Assessment of aflatoxin B1 myocardial toxicity in rats: mitochondrial damage and cellular apoptosis in cardiomyocytes induced by aflatoxin B1

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Abstract
Objective: The number of deaths from heart disease is increasing worldwide. Aflatoxin B1 (AFB1), a toxin produced by the fungi Aspergillus flavus and Aspergillus parasiticus, is frequently detected in improperly processed/stored human food products. While AFB1 hepatotoxicity and carcinogenic properties have been well addressed, its myocardial toxicity is poorly documented. This study aimed to investigate myocardial toxic activity of AFB1.
Methods: Ten rats were fed with AFB1 at a dose that did not result in acute toxic reactions for 30 days and 10 vehicle-fed rats served as controls. Transmission electron microscopy was used to assess mitochondrial damage in cardiomyocytes. The terminal deoxynucleotidyl transferase-mediated UTP nick-end labelling assay was performed to detect apoptosis of cardiomyocytes. Western blotting was performed to measure apoptotic proteins (i.e., active caspase-3, Bax, and Bcl-2) in heart tissue.
Results: AFB1 treatment resulted in mitochondrial membrane disruption and disorganization of cristae, which are indicators of mitochondrial damage. Myocardial cell apoptosis was significantly higher after AFB1 treatment (22.07% ± 3.29%) compared with controls (6.27% ± 2.78%, P < 0.05). AFB1 treatment enhanced expression of active caspase-3, Bax, and Bcl-2 in cardiac tissue.
Conclusion: Various adverse effects are exerted by AFB1 on the heart, indicating AFB1 myocardial toxicity.

Keywords
Aflatoxin B1, myocardial toxicity, mitochondria, cell apoptosis, caspase-3, Bcl-2

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Introduction

Aflatoxin B1 (AFB1), which is a toxin produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, has been extensively studied for its hepatotoxic and carcinogenic properties. AFB1 is classified as a Group I carcinogen by the International Agency for Research on Cancer (http://monographs.iarc.fr/ENG/Monographs/vol56/mono56.pdf). AFB1 can also affect the lungs and kidney, and shows immunosuppressive properties, such as inhibiting cell-mediated immune reactions, reducing natural killer cytolysis, and suppressing macrophage function.

Contamination of food products with AFB1, such as maize, cereals and nuts, is frequently found in Africa, Asia, and Latin America, where these commodities are dietary staples. In China, AFB1 is often detected in corn, rice, wheat flour, and cooking oil. A study by Mannaa et al. reported that treatment of rats with AFB1 resulted in oxidative stress in the heart. However, AFB1 cardiotoxicity has been poorly documented. Therefore, this study aimed to assess AFB1 myocardial toxicity in rats by examining aflatoxin B1-induced mitochondrial damage in cardiomyocytes, apoptosis of cardiomyocytes, and apoptotic protein expression in the heart.

Materials and methods

Chemicals and reagents

AFB1 was obtained from Sigma-Aldrich (Hong Kong, China) and dissolved at 1 mg/ml in dimethyl sulfoxide (DMSO, Sigma-Aldrich). HRP-conjugated anti-mouse or anti-rabbit secondary antibodies and RIPA lysis buffer were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against cleaved (active) caspase-3, Bax, Bcl-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Abcam (Shanghai, China). The DeadEnd Fluorometric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) Kit was purchased from Promega (Madison, WI, USA).

Animal experiment

The animal protocol was approved by the Medicine Research Committee of Qingdao University and all procedures were performed in compliance with the Guidance for the Care and Use of Laboratory Animals issued by the Ministry of Science and Technology, China. The LD50 of AFB1 significantly varies between female (approximately 18 mg/kg of body weight) and male rats (approximately 7 mg/kg of body weight). Therefore, only female Sprague–Dawley rats were used in this study. Rats (approximately 120 g) were purchased from the Animal Center, Qingdao University. The rats were fed with standard chow and kept in a pathogen-free laboratory for 2 weeks before AFB1 treatment started.

Rats were randomly divided into two groups: the control and the treatment group (10 animals/group). Because intake of AFB1 in humans occurs mostly through consumption of food contaminated with AFB1, we administered AFB1 in rats by gastric intubation. In a pilot experiment, a dose of 0.75 mg/kg of body weight of rats was determined, which did not result in acute toxic reactions (e.g., death, weight loss, jaundice, and ascites). Additionally, treatment with this dose did not cause gross pathological changes in the heart. Gastric intubation was carried out by an experienced animal technician to avoid the tube entering the trachea or damaging the oesophagus or stomach. In the treatment group, each rat received 0.75 mg/kg of body weight of AFB1 (in 0.15 ml DMSO) daily for 30 days, while each control rat received 0.15 ml DMSO daily.

