Reevaluation of the developmental toxicity of dieldrin by the use of fertilized Japanese quail eggs

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ABSTRACT

To reevaluate the toxicity of the organochlorine insecticide and persistent organic pollutant dieldrin and confirm its impact on development, an exposure trial using bird eggs was performed. Dieldrin at concentrations of 10–100 μg/g of egg was injected into the yolks of Japanese quail (*Coturnix japonica*) eggs. Hatchlings from the eggs were raised to sexual maturity and multiple tests to detect the harmful effects of dieldrin were conducted. Dieldrin at 100 μg/g decreased egg hatchability by 50.0% (vehicle control, 86.7%), although embryogenesis even in unhatched eggs treated with high doses of dieldrin was normal. In safely hatched chicks, dose-dependent early death with tonic seizure was observed and all birds exposed to 100 μg/g died within 3 days. Other significant alterations in hatchlings were enlargement of the whole brain, decreases in mRNA expressions of tryptophan hydroxylase in the brainstem and cholesterol side-chain cleavage in the male gonad, and increases in mRNA expressions of cytochrome P450 1A and 2C18 in the liver. For mature birds (males at 5 weeks and females at 10 weeks of age), impairment of eggshell formation such as reduced eggshell mass and eggshell thinning, increases in the body mass of males and the liver mass of females and increases in serum total cholesterol and triglyceride concentrations were observed. The results indicated that not only does the neurotoxicity of dieldrin bring early death, but its effects on reproductive and hepatic functions (detected as gene transcriptional changes in hatchlings) persist harmfully after maturity.

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1. Introduction

Use of the organochlorine insecticides aldrin, endrin and dieldrin (drins) is now banned in most developed countries. In Japan these compounds had been mainly used from the 1950s to the 1970s to exterminate crop pests; their production, sale and use were prohibited in 1981. However, their chemical stability and lipophilic properties have resulted in their long-term persistence in the natural environment and their accumulation in organisms. Even now, in Japan, the compounds are found in farm crops, environmental waters, sediments and wildlife (shellfish, fish and birds), although the levels are low (Ministry of the Environment, Government of Japan, 2006). Dieldrin was also applied to building lumber for termite control. Therefore, there is concern that the highly persistent dieldrin may leak out of the debris when buildings are demolished in the future.

Dieldrin exerts its major toxic effects on the central nervous system and the liver. Symptoms of acute central nervous system injury (such as tonic seizures, myoclonic jerks and mental disorders) and chronic hepatic dysfunction (enlarged liver and liver tumors)

have been well established for dieldrin as for other harmful organochlorine pesticides (Evangelista de Duffard and Duffard, 1996; U.S. Environmental Protection Agency, 2003). The neurotoxicity of dieldrin is considered to arise from its inhibitory effect on the chloride ion channel of the gamma-aminobutyric acid (GABA) A receptor (Bloomquist and Soderlund, 1985; Nagata and Narahashi, 1994; Narahashi et al., 1995; Vale et al., 2003), and blocking the inhibitory GABAA receptor appears to make central neurons hyper-excitable. It is also thought that the high lipophilicity and persistence of dieldrin can result in perinatal exposure and cause developmental effects on the embryo. Early deaths in chicks hatched from chicken (*Gallus gallus*) eggs exposed to dieldrin (Smith et al., 1970) and in pups born to female mice (*Mus musculus*) receiving dietary dieldrin during the 4 weeks prior to mating (Birgo and Bellward, 1975) were reported. Thus neurological dysfunction via GABAA receptor inhibition and eventual developmental abnormalities can also be predicted in exposed newborn animals. However, there have been very few studies of the consequences of embryonic exposure to dieldrin and its developmental toxicity is not fully understood.

The present study employed fertilized Japanese quail (*Coturnix japonica*) eggs and reevaluated the developmental toxicity of dieldrin injected into the eggs. Avian eggs offer a great advantage in the precise evaluation of the effects of embryonic exposure to test

chemicals because there is little mass transfer from outside the eggs during embryonic development. In addition, the signal transduction mechanisms involving hormones and neurotransmitters, of avian species are very similar to those of mammals in type and function. This is also true for metabolic processes in the liver such as conjugation and those involving cytochrome P450. Therefore, the findings obtained in this study can be compared in a meaningful way with literature data from mammals for pharmacological and toxicological interpretations. Chicks were observed for symptoms of toxicity from hatching until sexual maturity. Because dieldrin has been shown to include hepatic dysfunction among its toxic effects, hematological changes and gene expression in the liver were analyzed in this study. Early deaths of quail chicks by tonic seizure were observed at higher doses of dieldrin and therefore a further exposure trial was undertaken and analyses of gene expression in the brainstem of newly-hatched chicks were performed.

2. Materials and methods

2.1. Selection of fertilized eggs

Experiments were performed with the approval of the Animal Ethics Committee in the National Institute for Environmental Studies under guidelines contained in the Act on Welfare and Management of Animals in Japan.

