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Effects of *in vitro* and *in vivo* avermectin exposure on alpha synuclein expression and proteasomal activity in pigeons

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ABSTRACT

Avermectins (AVMs) are used worldwide in agriculture and veterinary medicine. Residues of avermectin drugs, causing toxicological effects on non-target organisms, have raised great concern. The aim of this study was to investigate the effects of AVM on the expression levels of alpha synuclein (α-Syn) and proteasomal activity in pigeon (*Columba livia*) neurons both *in vivo* and *in vitro*. The results showed that, the mRNA and protein levels of α-Syn increased in AVM treated groups relative to control groups in the cerebrum, cerebellum and optic lobe *in vivo*. Dose-dependent decreases in the proteasomal activity (*i.e.*, chymotrypsin-like, trypsin-like and peptidylglutamyl peptidehydrolase) were observed both *in vivo* and *in vitro*. The results suggested that AVM could induce the expression levels of α-Syn and inhibit the normal physiological function of proteasome in brain tissues and neurons. The information presented in this study is helpful to understand the mechanism of AVM-induced neurotoxicology in birds.
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1. Introduction

Avermectins (AVMs) are frequently used both as active components in some insecticidal and nematicidal products used in agriculture and as the most highly used agents in veterinary medicines for the prevention of parasitic diseases. Their wide use has resulted in serious environmental residue (Lankas and Gordon, 1989; Molinari et al., 2009; Novelli et al., 2012). Residues of AVM drugs or their metabolites in livestock feces can cause toxicological effects on non-target animals and other organisms and are thus of great concern among researchers (Kolar et al., 2008; Mladineo et al., 2006; Shen et al., 2011). Multiple toxic effects of AVMs have been identified, including neurological, immune, reproductive, and developmental toxicities (Ferri et al., 2013; Kennedy et al., 2014; Liu et al., 2014; Wise et al., 1997). Because they target the central nervous system, AVMs mainly cause neurotoxicity in a variety of non-target organisms, which may manifest as poor coordination, tremors, salivation, pupil dilation and coma in mammals (Muhammad et al., 2004; Wall and Strong, 1987); lethargy, swimming inhibition and paralysis in fishes (Palmer et al., 1987; Thain et al., 1997); and distinct types of paralysis in insects (Jackson, 1989). However, most studies have investigated the effects of AVMs on soil-dwelling invertebrates, freshwater organisms and livestock only. To date, research regarding the neural toxicological mechanism of AVMs in birds is scarce.

Maroteaux et al. (1988) cloned the synaptic protein alpha-synuclein (alpha-Syn, α-Syn) that localized to the nucleus and presynaptic nerve terminal in the electric ray Torpedo (*Torpediniformes*). After that, α-Syn homologues were found in humans, birds and rodents, with their molecular structure highly conserved between species (Jakes et al., 1994; Maroteaux and Scheller, 1991; Tiunova et al., 2000). α-Syn exists in different brain regions within the central nervous system, where it is expressed mainly in neurons and located in presynaptic nerve endings (Jakes et al., 1994). In 1993, a 35-amino acid peptide NAC [non-amyloid-β-component, NAC; non-A beta component of Alzheimer disease (AD) amyloid] was found in amyloid depositions of AD (Uéda et al.,

