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Effects of avermectin on heat shock proteins expression and histopathology in spleen tissues of pigeon

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ABSTRACT

Avermectin (AVM) is the active component of some insecticidal and nematicidal product used in agriculture and veterinary medicine for the prevention of parasitic diseases. Residues of AVM in environment have toxic effects on non-target aquatic and terrestrial organisms. Heat shock proteins (Hsps) are commonly used by environmental toxicologists as biochemical markers of exposure to various chemical and other stressors. The objective of this study was to investigate whether sub-chronic AVM exposure would alter the levels of stress proteins, Hsps in the pigeon spleen after 30, 60 and 90 days. Our results showed that Hsp60, Hsp70 and Hsp90, and their corresponding messenger RNA (mRNA) transcriptions (as well as Hsp30) significantly elevated, meanwhile, obviously histopathological changes were not observed in pigeons spleens after early AVM exposure. Then the expression of Hsps relatively decreased and obvious histopathological damages occurred in the spleen tissues with continued AVM exposure. So we suggest that the elevations of Hsps can be as a part of protective mechanism to reduce cellular damage, and important markers to help assess the toxicity induced by AVM. The reduction of Hsps in spleen implies that the tissues are damaged by long-term and excessive AVM exposure. Thus, the information presented in this study is believed to be helpful in supplementing data for further AVM toxicity study.

1. Introduction

Avermectin (AVM) is a mixture of not less than 80% of AVM B_{1a} and not more than 20% AVM B_{1b} derived from actinomycete *Streptomyces avermitilis* [1]. Because of its efficiency and broad-spectrum effectiveness, it has been widely used in agriculture [2], and it also used for treatment and prevention of parasitic diseases of animals in the field of veterinary medicine [3]. As its lipophilicity and water-insoluble, the AVM is largely remained in the environment, which result in serious pollution and health threaten [4]. Various studies have expressed that residues of AVM or its metabolites have toxic effects on non-target organisms. For example, AVM are highly toxic to some freshwater organisms such as Daphnia magna and fish (e.g. rainbow trout) [5]; AVM showed significant toxicity on the growth of earthworms with increasing concentrations up to 5 mg/kg [6]; and exposure to subcytotoxic levels of AVM could significantly reduce neurites process outgrowth and decrease the average length of neurites in differentiating N2a cells, resulting in neurotoxicity to mouse [7].

Heat shock proteins (Hsps) are a group of highly conserved cellular stress proteins present in every organism from bacteria to man. Hsps play a pivotal role in the protection and maintenance of several vital cellular functions [8]. They act as molecular chaperones by binding to other cellular proteins, assisting intracellular transport and facilitating the folding of proteins into appropriate secondary structures, thereby preventing aggregation [9]. Upon exposure to adverse environmental conditions, such as, heat shock stress, pathogen infection, oxidative stress, heavy metals and xenobiotics stresses [10], these stress responses entail the rapid synthesis of Hsps. The overexpression of one or more Hsp genes confers protection against subsequent stress [8]. Hsps can be classified on molecular weight with the predominant classes being Hsp110, Hsp90, Hsp70, Hsp60 and the small Hsps [11]. Hsps have been acted as potential molecular biomarkers for effect of environmental toxicology [12]. For example, five full-length cDNAs of Hsps genes (Fo-Hsp90, Fo-Hsp70, Fo-Hsp60, Fo-Hsp40 and Fo-Hsp28.9) were induced by exposure to sublethal concentrations of the

Abbreviations: AVM, avermectin; Hsp, heat shock protein; mRNA, messenger RNA.

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insecticide AVM in *Frankliniella occidentalis* at different stages [13]; the expression of Hsp 60, Hsp70, and Hsp90 is induced by neurotoxic insecticides of chlorpyrifos and esfenvalerate in Chinook salmon (*Oncorhynchus tshawytscha*) [14].

Pigeons are one of the recommended species to assess environmental pollution (OECD, 2010). The toxicity results of preliminary experiments completed in our laboratory revealed that oxidative stress, histopathological damages, inflammatory and apoptosis occured in pigeon after AVM exposure [15–19]. However, little is known regarding the effects of AVM on Hsps expression and histopathological changes in the pigeon spleen. In the present study, the variatio of Hsps (Hsp30, Hsp60, Hsp70, and Hsp90) levels in the pigeon spleen after AVM-exposure were studied using Western blotting (WB) and a fluorescence quantitative real-time polymerase chain reaction (FQ RT-PCR). Besides, histopathological changes were also observed in pigeon spleen induce by AVM.

2. Materials and methods

2.1. Chemicals

AVM (98% pure, containing 92% 1a) was purchased from the New Technology Development Company, CHINA AGR UNIV. TRIzol reagent was purchased from manufacturer of Invitrogen, America. Oligo dT primers and Superscript II reverse transcriptase were provided by Promega, China.

