reliable diagnostic techniques to determine the cause of death in order to rapidly start mitigation procedures. Currently, 2 main diagnostic techniques are routinely employed in our laboratory protocol to detect OP and CA pesticides in birds and mammals, namely a multi-residue GC/MS screening and whole brain cholinesterase activity measurement. A diagnosis based on depressed whole brain cholinesterase activity requires the knowledge of normal brain cholinesterase activity values of a wide range of wild bird species.

The purpose of the present study was to determine the normal whole brain cholinesterase activities in domestic birds in Israel. Although numerous studies of a similar nature were published in the last century in the UK and North America, the cholinesterase activity values do not always match, mainly due to slight variations in the employed assay, varied storage conditions, variable food consumption habits and diverse habitats (2, 11, 14, 19, 20). Therefore, the reference values presented in this study (Tables 2 and 3) will enable ecotoxicologists in Israel to interpret results with greater confidence and reliability. The most prominent anticholinergic pesticides detected in birds of prey and other birds were the CA, aldicarb and methomyl (Table 1-3). Meat and wheat baits containing aldicarb or methomyl were often found near the bird's carcasses, which were, in several documented cases, scattered by local farmers intending to poison mammalian pests.

Birds' carcasses with insecticide residues in their proventriculus and gizzard, exhibited more than a 50% decrease of whole brain cholinesterase activity compared to their corresponding normal values (Tables 2 and 3). These findings agree with the widely accepted conservative threshold of at least 50% depression in whole brain cholinesterase activity as a reliable diagnostic criterion of death from cholinesterase poisoning, even though depression of more than 70% is routinely reported for birds dying from OP and CA insecticides (19).

In contrast to OP poisoning, cholinesterase reactivation is a major concern for false negative findings in cases of CA poisoning (15). Dependent on the periods of time and the temperature the carcass remained in the field, a pronounced reactivation of carbamylated cholinesterase might occur that would mask CA poisoning (15). Therefore it is advisable to immediately store the carcass on ice until the samples can be placed in a refrigerator or freezer.

In conclusion, this present study documents normal whole brain cholinesterase activity values and reports the depressed values of bird species known to be exposed to OP and CA insecticides. The tabulated values will enable the evaluation of suspected cases of OP or CA exposure of wild birds in Israel. Expanding the reference values to include more wild bird species and also mammals is currently under progress in our laboratory.

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