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Short Communication

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Acetylcholinesterase activity in the brain of wild birds in Korea—2014 to 2016

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ABSTRACT

Acetylcholinesterase (AChE) activity level can be used as a diagnostic marker for anticholinesterase pesticide poisoning. In this study, we aimed to establish a baseline level of normal brain AChE activity in wild birds. AChE activity was measured in the brains of 87dead wild birds (26 species). The level of AChE activity ranged from 6.40 to 15.9 μmol/min/g of brain tissue in normal wild birds. However, the brain tissue AChE activity level in wild birds exposed to organophosphate (OP) pesticide was 48.0%–96.3% of that in the normal birds. These results may serve as reference values to facilitate routine diagnosis and monitoring of OP-poisoned wild birds.

Keywords: Acetylcholinesterase; organophosphates; poisoning; reference range; wild birds

Organophosphates (OPs), the most widely used pesticide due to their strong insecticidal effect, are a potent acetylcholinesterase (AChE) inhibitor [1-4]. OP pesticides competitively bind to AChE and inhibit the hydrolysis of acetylcholine leading to the accumulation of acetylcholine at various receptors in the nervous system [2,3,5,6]. Overstimulation of acetylcholine receptors can produce fatal neurotoxic effects [7], such as respiratory failure [3].

According to reports released by the Korean Animal and Plant Quarantine Agency (APQA, Republic of Korea), a total of 182 dead wild birds of 27 species have been examined between 2010 and 2013. Pesticides were detected in 59% of those birds and organophosphorus-based pesticides were the most common type detected [8].

As wild birds can be exposed to OPs via ingestion of pesticide-contaminated water or food, the diagnosis of pesticide poisoning relies on the detection of pesticide residues in the stomach contents, typically by using gas chromatography or mass spectrometry [9,10]. However, there are difficulties in the diagnosing pesticide poisoning as the cause of death when insecticide concentrations are very low or when there is very little stomach content available for examination. However, determination of brain AChE activity in conjunction with the chemical detection of pesticide residues can be useful diagnostic markers for anticholinesterase pesticide poisoning. It is widely accepted that brain AChE activity is stable at normal body temperatures and is little changed during the initial post-mortem period, and

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Yi H; Data curation: Bang JH; Formal analysis: Park SW; Funding acquisition: Kang HG; Project administration: Yi H; Resources: Jang I, Bae YC; Supervision: Kim YS; Validation: Kim H, Kim S; Visualization: Ku HO; Writing - original draft: Bang JH; Writing - review & editing: Woo GH.

dead birds with brain AChE activity lower by more than 50% of their normal brain AChE can be diagnosed with OP poisoning as the cause of death [4,11].

As the reference values reported by different laboratories in different countries do not always correspond, there is a need to obtain Korean-specific reference values for wild birds. The purpose of this study was to establish a brain AChE activity reference range in non-exposed wild birds in Korea that can be used in the field diagnosis of wild bird mortality from OP poisoning.

Brain tissues of wild birds obtained between 2014 and 2016 were kindly provided by the Avian Disease Division of the Korean Animal and Plant Quarantine Agency (APQA). The AChE activity level in the tissue samples was measured by using, according to the manufacturer's instructions, the Acetylcholinesterase Activity Colorimetric Assay Kit (Biovision, USA), which is based on a modification of Ellman's method [12]. Briefly, 500 mg of brain tissue were homogenized in 2 mL of EZLys Tissue Protein Extraction Reagent (Biovision, USA) with ceramic beads in a 7 mL tube for 15 sec using a Precellys 24 homogenizer (Bertin, France). Ten microliters of the homogenized solution were then diluted 20 times after centrifuging at 10,000 × *g* for 5 min at 4°C in a Labogene 1580R centrifuge (LaboGene, Denmark). Subsequently, 3 µL of the solution were mixed with 97 µL of reaction mix (containing 92 µL of assay buffer, 2 µL of choline oxidase enzyme mix, 2 µL of AChE probe, and 1 µL of AChE substrate) in a 96-well plate. The optical density of the sample solution was measured using a Flexstation3 microplate reader (Molecular Devices, USA) in kinetic mode for 25 min at 37°C. The AChE activity level was calculated as AChE activity = B / (ΔT × V) × D, where B represents the amount of choline from the end point of the choline standard curve, ΔT is the difference between two chosen reaction times (such as 10 min to 15 min), V represents the sample volume added to the reaction plate, and D represents the sample dilution factor. The result represented the AChE enzyme activity in the sample and is expressed in nmol/min/mL of brain tissue. Three replicates were performed per sample to confirm the AChE activity level.