At the end of the experimental period, rats were anesthetized with intraperitoneal
injection of sodium pentobarbital (50 mg/kg of body weight), and sacrificed by cervical decapitation. The heart was rapidly removed and cut into two halves transversely. The halves were randomly processed as follows: 1) snap-frozen in liquid nitrogen and stored in a −80°C freezer for protein extraction and analysis; 2) fixed with 10% formalin for analysis of cellular apoptosis; and 3) immediately processed for mitochondrial structure analysis by transmission electron microscopy (TEM).

**Mitochondrial structure analysis by transmission electron microscopy**

TEM was performed to assess mitochondrial structure in cardiomyocytes. A piece of 1 × 1 mm of cardiac tissue was dissected from the left ventricular region and fixed at room temperature for 2 hours in 1.25% formaldehyde, 2.5% glutaraldehyde, and 0.03% picric acid in 0.1 M cacodylate buffer. Subsequently, the tissue was rinsed thoroughly with 0.1 M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide for 2 hours, and rinsed with ddH2O. The sections were treated with 2% uranyl acetate in the dark for 2 hours. After dehydration through a series of ethanol, the sample was embedded with resin-embedding medium and examined under a transmission electron microscope. Mitochondrial structure of cardiomyocytes in a typical cylindrical shape was observed and imaged.

**HE staining and the TUNEL assay**

Cardiac tissue fixed with 10% formalin was rinsed with PBS and dehydrated with a series of ethanol. After ethanol was replaced with xylene, the tissue was embedded in paraffin. Sections (5 μm) were cut and mounted on glass slides. The sections were then de-paraffinized/rehydrated as described previously. The primary antibodies used were as follows: active caspase-3 antibody, Bcl-2 antibody, and Bax antibody diluted to 1:1000. GAPDH antibody was diluted to 1:5000. The relative levels of active caspase-3, Bcl-2, and Bax were compared between non- and AFB1-treated animals. The density of each protein band was measured using Image J software (NIH, Bethesda, USA), and the relative level was performed by following the standard protocol (http://www.ihcworld.com/_protocols/special_stains/h&e_ellis.htm). Apoptosis of cardiomyocytes was measured using the DeadEn Fluorometric TUNEL Kit according to the manufacturer’s instructions. Apoptotic cells were observed under a microscope. Apoptotic and total cell numbers under each field (10×) were counted. For each sample, cells in 3–5 randomly selected fields were counted. The cellular apoptotic rate was calculated using the following formula: average number of positively stained apoptotic cells/average total number of cells ×100%. A cell count was performed in a blind manner.

**Western blotting**

Protein levels of active caspase-3, Bax, and Bcl-2 in cardiac tissue were determined by western blotting. A total of 100 mg of frozen cardiac tissue was homogenized with 200 μl of RIPA lysis buffer containing a proteinase inhibitor cocktail (1:100 dilution, Sigma-Aldrich). After centrifugation at 15,000 g for 20 min at 4°C, the supernatant was collected and the protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Co., Ltd, Shanghai, China) following the manufacturer’s instructions. Total protein (50–100 μg) was separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and electronically transferred onto a nitrocellulose membrane. The blotting was performed as described elsewhere. The primary antibodies used were as follows: active caspase-3 antibody, Bcl-2 antibody, and Bax antibody diluted to 1:1000. GAPDH antibody was diluted to 1:5000. The relative levels of active caspase-3, Bcl-2, and Bax were compared between non- and AFB1-treated animals. The density of each protein band was measured using Image J software (NIH, Bethesda, USA), and the relative level was
calculated by the following formula: the density value of the target protein band/the density value of the GAPDH band.

**Statistical analysis**

All data are expressed as mean ± standard deviation. Statistical analysis of the data was

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**Figure 1.** HE staining of heart tissue

Representative photomicrographs of HE staining of heart tissue from control (panel a) and experimental animals (AFBI-treated, panel b) are shown. Normal arrangement of myocardial fibres was observed in the control and AFB1-treated samples, and no apparent abnormalities were observed after AFB1 treatment (panel b).

**Figure 2.** Transmission electron microscopic analysis of mitochondrial structure in myocardial cells

Compared with mitochondrial structure in control animals (panel a), AFB1 treatment resulted in damage to mitochondria (panel b), including disruption of the mitochondrial membrane (red arrows) and disorganization of cristae (red arrow heads).
performed using the Student’s *t* test. A value of *P* < 0.05 was considered statistically significant.

**Results and Discussion**

Mitochondria, as energy producers, are particularly important for cardiomyocytes because they are estimated to produce 90% of ATP, involved in ion homeostasis, free radical production, and ultimately cell death.\(^{18,19}\) Impaired mitochondrial activity can result in cardiomyopathy.\(^{20,21}\) In our study, HE staining showed no apparent abnormalities in heart tissue from AFB1-treated animals (Figure 1). We then examined the effect of AFB1 on mitochondrial structure in cardiomyocytes. Treatment with AFB1 led to disruption of the mitochondrial membrane and disorganization of cristae (Figure 2). Cardiomyocytes have a large energy requirement. Therefore, any change in cellular architecture, especially organization of mitochondria, could impair cellular energetics, and consequently, cellular function.\(^{22-24}\) Additionally, mitochondria play an important role in cellular apoptosis. Therefore, mitochondrial damage caused by AFB1 might eventually result in dysfunction of cardiomyocytes.