2.2. Pigeons and tissues collection

Eighty pigeons were purchased from the factory of XiangYu (Harbin City, China) and were randomly allocated into 4 equal groups: a control group, a low-dose group, a medium-dose group and a high-dose group fed the basal diet spiked with 0, 20, 40 and 60 mg AVM/kg diet respectively. Bue to this paper highlights on the toxicological effects of AVM, rather than the residual of AVM in the environment, the contents of AVM used in this study was determined based on the method of Helen and Emest [20] after a subacute toxicity testing was done to calculate the dietary LC_{50}. Briefly, the pigeons were maintained in our animal facility, kept under a 12 L:12 D photoperiod and a temperature of 24 ± 2 °C with 50% humidity, and given free access to a commercial feed and water *ad libitum*. The pigeons (six/group) were euthanized by cervical dislocation after 30, 60 and 90 days AVM exposure respectively. The spleen tissues were excised immediately on an ice-cold plate and washed in a physiological saline solution, and then divided into two portions, one stored at −80 °C for cDNA and protein analysis and the other fixed in 4% buffered formaldehyde for histopathological examination. The experiment was undertaken in accordance with the guidelines of the regional Animal Ethics Committee and was approved by the Institutional Animal Care and Use Committee of Northeast Agriculture University.

2.3. Histopathological examination

The samples were dehydrated through a graded series of ethanol, cleared in xylene and embedded in paraffin. Sections that were 5 μm thick were prepared from paraffin blocks using a microtome. And then stained with hematoxylin and eosin (H&E). The sections were examined under a Leica DME 100 light microscope.

Histological alterations were scored as (−), no histopathology; (+), histopathology in <20% of the fields; and (++), histopathology in 20–60% of the fields. This categorization was modified from methods described by [21–23].

2.4. Fluorescence quantitative real-time PCR

2.4.1. The extraction of total RNA

Total RNA was purified from the spleen tissues (50 mg tissue; $n = 6$/group) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The dried RNA pellets were resuspended in 50 μl of diethyl-pyrocarbonate-treated water. The concentration and purity of the total RNA were determined spectrophotometrically at 260/280 nm [24]. First-strand cDNA was synthesized from 5 μg of total RNA using oligo dT primers and Superscript II reverse transcriptase according to the manufacturer's instructions. Synthesized cDNA was diluted five times with sterile water and stored at −80 °C before use.

2.4.2. Design of primers and real-Time PCR (qPCR)

The primers of Hsp30, Hsp60, Hsp70 and Hsp90 (Table.1) were designed by Premier Software (PREMIER Biosoft International, USA) for real-time PCR (RT-PCR). General PCRs were first performed to confirm the specificity of the primers. The PCR products were electrophoresed on 2% agarose gels, extracted, cloned into the pMD18-T vector, and sequenced. Quantitative real-time PCR was performed on an ABI PRISM 7500 Detection System (Applied Biosystems, USA). Reactions were performed in a 20 μl reaction mixture containing 10 μl of 2× SYBR Green PCR Master Mix, 2 μl of diluted cDNA, 0.6 μl of each primer (10 μM), and 6.8 μl of PCR-grade water. The PCR procedure for Hsp30, Hsp60, Hsp70, Hsp90 and β-actin consisted of heating the reaction mixture to 52 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, 95 °C for 15 s and 60 °C for 20 s. The melting curve analysis showed only one peak for each PCR product. Electrophoresis was performed with the PCR products to verify primer specificity and product purity. A dissociation curve was run for each plate to confirm the production of a single product. The amplification efficiency for each gene was determined by using the DART-PCR program [25]. The mRNA relative abundance was calculated according to the method of Pfaffl [26], accounting for gene-specific efficiencies and was normalized to the mean expression of β-actin.

2.5. Western blotting

Protein extracts were subjected to SDS–polyacrylamide gel electrophoresis under reducing conditions on 12% gels. Separated proteins were then transferred to polyvinylidene fluoride membranes using a tank transfer for 2 h at 200 mA in Tris–glycine buffer containing 20% methanol. Membranes were blocked with 5% skim milk for 12 h and incubated 1 h at 37 °C with diluted primary pigeon antibody (polyclonal antibody by our lab) Hsp60 (1:1500), Hsp70 (1:500), and Hsp90 (1:500), followed by a horseradish peroxidase (HRP)-conjugated secondary antibody against rabbit IgG (1:1500, Santa Cruz, CA, USA). To verify equal loading of samples, the membrane was incubated with monoclonal β-actin antibody (1:1000, Santa Cruz, CA, USA), followed by a HRP-conjugated goat anti-mouse IgG (1:1500). The signal was detected by X-ray films (Trans Gen Biotech Co., China). The optical density (OD) of each band was determined by Image VCD gel imaging system, and the Hsp60, Hsp70, and Hsp90 expression were detected as the ratio of OD of Hsp90, Hsp70, Hsp60, and OD of β-actin, respectively.

2.6. Statistical assays

Statistical analysis of all data was performed using SPSS for Windows (version 13; SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and least significant difference (LSD) test were used to test the useful indexes in treatment and control groups. It was considered statistically different when $P < 0.05$.