Eighty-seven wild birds suspected of being poisoned by pesticides were submitted to APQA between 2014 and 2016. The monthly distribution of the number of submitted wild birds between 2014 and 2016 showed a peak (representing 87% of the submitted birds) during the January to March period (**Fig. 1**). In the present study, the brain tissue AChE activity was measured in the brain tissue of each of the 87 wild birds (26 species).

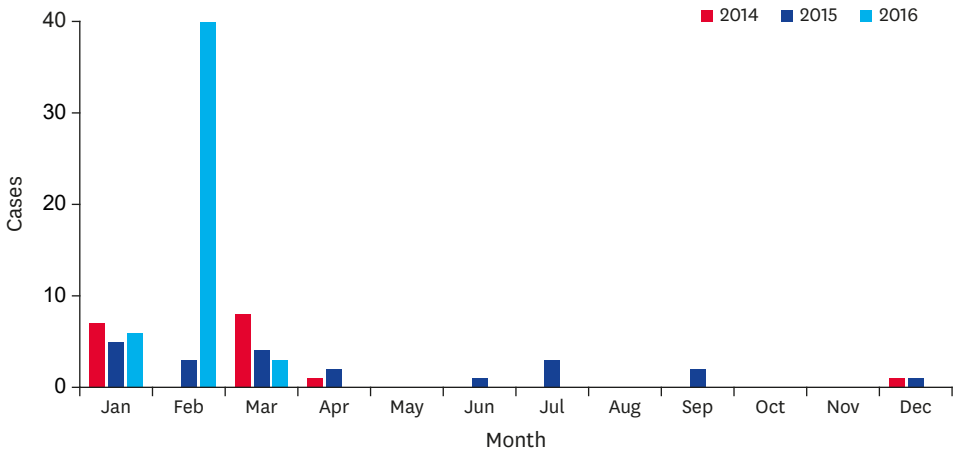

Fig. 1. Monthly distribution of avian carcasses submitted to the Korean Animal and Plant Quarantine Agency from 2014 to 2016. (Seventeen cases in 2014, twenty-one cases in 2015, and forty-nine cases in 2016).

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Table 1. Intraday- and interday-validation of the AChE activity assay in brain tissues of wild birds

Species	Intraday assay				Interday assay					
	AChE activity*	Mean*	SD	CV (%)	AChE activity*			Mean*	SD	CV (%)
					Day 1	Day 2	Day 3			
Spot-billed duck (*Anas poecilorhyncha*)	4.35	4.98	0.55	11.0	4.98	6.27	6.25	5.84	0.74	12.7
	5.31									
	5.29									
Korean native chickens (*Gallus gallus domesticus*)	8.30	8.28	0.44	5.30	8.28	9.89	8.73	8.96	0.83	9.24
	7.84									
	8.71									
Magpie (*Pica pica serica*)	5.89	5.95	0.22	3.68	5.95	7.15	6.39	6.50	0.61	9.36
	6.19									
	5.76									

AChE, Acetylcholinesterase; SD, standard deviation; CV, coefficient of variation.
*Value units are μmol/min/g tissue.

The repeatability of the AChE activity assay was evaluated in spot-billed duck, magpie, and native chicken (**Table 1**). In the intraday assay assessment, the coefficients of variation of brain AChE activity in each sample of spot-billed duck, magpie, and native chickens were 11.0%, 5.30%, and 3.68%, respectively (**Table 1**). In the interday assay assessment, the coefficients of variation of brain AChE activity in spot-billed duck, magpie, and native chicken were 12.7%, 9.24%, and 9.36%, respectively. Overall, the coefficient of variation of the intraday assay was equal to or less than 10%, whereas that of the interday assay was less than 15%.

The stomach contents of the birds were analyzed by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) to determine pesticide residue levels, and dead birds with a high pesticide level were considered to have died from pesticide poisoning. Twenty-two bird stomachs contained pesticide residues while the other 65 birds were identified as negative for the presence of pesticide residue (unpublished data). The species of wild birds with stomach pesticide residues were crows, magpies, pigeons, mallards, bean geese, native chickens, and spot-billed ducks.

The AChE activity levels in the brain of pesticide-unexposed wild birds ranged from 6.40 ± 2.19 to 15.9 ± 4.10 μmol/min/g brain tissue, and the results showed differences among the species sampled (**Table 2**). In a previous study, the range in brain AChE activity in wild birds was reported to be 7.4–31.2 μmol/min/g, with differences among the species sampled and experimenters [1,13,14]. In the present study, the brain AChE activity in unexposed mallard ducks was 9.06 ± 1.10 μmol/min/g tissue, whereas, in normal mallard duck brains, Hill reported it to be 12 ± 1.3 μmol/min/g [11], and Shimshoni showed it to be 8.7 ± 1.2 μmol/min/g [4]. In our chicken samples, brain AChE activity was 15.9 ± 4.10 μmol/min/g in unexposed birds, whereas, Shimshoni reported it to be 13.6 ± 1.2 μmol/min/g [4].

In the present study, we observed that the brain AChE activity level in OP-exposed birds was 48.0% to 96.3% of that of normal birds. Shimshoni reported that the AChE activity level of chickens in Israel that had been exposed to diazinon was 3 ± 2.7 μmol/min/g [4], whereas, in the present study, Korean chickens exposed to monocrotophos and phosaphamidon had an AChE activity level of 0.69 ± 0.35 μmol/min/g.

Although a number of similar studies have been conducted worldwide, reference values of AChE activity in bird brains are not always consistent, mostly due to the temperature at measurement, the measured part, and differences between experimenters and laboratories.

Table 2. Summary of AChE activities of avian samples suspected as OP pesticide-exposed and unexposed samples

Species	Scientific name	OP-exposed			OP-unexposed		
		Average	Range	No.*	Average	Range	No.*
Spot-billed duck	*Anas poecilorhyncha*	2.93 ± 0.75	1.96–3.88	6	9.56 ± 6.74	4.79–14.3	2
Mallard	*Anas platyrhynchos*	1.29 ± 1.09	1.22–2.67	4	9.06 ± 1.10	8.14–10.3	3
Dove	*Columbidae*	4.05 ± 4.07	1.17–6.93	2	9.94 ± 2.60	9.01–12.2	15
Carrion crow	*Corvus corone orientalis*	0.57 ± 0.04	0.54–0.59	2	6.61 ± 0.49	6.11–7.14	5
Magpie	*Pica pica sericea*	5.43 ± 3.49	2.96–7.89	2	10.4 ± 2.03	10.5–12.0	4
Korean native chicken	*Gallus gallus domesticus*	0.69 ± 0.35	0.44–0.94	2	15.9 ± 4.10	13.0–18.8	2
Bean goose	*Anser fabalis*	1.17 ± 1.10	0.39–1.95	2	-	-	-
Brown-eared bulbul	*Microscelis amaurotis*	2.01	-	1	-	-	-
Hooded crane	*Grus monacha temminck*	3.38	-	1	-	-	-
Great tit	*Parus major*	-	-	-	11.8 ± 1.83	8.80–14.3	9
Kestrel	*Falco tinnunculus interstinctus*	-	-	-	8.66 ± 1.04	7.47–9.91	5
Gray heron	*Ardea cinerea*	-	-	-	9.53 ± 3.94	5.25–13.0	3
Red-throated diver	*Gavia stellata*	-	-	-	11.5 ± 0.46	11.2–11.9	2
Common kingfisher	*Alcedo atthis*	-	-	-	9.86 ± 0.08	9.80–9.91	2
Gray starling	*Sturnus cineraceus*	-	-	-	6.4 ± 2.19	4.85–7.95	2
Upland buzzard	*Buteo hemilasius*	-	-	-	7.37	-	1
Black kite	*Milvus migrans*	-	-	-	8.21	-	1
Ancient murrelet	*Synthliboramphus antiquus*	-	-	-	12.0	-	1
Pheasant	*Phasianus colchicus*	-	-	-	11.0	-	1
Cinereous vulture	*Aegypius monachus*	-	-	-	2.09	-	1
Hazel grouse	*Tetrastes bonasia vicinitas*	-	-	-	10.8	-	1
Woodpecker	*Picidae*	-	-	-	7.23	-	1
Rook	*Corvus frugilegus*	-	-	-	6.53	-	1
Rufous turtle dove	*Streptopelia orientalis*	-	-	-	8.83	-	1
Baikal teal	*Anas formosa*	-	-	-	-	-	1
Black-tailed gull	*Larus crassirostris*	-	-	-	1.19	-	1
Total				22			65

AChE, acetylcholinesterase; OP, organophosphate.
*Number of birds.

Because of these differences, standardization of the AChE assay method, presently unique to each laboratory, is required [4]. It has been suggested that a decrease of 20% in normal brain AChE activity is indicative of exposure to anticholinesterase pesticides (OPs and carbamates), and that dead birds with a brain AChE activity lower by more than 50% of their normal brain AChE can be diagnosed with anticholinesterase poisoning as the cause of death [4,5,14].

In this study, we employed a method to measure the AChE activity in the brain of dead wild birds and evaluated the repeatability of the method in an attempt to improve the reliability of diagnosing OP poisoning. In addition, we investigated differences in AChE activity between unexposed and pesticide-exposed individuals. Those results can form a basis for future accurate diagnoses of OP poisoning in wild birds in Korea. A limitation of this study was that the activity of AChE was assayed in a relatively small number of birds. However, the brain AChE activity reference values for wild birds presented in this study could facilitate routine diagnosis and monitoring of exposure to AChE inhibitors in wild birds in Korea.

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