![Figure 3. Promotion of apoptosis of myocardial cells by AFB1](image)

Panels a and b show representative images of a TUNEL assay for apoptotic cells from control and experimental animals, respectively. The percentage of apoptotic cells in AFB1-treated rats was significantly higher than that in control rats (panel c). All nuclei were stained blue by DAPI. Arrows indicate apoptotic cells (brown). *P* < 0.05 compared with controls (n = 6).
We then analysed the effect of AFB1 on myocardial cell apoptosis. As shown in Figure 3, the percentage of apoptotic myocardial cells in AFB1-treated animals (22.07% ± 3.29%) was significantly higher than that in the control group (6.27% ± 2.78%, $P < 0.05$, $n = 6$). Apoptosis is a fundamental process in cell biology, and plays an important role in tissue/organ development, physiological adaptation, and pathogenesis of various diseases. Myocardial cell apoptosis has been implicated in different cardiovascular disease conditions, including myocardial infarction, heart failure, and arrhythmias. Despite the fact that animal studies have shown that cardiomyocytes can be regenerated from pre-existing cardiomyocytes or stem cells, in humans, cardiomyocyte renewal is extremely low. Therefore, cardiac regeneration and repair remain a major challenge in the clinical setting. AFB1 induces apoptosis of cardiomyocytes, providing further evidence for AFB1 cardiotoxicity.

We measured the levels of apoptotic proteins (i.e., the active form of caspase-3, Bcl-2, and Bax) in heart tissue. Western blotting analysis showed elevated levels of the active form of caspase-3, Bcl-2, and Bax protein in heart tissue (Figure 4). Caspase-3 and Bax are pro-apoptotic proteins, while Bcl-2 is an anti-apoptotic regulator. Cleavage caspase-3, a central player in apoptosis, can be activated by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. Caspase-3 activates an endonuclease termed caspase-activated DNase, which triggers DNA fragmentation during apoptosis. Therefore, elevated expression of caspase-3 and Bax, together with mitochondrial structural damage, might be responsible for the increased cellular apoptosis induced by AFB1. In our study, there appeared to be a paradox that Bcl-2 expression was increased because Bcl-2 inhibits apoptosis. We speculate that augmented expression of Bcl-2 might be a cellular protective reaction against AFB1-induced apoptosis. Notably, Bcl-2 can be cleaved by caspases to generate Bax-like pro-apoptotic fragment. However, in this study, we used an antibody against Bcl-2 that did not detect this cleaved fragment.

![Western blotting analysis of active caspase-3, Bax, and Bcl-2 in heart tissue](image)

**Figure 4.** Western blotting analysis of active caspase-3, Bax, and Bcl-2 in heart tissue
Representative blots for each protein are shown in the top panels. The relative levels of active caspase-3, Bax, and Bcl-2 were significantly higher in AFB1-treated samples than in control samples (bottom panels). Lane 1: control sample; lanes 2 and 3: two independent experimental samples. $^*P < 0.05$ compared with controls ($n = 6$).
How AFB1 exerts myocardial toxic effects is unclear. Ingestion of AFB1 results in elevation of serum nitric oxide (NO), TNF-α, and IL-1α levels in rats. High NO concentrations can induce apoptosis of cardiomyocytes. Leist et al. discovered that when mitochondrial ATP generation was preserved under high NO concentrations, cellular apoptosis occurred. However, once ATP generation was inhibited by high NO concentrations, and with loss of the energetic supply, cardiomyocytes then underwent necrosis. TNF-α and IL-1α are pro-inflammatory cytokines, and TNF inducing cardiomyocyte apoptosis has been well established. Despite these data, the exact mechanisms of AFB1 cardiotoxicity remain to be explored.

In conclusion, to the best of our knowledge, we show for the first time that AFB1 induces damage of mitochondria in cardiomyocytes, promotes apoptosis of cardiomyocytes, and regulates the expression of apoptosis-related proteins, highlighting the cardiac toxicity of AFB1. Considering these findings and the facts that AFB1 contamination of food products frequently occurs and death caused by heart disease is increasing, epidemiological studies should be conducted to determine if there is any link between AFB1 food contamination and heart disease. The main limitations of this study are that the function of mitochondria was not further studied and the detailed mechanisms of AFB1-induced myocardial toxicity were not addressed.

Declaration of conflicting interest

The Author(s) declare that there is no conflict of interest.

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