Forty pairs of Brazilian Brown male (Br, maintained in our laboratory) and White Egg female (WE, originally from the Nippon Institute for Biological Science, Yamanashi, Japan and maintained in our laboratory) Japanese quails (*Coturnix japonica*) were prepared and maintained to supply fertilized eggs. From pairs which regularly produced 10 or more eggs each per 2 week period, at least 10 eggs were taken and incubated prior to the study and their fertility and embryo viability on day 10 of incubation were checked. Eggs for the study were collected from those pairs that exhibited embryo viability of 80% or more within the 2 weeks before the beginning of incubation and were stored at 12–13 °C. As the average egg mass from pairs of Br males and WE females was approximately 10.5 g in our preliminary study, eggs weighing 10 to 11 g were chosen from the stored eggs and divided into treatment groups. Thirty-five eggs in each group were randomly selected to minimize the differences between the averages of egg masses for the groups.

2.2. Administration of test compound

Dieldrin (Accu Standard, Inc. New Haven, CT, USA) was dissolved in dimethylsulfoxide (DMSO) and, on the day before treatment, the DMSO solution was diluted with corn oil to give appropriate concentrations for administration. Doses of dieldrin were adjusted to 0 (vehicle alone, 1% DMSO in corn oil), 10, 30 and 100 µg/g per egg. On the day of treatment, eggs were filed at the blunt end with an electric micro grinder to make a small hole in the eggshell but to leave the shell membrane untouched. Test solution (20 µL) was injected into the yolk using a 25-gauge needle attached to a Hamilton syringe. After injection, each hole was sealed with melted paraffin wax. Thirty successfully injected eggs were incubated at 37.8 °C and 60% relative humidity with a turning cycle of once an hour. On day 10 of incubation, eggs from each group were checked for embryo viability.

2.3. Handling of birds

Quails were reared according to our protocol (Kamata et al., 2009a,b). Briefly, eggs were moved to hatching boxes on day 15 of incubation and hatched chicks were collected on days 17 and 18 and moved to brooders in mixed sex groups for each treatment. As F1 offspring from Br male and WE female can be genetically sexed by their plumage color (Kamata et al., 2006a), 7 female and 7 male chicks from each treatment group were

randomly selected at 3 days of age. The rest of the chicks were sacrificed and examined for gross morphological characteristics. Samples of the liver and gonad were removed for analysis of gene expression and kept at −80 °C until analysis. Quails were fed commercial diets (PLD-CHICK for chicks and PLD from 5 weeks of age, Oriental Yeast Co. Ltd., Chiba, Japan) and kept under optimal air conditions (Kamata et al., 2009a,b). They were divided according to sex 10 days after hatching. Female quails were individually moved to single cages at 5 weeks of age. The eggs laid by each female quail were collected and the number and masses of the eggs were recorded.

Male and female quails at 5 and 10 weeks of age, respectively, were necropsied following blood removal from the jugular vein. Serum was collected after centrifugation of the blood and kept at −80 °C until being hematologically analyzed. The liver, testis and oviduct were removed and weighed and oviduct length was measured. Small pieces of the liver and reproductive organs were also removed and frozen at −80 °C prior to analysis for gene expression.

As early deaths of hatchlings by tonic seizure within 3 days after hatching were observed at higher doses of dieldrin, a further exposure trial was performed following the same procedures in an attempt to determine the mechanistic basis of the seizures. Chicks treated in ovo with 30 µg dieldrin/g per egg or with the vehicle alone were sacrificed at 18 days from the onset of incubation (within 2 days after hatching) and the whole brain was removed and weighed. Samples of the brainstem were kept at −80 °C for analyses of gene expression.

2.4. Eggshell properties

The eggshell strength of collected eggs was measured as the fracture resistance of the shell (N) using a digital force gauge (CPU-9500, Aikoh Engineering Co. Ltd., Gifu, Japan) and a vertical test stand wherein an egg was compressed at the equator between two stainless steel surfaces. After measurement of shell strength, the egg was cut around its meridian, emptied and dried together with its shell membrane. The resultant eggshell was weighed and the shell thickness was measured with a micrometer (BMD-25DM, Mitutoyo Corporation, Kanagawa, Japan) at 5 points along the meridian: the blunt end of the egg, the mid-point of the blunt end and equator, the equator, the mid-point of the equator and sharp end, and the sharp end.

2.5. Gene expression

Total RNA was extracted from chick gonads, brains, livers and oviduct uteri with a SV Total RNA Isolation System (Promega, Madison, WI, USA) to determine the amount of transcript from the genes; the quantity was determined by spectrophotometry at 260 nm. One hundred nanograms total RNA with 0.5 µg oligo dT_{15} primer in a final volume of 25 µL was reverse transcribed at 37 °C for 60 min using M-MLV Reverse Transcriptase (Promega) and the resultant cDNA was diluted 4 times with nuclease-free water. For amplification of each target mRNA, 2 µL of a diluted cDNA solution was amplified by GoTaq DNA Polymerase (Promega), together with 0.5 µM each of the specific primer combinations designed for each gene in a total volume of 50 µL. The PCR conditions were an initial denaturing step at 94 °C for 2 min, followed by 30–40 additional cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s.

Primers used with samples of the liver or reproductive organs were designed from the base sequences of the genes represented by the GenBank accession numbers in Table 1 so that they would anneal at 60 °C. However, as the base sequences of genes measured here in the brainstem had not been identified for Japanese quail, primers for these analyses were designed from the chicken genes represented by the accession numbers in Table 2. Amplified products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied