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# The effects of avermectin on amino acid neurotransmitters and their receptors in the pigeon brain

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ABSTRACT

The objective of this study was to examine the effects of avermectin (AVM) on amino acid neurotransmitters and their receptors in the pigeon brain. Four groups two-month-old American king pigeons ($n = 20$/group) were fed either a commercial diet or an AVM-supplemented diet (20 mg/kg·diet, 40 mg/kg·diet, or 60 mg/kg·diet) for 30, 60, or 90 days. The contents of aspartic acid (ASP), glutamate (GLU), glycine (GLY), and γ-aminobutyric acid (GABA) in the brain tissues were determined using ultraviolet high-performance liquid chromatography (HPLC). The expression levels of the GLU and GABA receptor genes were analyzed using real-time quantitative polymerase chain reaction (qPCR). The results indicate that AVM exposure significantly enhances the contents of GABA, GLY, GLU, and ASP in the cerebrum, cerebellum, and optic lobe. In addition, AVM exposure increases the mRNA expression levels of γ-aminobutyric acid type A receptor (GABAAR), γ-aminobutyric acid type B receptor (GABABR), *N*-methyl-D-aspartate 1 receptor (NR1), *N*-methyl-D-aspartate 2A receptor (NR2A), and *N*-methyl-D-aspartate 2B receptor (NR2B) in a dose- and time-dependent manner. Moreover, we found that the most damaged organ was the cerebrum, followed by the cerebellum, and then the optic lobe. These results show that the AVM-induced neurotoxicity may be associated with its effects on amino acid neurotransmitters and their receptors. The information presented in this study will help supplement the available data for future AVM toxicity studies.



## 1. Introduction

Avermectins (AVMs) are streptomycete-derived macrocyclic lactones that were originally isolated as antiparasitic agents. AVMs are extensively used in agriculture and animal husbandry [1]. However, the increasing usage of AVM has made AVMs a potential risk to the non-target organisms, such as insects, beetles, fleas, shrimp, and several types of fish [2,3]. In recent years, the number of studies on AVM toxicity has increased, and AVMs have been demonstrated to be harmful to vertebrates [4]. The effect of AVMs on birds cannot be neglected. Although the data on the toxic effects of AVM on bird are limited, prior studies have indicated the adverse roles of AVMs in several types of birds [5], and the median lethal doses for the American quail and ducks are 102 and 383 mg $kg^{-1}$, respectively [5]. Driniaev et al. [6] showed that AVM exhibits cytostatic and cytotoxic effects on neuroblastoma B 103 cells. In addition, in a previous study (unpublished), we showed that pigeons that ingested an excessive amount of AVMs suffered from damage to their nervous systems and exhibited various symptoms, including ataxia, tremor, mental depression, limb twitching, lack of flight, coma, and death.

Neurotransmitters, as messengers in chemical synaptic transmission, play an important role in information transfer in the nervous system. Amino acid neurotransmitters are widely distributed in the central nervous system (CNS), especially in brain tissues and the cerebrospinal fluid. Amino acid neurotransmitters can be divided into two types: excitatory amino acids (including GLU and ASP) and inhibitory amino acids (including GABA and GLY). Combined with amino acid receptors, amino acid neurotransmitters can exert crucial effects on synaptic transmission, such as sensory information conduction in the CNS. The amino acid receptors,

*Abbreviations:* AVM, avermectin; CNS, central nervous system; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; NR1, NMDA receptor 1; NR2, NMDA receptor 2; NR2A, NMDA receptor 2A; NR2B, NMDA receptor 2B; GABAAR, GABA A receptor; GABABR, GABA B receptor; HPLC, high-performance liquid chromatography; qPCR, real-time quantitative polymerase chain reaction; DART-PCR, data analysis for real-time PCR; ASP, aspartic acid; GLU, glutamate; GLY, glycine.

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which include *N*-methyl-D-aspartate receptors (NR1, NR2A, NR2B), and GABA receptors (GABAAR, GABABR), play important roles in the CNS. After injury, the levels of amino acid neurotransmitters in brain tissues are changed significantly, and the balance between excitatory and inhibitory neurotransmission is disturbed [7]. Some prior studies have suggested that AVMs can promote GABA release and bind to its receptors to result in $Cl^-$ influx, hyperpolarization of the cell membrane, and inhibition of neural cells [8,9]. In addition, AVMs can induce the opening of GLU-gated $Cl^-$ channels, increase membrane $Cl^-$ permeability, and reduce nerve conduction, which results in excitotoxicity, apoptosis, and necrosis [10,11]. As mentioned above, AVMs can influence the release of some neurotransmitters and their receptors in invertebrate phyla. However, the role of AVMs in the regulation of neurotransmitters and their receptors in vertebrates, particularly birds, is not well elucidated. It is been known that excessive AVM intake can induce damage in pigeon brains. Thus, the study of the role of AVMs in pigeon brain involving signaling through neurotransmitters and their receptors may provide insights into the possible mechanism of AVM toxicity in pigeon.

## 2. Materials and methods

### 2.1. Animal models and sample collection

All of the protocols were approved by the Experimental Animal Protection and Use Committee of Northeast Agricultural University of China. Eighty-two-month-old American king pigeons (purchased in Harbin, China) were randomly divided into four groups and were respectively fed either a commercial diet (control group) or an AVM (consisting of 98% avermectin B1 and 92% B1a)-supplemented diet (20 mg/kg·diet; low-dose group), 40 mg/kg·diet (moderate-dose group), or 60 mg/kg·diet (high-dose group) for 30, 60, or 90 days. Feed and tap water were supplied *ad libitum*. After 30, 60, or 90 days of treatment, the pigeons were subjected to euthanasia with sodium pentobarbital, and their brains were quickly removed. The tissues were blotted, rinsed with ice-cold sterile deionized water, frozen immediately in liquid nitrogen, and stored at −80 °C until required.

### 2.2. HPLC analysis of the concentrations of amino acid neurotransmitters in brain tissues

The HPLC procedures used in this study were similar to those described in a previously published HPLC method with a modified amino acid detection method [12], which is described below.

#### 2.2.1. Sample processing and chromatographic conditions

From each brain, 40 μl of tissue fluid was mixed with 40 μl of acetonitrile, and the mixture was centrifuged for 10 min at 12,000 r $min^{-1}$ and at −4 °C. The supernatants were treated with 40 μl of a 0.5 mol $L^{-1}$ $NaHCO_3$ solution and with 10 μl of 0.5% (V:V) 2,4-dinitoflruorobenzene for derivation in a thermostatic water bath at 65 °C for 55 min in the presence of 10 μl of the sample. Under optimal derivation conditions, the derivative rate of the four types of amino acid neurotransmitters was 100%. The chromatographic conditions were as follows: the chromatographic column was a Diamonsil C18 column (4.6 mm × 200 mm, 5 μm), the mobile phase A was a 0.05 mol $L^{-1}$ sodium acetate buffer solution (pH = 5.8), the mobile phase B was an acetonitrile–water solution (1:1 V:V), the flow rate was 1 mol $L^{-1}$, the detection wavelength was 350 nm, and the column temperature was 20 °C. During gradient elution, the mobile phase B was changed from 13% to 48% over a period of 10 min and to 85% over a period of 18 min. A total of 10 μl of the sample was analyzed and quantified using an external standard method.

#### 2.2.2. Modified HPLC procedures

The four types of amino acids (1–100 mg $L^{-1}$) that were examined exhibited a good linear correlation with the peak area. The correlation coefficients were 0.9980 for GABA, 0.9975 for GLY, 0.9907 for GLU, and 0.9979 for ASP. The minimum detection limits were 0.1 mg $L^{-1}$ for GABA, 0.1 mg $L^{-1}$ for GLY, 0.5 mg $L^{-1}$ for GLU, and 0.5 mg $L^{-1}$ for ASP. The recoveries were 1.0179 for GABA, 0.9906 for GLY, 0.7686 for GLU, and 0.9195 for ASP. A precision test showed that the within-day coefficients of variation were 3.83%, 5.36%, 3.79%, and 3.55%, respectively, and the between-day coefficients of variation were 3.47%, 3.70%, 3.99%, and 3.61%, respectively. The detection was completed within 18 min. The retention times were 8.1–8.4 min for ASP, 10.2–10.5 min for GLU, 15.1–15.3 min for GLY, and 16.80–17.1 min for GABA and were stable (Figs. S1 and S2). The chromatograms showed that the four types of amino acids were well separated from other substances. The standard preparations were stable at room temperature for 24 h and at 4 °C for 30 days. The brain homogenate specimens were stable at −80 °C for 30 days (Figs. S1 and S2).

### 2.3. qPCR for the analysis of amino acid receptor expression in brain tissues

The total mRNA from the brains samples was isolated using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). The RNA preparation, qPCR procedure, and relative mRNA abundance qualification were the same as previously described [13]. The chicken β-actin gene was used as an internal reference. The primers (Table S1) used for all of the assayed genes were designed using the Primer Premier software (PREMIER Biosoft International).

### 2.4. Statistical analysis

The statistical analyses of all of the data were performed using the SPSS 13.0 computer software, and all of the data were analyzed by one-way ANOVA. All of the data are expressed as the means ± S.D. Differences with $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Clinical data

None of the experimental animals suffered from other diseases or AVM poisoning-induced lethality. The pigeons in the control group presented good physical development and dental status, normal drinking and diet, bright feathers, and normal stool. Compared with the control group, the pigeons in the low-dose group did not show specific abnormalities in the early phase. After 60 days of AVM exposure, the poisoned pigeons in the low-dose group showed signs of depression, such as reduced activities and food intake and thin bodies. The pigeons in the moderate-dose group exhibited signs of obvious depression, anorexia, reduced movement, lethargy, and loose dim feathers. After 45 days of exposure, the pigeons could not stand steadily and experienced slight ataxia. The pigeons in the high-dose group displayed signs of severe depression, obvious anorexia, emaciation, and thin etiolated and uncombed dim feathers. Thirty days after exposure, the pigeons exhibited obvious nervous system symptoms. Increased AVM intake and prolonged exposure resulted in the gradual aggravation of nervous system inhibition, which resulted in the pigeons showing unstable standing, ataxia, lying on their sides, and spasms.