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Protective Effects of Selenium on Aflatoxin B_1-induced Mitochondrial Permeability Transition, DNA Damage, and Histological Alterations in Duckling Liver

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Abstract Aflatoxin B_1 (AFB_1) is a mycotoxin that causes cytotoxicity through oxidative damage to its target organs. The liver is the first target of AFB_1 damage. The aim of this study was to evaluate the protective effect of selenium on AFB_1-induced hepatic mitochondrial damage in ducklings using molecular biological and histopathological techniques. Aflatoxin was administered via intragastric intubation (0.1 mg/kg body weight), daily for 21 days. The experimental group also received intragastric sodium selenite (1 mg/kg body weight), while the control group was given the same volume of dimethyl sulfoxide (DMSO). Sequence analysis of the mitochondrial DNA D-loop region showed that AFB_1 induced damage. All AFB_1-administrated ducklings were identified as having D-loop mitochondrial DNA mutations. Mutations were detected in two ducklings that had received both AFB_1 and selenium. Mitochondrial swelling assays showed that opening of the mitochondrial permeability transition pores was increased in ducklings that had received AFB_1 for 14 and 21 days ($P<0.05$). Selenium significantly attenuated these adverse effects of AFB_1. After AFB_1 exposure, histological alterations were observed, including fat necrosis, steatosis, and formation of lymphoid nodules with infiltrated lymphocytes. These histological abnormalities were also attenuated by treatment with selenium. The overall data indicated that selenium exerts a potent protective effect against AFB_1-induced hepatic mitochondrial damage, possibly through its antioxidant activity.

Keywords Aflatoxin B_1 · Selenium · Mitochondrial DNA · Mitochondrial permeability transition

Introduction

Aflatoxins (AF) are secondary toxic fungal metabolites produced by the fungi *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nominus* [1]. There are four naturally occurring aflatoxins; aflatoxin B_1 (AFB_1) is the most highly toxic form. Humans and animals can be exposed to AFB_1 both directly and indirectly, and AFB_1 exposure is a major risk factor for human liver cancer [2]. Several studies have characterized AFB_1-induced oxidative damage and its role in cytotoxicity in the liver [3, 4]. AFB_1 can produce reactive oxygen species (ROS), which cause oxidative stress by damaging cells and DNA [5]. Previous studies have suggested that AFB_1-induced toxicity can be prevented by antioxidants such as silymarin [6], green tea [7], and pentoxifylline [8].

Selenium (Se), an essential trace nutrient, plays an important role in oxidant defense [9]. Se can effectively protect the thymus from AFB_1-induced adverse effects [10]. Previous studies from our laboratory have shown that Se can ameliorate the negative effects of aflatoxin B_1 on the hepatic mitochondrial respiratory control ratio (RCR) and hepatic mitochondrial antioxidant function [11, 12]. In addition, ROS have been elucidated to induce genetic alterations leading to DNA damage and mitochondrial permeability alterations. We speculated that addition of Se could alleviate AFB_1-induced hepatic mitochondrial toxicity. Therefore, the present study was designed to investigate the ability of Se to mitigate AFB_1-

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induced genotoxicity and hepatic mitochondrial toxicity in an in vivo duckling model.

Materials and Methods

Chemicals and Reagents

Aflatoxin B_1, D-mannitol, hydroxyethylpiperazine ethane sulfonic acid (HEPES), ethylene glycol tetraacetate (EGTA), and bovine serum albumin (BSA) were purchased from Sigma (USA). Taq enzyme and pMD18-T were obtained from TaKaRa (China). Kits used for gel purification were from Omega (USA). All other chemicals were analytical grade.

Animals and Treatments

All procedures used in this study were approved by the Ethics Committee of South China Agricultural University. Male ducklings (weighing 180–200 g) were used as experimental animals. A total of 90 ducklings were randomly divided into three groups (n=30/group): AFB_1, AFB_1 treated with Se (AFB_1-Se), and control. The ducklings in group AFB_1-Se were given AFB_1 (0.1 mg/kg body weight) and sodium selenite (1 mg/kg body weight) through intragastric intubation. The added content of Se in group AFB_1-Se was based on our previous study [11, 12]. The AFB_1 group received intragastric AFB_1 (0.1 mg/kg body weight) dissolved in dimethyl sulfoxide (DMSO). The control group was given the same volume of DMSO with no AFB_1. This was repeated daily for a total of 21 days.