Abbreviations: AVM, avermectin; AVMs, avermectins; α-Syn, alpha synuclein; NAC, non-amyloid-β-component;; AD, Alzheimer disease; NACP, non-amyloid-β-component precursor; PD, Parkinson's disease; MSA, multiple system atrophy; DLB, Dementia with Lewy bodies; UPP, ubiquitin proteasome pathway; E1, ubiquitin activating enzyme; E2s, ubiquitin transferase; E3s, ubiquitin ligase; DUBs, deubiquitinating enzymes; DMSO, dimethyl sulfoxide; LC_{50}, lethal concentration 50; qPCR, quantitative real-time PCR; PBS, phosphate-buffered saline; FBS, fetal bovine serum; cDNA, complementary DNA; M-MLV, Moloney murine leukemia virus; mRNA, messenger RNA; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel-electrophoresis; PVDF, polyvinylidene fluoride; HRP, horseradish peroxidase; PBST, phosphate-buffered saline with Tween-20; OD, optical density; Ala, alanine; Phe, phenylalanine; Leu, leucine; Glu, glutamic acid; Ser, serine; Thr, threonine; Arg, argnine; ELISA, enzyme-linked immunosorbent assay; SPSS, Statistical Product and Service Solutions; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; NADPH, Nicotinamide Adenine Dinucleotide Phosphate; ATP, Adenosine Triphosphate; SH-SY5Y, human neuroblastoma cell line; PC12, pheochromocytoma

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1993). Further work identified α-Syn as the NAC precursor (NACP), prompting researchers to focus on the relationship between α-Syn and nerve cell pathological damage (Yoshimoto et al., 1995). A variety of factors can promote abnormal α-Syn expression, including gene mutations and deletions; changes in temperature, pH and concentration; heavy metal ions; and some chemical dyes (Goodwin et al., 2013; Yasuda et al., 2013). In addition, pesticides such as rotenone and paraquat can also induce expression of endogenous α-Syn and promote the formation of α-Syn aggregates, resulting in cell toxicity (Chorfa et al., 2013). Abnormal α-Syn expression was linked to multiple types of nerve cell toxicity and was eventually confirmed to be associated with a variety of neurodegenerative diseases such as Parkinson's disease (PD), AD, multiple system atrophy (MSA) and dementia with Lewy bodies (DLB) (Cookson, 2009; Shults et al., 2005; Uéda et al., 1993).

Eukaryotic cells have two major protein degradation pathways: the lysosomal pathway, which degrades extracellular proteins brought into the cell by endocytosis and the ubiquitin-proteasome pathway (UPP), which regulates intracellular protein levels and eliminates damaged, misfolded and mutant proteins to maintain protein quality control in the cytoplasm and nucleus (Glickman and Ciechanover, 2002). The UPP is composed of ubiquitin, a variety of enzymes [ubiquitin activating enzyme (E1), ubiquitin transferase (E2s), ubiquitin ligase (E3s) and deubiquitinating enzymes (DUBs)] and the 26 S proteasome. Polyubiquitinated proteins are recognized and subsequently degraded by the 26S proteasome. This ATP-dependent proteolytic complex consists of a 20S core particle and one or two 19S regulatory particle(s) (McKinnon and Tabrizi, 2014). There are three different proteolytic sites within the 20S subunit, namely the chymotrypsin-like, trypsin-like and peptidyl-glutamyl hydrolase activity sites, which each degrade specific proteins selectively (Groll et al., 2000). One 19S subunit contains six ATPases along with the first receptor for multi-ubiquitylated proteins and controls the entry of substrates into the 20S cylindrical core (Ravikumar et al., 2003). Environmental exposure to insecticides can cause proteasome dysfunction in organisms, which may result in neurotoxicity (Martyniuk et al., 2010; Mostafalou and Abdollahi, 2013).

According to the OECD Guidelines for the Testing of Chemicals, pigeon is a recommended species for bird toxicology experiments. Previous work by our lab showed that the King pigeon is sensitive to AVM exposure. After sub-chronic AVM exposure, obvious symptoms of central nervous system damage similar to those of human neurodegenerative diseases were found, including unkempt and loose feathers, depression, loss of appetite, decreased activity, unstable standing and ataxia (Chen et al., 2014). Based on these results, we sought to determine whether AVM-induced neurotoxicity affects α-Syn expression or proteasomal activity and whether these factors are related. Therefore, in this study, we examined α-Syn expression and proteasomal activity in brain tissues of King pigeon in a sub-chronic AVM toxicity model. The above indices were also detected in King pigeon nerve cells in an *in vitro* AVM toxicity model, and the possible mechanisms underlying these changes were discussed. Revealing the effect of AVM on α-Syn expression and proteasomal activity in birds' central nervous system will not only help unveil the pathogenesis of AVM-induced neurotoxicity but will also provide useful clues to the environmental impact of AVM.

2. Materials and methods

2.1. Preparation of animals

Eighty 60-d-old American King pigeons (*Columba livia*) were supplied by Harbin Zoo, Harbin, China. They were housed in the animal facility for 7 days prior to each experiment and fed a standard pigeon diet. The animal room was maintained at 24 ± 2 °C with 50% humidity and time-controlled lighting (12 h light /12 h dark).

2.2. Chemicals

AVM (98.0% pure, containing 92% AVM_{1a}) was purchased from the New Technology Development Company, China Agricultural University. Stock solutions of AVM (6 mg ml^{-1}) were prepared in analytical grade acetone (Sigma-Aldrich, USA) in the dark. The stock solution was sprayed onto the standard pigeon diet at a proportion of 60 mg AVM:1 kg diet, and the pesticide diet was dried for approximately 12 h at room temperature in the dark before use. For *in vitro* experiments, stock solutions of AVM (20 mg ml^{-1}) were prepared in analytical grade DMSO (Sigma-Aldrich, USA) in the dark.

2.3. Animal treatment

Animals were randomly allocated into four equal groups, a control group, a low-dose group, a middle-dose group and a high-dose group, that were fed the basal diet spiked with 0, 20, 40 and 60 mg AVM/kg diet, respectively. The method for challenge dose selection was as follows: First, according to the guideline for toxicity tests in terrestrial ecotoxicology (Kendall et al., 2002), a subacute toxicity test was conducted in the pigeons. The birds were given contaminated food, and the dietary LC50 (the concentration of contaminant in the diet which caused mortality in 50% of the treated population) was calculated. The detailed method has been published in Chinese (Li et al., 2003). Then, a sub-chronic toxicity test was performed according to the method of Cunny and Hodgson (Cunny, 2004). The pigeons were given free access to standard chow and water. The pigeons (six/group) were killed by cervical dislocation after 30, 60 or 90 day of AVM exposure. The cerebrums, cerebellums and optic lobes were excised immediately on an ice-cold plate and washed in a physiological saline solution. The different brain portions were identified following the illustration of Kradong (1995). The tissues were divided into two portions, one for qPCR analysis and the other for protein examination.

2.4. Preparation and pharmacological treatment of brain neuron primary culture

Fertilized eggs of the King Pigeon (Harbin Zoo, Harbin, China) were collected and hatched in an artificial hatching incubator (38 °C, 50% humidity). Pigeon brain neuron cultures were prepared according to the method described by Freshney, with modifications (Freshney, 2000). Briefly, at embryonic development days 7–8, embryos were removed carefully from eggs under aseptic conditions and collected in phosphate buffered saline (PBS, 0.1 M phosphate buffer with 0.85% NaCl, pH 7.2) at room temperature. The brain tissues were transferred to a Petri dish containing PBS and washed two or three times. After removing the meninges and vessels in PBS, the tissues were cut into small pieces (approximately 1–2 mm^3) and transferred into a sterile test tube to be incubated in PBS containing 0.125% trypsin (pH 7.2, Sigma-Aldrich, USA) at 37 °C for 15 min. Next, the tissues were filtered through a sterile stainless steel mesh with a pore size of 200 μm, and a mixed medium culture solution [NEUROBASAL (Gibco, USA) containing 1 × Gluta MAX (Gibco, USA) and 20% fetal bovine serum (FBS Gibco, USA)] was added to stop the enzymolysis by trypsin. The filters were collected and centrifuged at 1000 rpm for 5 min, and the supernatant was aspirated. The tissue pellet was triturated with a fire-polished Pasteur pipette into suspension in the same

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