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Cytotoxic effects of Avermectin on human HepG2 cells in vitro bioassays☆

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ABSTRACT

Avermectin (AVM) has been widely used in agriculture and animal husbandry based on its broad spectrum of effective anthelmintic activity and specificity targets. However, AVM induction of cytotoxicity in human liver is largely unknown. In this study, we investigate the cytotoxic effects of AVM on HepG2 cells in vitro. The results revealed that AVM inhibited the viability of HepG2 cells and enhanced apoptosis. Established assays of cytotoxicity were performed to characterize the mechanism of AVM toxicity on HepG2 cells. Typical apoptosis morphological changes were shown in AVM-treatment cells including chromatin condensation and DNA fragmentation. We demonstrated that AVM-induced apoptosis of HepG2 cells were mediated by generated ROS. Moreover, a decrease in mitochondrial membrane potential (*MMP*) and up-regulating the Bax/Bcl-2 ratio, resulted in a release of cytochrome-c as well as activation of caspase-9/-3. In conclusion, our experimental results show that AVM has a potential threat to human health which may be induce apoptosis of human hepatocyte cells via caspase-dependent mitochondrial pathways.

1. Introduction

Pesticide pollution in the environment has been increasing due to their extensive use in agriculture. Avermectin (AVM) is a macrocyclic lactone compound was first discovered in the extracts of the fungus *Streptomyces avermitilis* (Burg et al., 1979; Campbell, 1989). Avermectin B_1, the main component of AVM, has been widely used as pesticides to control agricultural pests or as anthelmintic against parasites of livestock, both internal and external (And and Dybas, 1991; Evans et al., 1995; Novelli et al., 2012). Due to its well-known character of potentiating the activity of glutamate-gated chloride ion channels or gamma-aminobutyric acid (GABA) receptor, AVM mainly acts on the nervous system based on specificity targets. Meanwhile, because of the restriction of GABA-sensitive neurons to the mammalian central nervous system, AVM has been generally supposed to be of low toxicity for the health of adults. However, it is actually a serious potential risk to the health of human, since AVM undergoes little metabolism within the target or non-target organism.

In recent years, a larger number of studies showed non-target organisms, such as Chinese hamster ovary cells and male rat hepatocytes (El, 2010; Molinari et al., 2009), have adverse reactions to AVM, whereas the cytotoxic effects and molecular mechanism of AVM on human liver cells have not been well characterized. Meanwhile, the main sources of contamination by pesticide in humans is drug residues on our food and accumulation. And the liver in the human organism is primarily responsible for the metabolism of toxic substances, including pesticide residues in food. Therefore, we have implemented a study of cytotoxic and apoptotic effects on human liver cells, which is aimed at evaluating the safety of the widely used pesticides, and the conclusion indicates that AVM has significant cytotoxicity.

Apoptosis, as an important mechanism of cell death, plays a key role to maintain balance of diverse biological processes and eliminate the unwanted or damaged cells in multicellular organisms (Degterev and Yuan, 2008). The apoptosis process is strictly regulated, and its dysregulation would result in pathological conditions including various diseases and cancer (Ola et al., 2011). We have known that two major pathways contained in apoptosis, intrinsic

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(mediated by mitochondrial) and extrinsic (mediated by death receptors). In the intrinsic pathway, the release of death factors into the cytosol from the mitochondria and the regulation of Bcl-2 family proteins would contribute to the triggering of apoptosis which induced by some intracellular signals, such as DNA damage (Gross et al., 1999; Schleich and Lavrik, 2012). For instance, the mainly death factor cytochrome-c once released into the cytosol and Apaf-1, in the presence of ATP or dATP, are required for the activation of caspase-9 and subsequently effector caspases-3. Finally, the activated effector caspases leading irreversibly to cell death. Meanwhile, the rise of intracellular reactive oxygen species (ROS) generation resulted in a release of cytochrome-c and a parallel loss of cardiolipin content (Petrosillo et al., 2003). Besides, Bcl-2 family proteins play a key role in apoptotic process to regulate the release of cytochrome-c. Among them, Bax/Bcl-2 is the key factors in regulating apoptosis, a lot of research think of it as the prognosis of apoptosis and tumor markers (Perfettini et al., 2004). If cells under the abnormal condition, they will be subjected to apoptosis, and while apoptosis continuance to organism, formation damage cannot be estimated any more.

In the present study, Human HepG2 cells were used as a model to evaluate the toxicological effects of AVM for human liver, and we found the AVM has significant cytotoxicity to it in vitro. Established assays of cytotoxicity were performed to verify the molecular mechanism of action of AVM against HepG2 cells, which was shown as it can induce apoptosis. Typical apoptosis morphological changes were shown in AVM-treatment cells including chromatin condensation and DNA fragmentation. We also demonstrated that AVM-induced apoptosis of HepG2 cells were mediated by generated ROS. Moreover, a decrease in mitochondrial membrane potential (*MMP*), up-regulating the Bax/Bcl-2 ratio, resulted in a release of cytochrome-c as well as activation of caspase-9/-3. Take together, a caspase-dependent mitochondrial pathways contributed to AVM-induced apoptosis in HepG2 cells.

2. Materials and methods

2.1. Chemicals and antibodies

Avermectin(AVM, 98% pure, containing 95% B_{1a}) was purchased from Shanghai Pesticide Research Institution (shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Phenylmethylsulfonyl fluoride (PMSF), RIPA lysis buffer, Rhodamine123 (Rh-123), 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) and were obtained from Sigma–Aldrich (St. Louis, MO, USA). All primary antibodies were obtained from Cell Signaling Technology (CST, Beverly, MA, USA). And the horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was obtained from Sangon Biotech Co., Ltd (Shanghai, China). The Enhanced Chemiluminescence (ECL) reagent were obtained from Pierce (Rockford, IL, USA). Other common chemicals were purchased from Shanghai Titanchem Co. Ltd. (Shanghai, China).

2.2. Cell culture

Human HepG2 cells (ATCC, HB-8065, USA) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS and 1% antibiotics, and incubated at 37 °C in a 5% CO_2 incubator. After treatment, the HepG2 cells were harvested by trypsinization.

2.3. Cell viability assay

The cytotoxic effects of AVM on the HepG2 cells' viability was performed by MTT assays as previously described (Zhang et al., 2016). Exponentially growing HepG2 cells (1×10^5 cells/mL) were seeded onto the 96-well plates overnight and treated with specified concentrations of AVM for specified times mentioned in the figure legends. After 24 h and 48 h of treatment, 20 μL MTT reagent (5 mg/mL) was added to each well. After incubation for 4 h at 37 °C in the dark, the supernatants of culture media was aspirated and 150 μL DMSO was added to each well to dissolve the formazan crystals. Then, the reading of absorbance was taken at 492 and 630 nm by a Synergy H1 microplate reader (Bio-Teck, Winooski, VT, USA).

2.4. DNA damage assay

The alkaline comet assay can be used for detecting the DNA damage, was performed according to a previously described (Yu et al., 2016). After HepG2 cells were treated by 0, 5, 10, 20 and 40 μM AVM for 12 h, the cells were collected. We washed the cells with PBS (pH 7.4) for three times to remove AVM, and treated cells (2×10^4 cells) were then suspended in 1% molten low-melting-point agarose (1:5) at 37 °C to form the mono-suspension which was cast on a microscopic slide. The agarose was curdled at 4 °C for 15 min and the slides were immersed in fresh lysis solution (10% DMSO, 1% Triton X-100, 10 mM Tris–HCl, 100 mM EDTA, 2.5 M NaCl, pH 10) at 4 °C for 30 min. After lysis, the slides were washed in distilled water three times and immersed in fresh alkaline electrophoresis solution (1 mM EDTA, 300 mM NaOH, pH 13) at 4 °C for 10 min. An electric field was then applied at 20 V (1 V/cm) for 10 min. The slides were neutralized to Tris buffer (0.4 mM, pH 7.5), stained with PI reagent (20 μg/mL) for 5 min, and then examined by fluorescence microscopy (Lecois, DM3000, GER). The extent of DNA damage was measured by using an image analysis system (CASP, www.casp.of.pl).

2.5. Chromatin condensation detection

After the HepG2 cells were treated by 0, 5, 10, 20 and 40 μM AVM for 24 h, cells were fixed with 4% paraformaldehyde solution for 10 min at 4 °C. The fixed cells were washed two times with cold PBS (PH7.4) and then stained with 1 μL of Hoechst 33,258 (5 mg/mL) in 1 mL cold PBS (PH7.4) and incubated for 10 min at 37 °C. The morphology of the AVM-treatments HepG2 cells was examined and photographed by fluorescence microscopy (Lecois, DM3000, GER).

2.6. Apoptosis assay

We used the AF488 Annexin V/Dead cell apoptosis kit (Invitrogen™) to estimate the apoptosis. HepG2 cells were sowed in six-well plates (2×10^5 cells/mL) in 2 mL of complete DMEM, and then processed with 5 different AVM concentrations (0, 5, 10, 20 and 40 μM) in 12 h. In the apoptosis assay, we centrifuged cells at $100 \times g$ for 5 min and then washed them with cold PBS (PH7.4) for three times. Later, the treated cells were labeled with PI and Annexin V-FITC for 10 min before being analyzed by flow cytometry (B.D. FACS Calibur). Data analysis was performed using the *Flowjo* software program.

2.7. Determination of the mitochondrial membrane potential

Mitochondrial membrane potential (*MMP*) is regarded as an early apoptotic sign for cells (Koya et al., 2000). The changes in mitochondrial membrane potential (*MMP*) were determined on the basis of mitochondrial retention of the fluorescent dye Rhodamine123(Rh-123) (Juan et al., 1994). Reduction in the fluorescence intensity shows a decrease of *MMP*. Following the treatment of AVM with concentrations of 0, 5, 10, 20 and 40 μM for 6 h, HepG2 cells were acquired. We washed the cells with PBS (pH 7.4) for three times to remove AVM and stained them with Rh-123 at

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