Mitochondrial Preparation and DNA Extraction

Mitochondria were isolated from duckling liver by differential centrifugation as described by Tang [13], with modifications. On the 14th and 21st days of administration, five randomly selected ducklings from each group were sacrificed and the liver tissues were obtained. Excised liver was washed in ice-cold initial liver mitochondria isolation buffer A (220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES, 1 mM EGTA, 0.5 mg/mL BSA, pH 7.0) then chopped in ice-cold buffer A (6×50 mL) and centrifuged at 1,000×g for 10 min at 4 °C. The supernatant was centrifuged again at 1,000×g for 10 min at 4 °C. The pellet was suspended in 100 mL (2×50 mL) buffer B (220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES, 0.5 mg/mL BSA, pH 7.0) and centrifuged for 10 min at 10,000×g. The final pellet was resuspended in buffer C (220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES, pH 7.0). The protein concentration of the final mitochondrial pellet was determined with Bradford's assay using BSA. All specimens were immediately fresh frozen and stored at −80 °C. The mitochondria were evaluated for mitochondrial displacement loop (D-loop) mutation and mitochondrial permeability, and the liver tissue was evaluated for morphological appearance. Mitochondrial DNA (mtDNA) was extracted from mitochondria isolated from all three groups on the 21st day. The protocol was described previously [14].

Mutation Analysis for D-loop Region of mtDNA

Sequences for Jiancheng duck mitochondrial D-loop gene were retrieved from NCBI (GenBank FJ167857). mtDNA fragments containing the D-loop region were amplified using forward primer DLF (5′-AGCTAGAATAGCCTAA TAATGCTCT-3′) and reverse primer DLR (5′-TGCATG TATATGTCTAGCAAAAACC-3′) and DNA polymerase (TaKaRa, China) by the following polymerase chain reaction (PCR) protocol: initial denaturing at 94 °C for 3 min followed by 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s for 35 cycles and a final extension at 72 °C for 5 min. The PCR products were gel purified and subcloned into pMD18-T vector (TaKaRa, China). This plasmid was transformed into *Escherichia coli* DH5α cells. Transformants were screened through colony PCR. The sequence of the D-loop region of the mtDNA was finally confirmed by sequencing of the clone. The mutations of the mitochondrial D-loop were analyzed by ClustalW (available at EBI, http://www.ebi.ac.uk/Databases/index.html).

Mitochondrial Swelling Assay

Activation of the mitochondrial permeability transition pore was determined by AFB_1-induced swelling of isolated liver mitochondria. Opening of the pore causes mitochondrial swelling, which is measured spectrophotometrically as a decrease in absorbance at 540 nm. Mitochondria (3 mg of protein/mL) were resuspended in the swelling buffer, which contained 125 mM sucrose, 90 mM D-mannitol, 5 mM HEPES (pH 7.4) to a final protein concentration of 300 μg/mL. The percentage of absorbance decrease was calculated according to the following equation:

$$\%\text{Decrease} = (A_0 - A_{\min})/A_0 \times 100$$

where A_0 and $A_{\min}$ are the absorbance determined at different times.

Mitochondrial swelling was also confirmed by transmission electron microscopy (TEM). Sample treatments, fixation, embedding procedures, and ultrathin sectioning have been described previously [15]. In brief, hepatic tissues not exceeding 1 mm^3 of the volume of the fixing and washing solutions

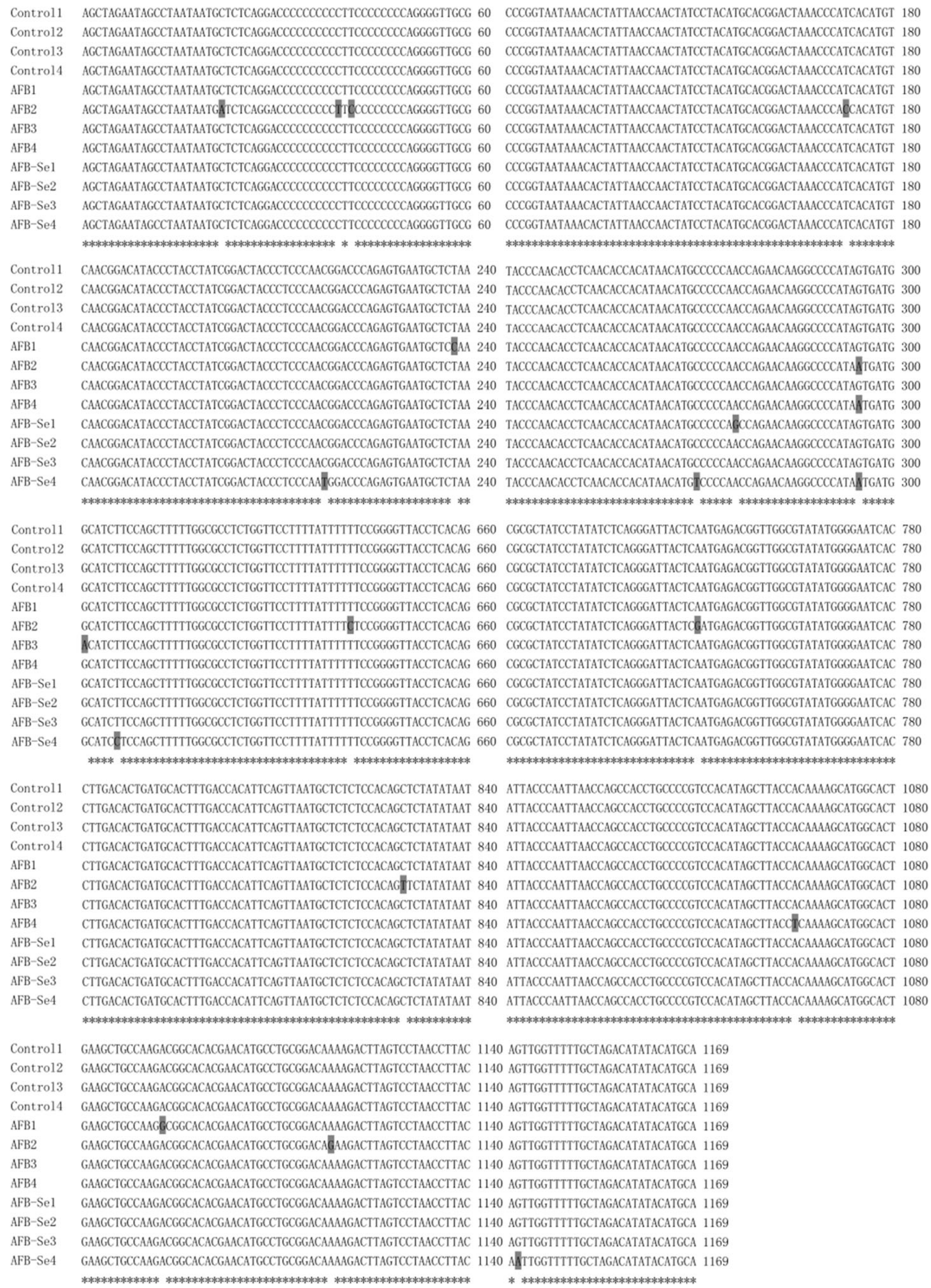

Fig. 1 Nucleotide multiple sequence alignments of the mtDNA D-loop region. The nucleotide sequences of the AFB_1 and AFB_1-Se groups were compared with the sequence from the control group. The single nucleotide polymorphisms are highlighted in *gray*

were fixed with 2 % glutaraldehyde in 0.1 M PBS at pH 7.4 for 2 h at 4 °C. After a rapid rinse in PBS, samples were fixed again for 2 h in reduced osmium solution, prepared by mixing one volume of 1 % aqueous osmium tetroxide at room temperature. Samples were progressively dehydrated, embedded in araldite, cut into ultrathin sections (60–80 nm), stained in uranyl acetate and lead citrate and then examined by JEM-1200EX (JEM, Japan).

Histopathologic Examination

Previously harvested liver tissue was fixed in 10 % neutral formalin and embedded in paraffin. Sections of 5 μm were obtained, deparaffinized, and stained with hematoxylin and eosin (H&E). The liver tissue was examined, evaluated, and photographed in random order under blind conditions using standard light microscopy (Zeiss, Germany).

Statistical Analysis

The statistical significance of differences between groups in these studies was performed using one-way analysis of variance (ANOVA) test of SPSS for Windows (version 15.0, SPSS Inc., Chicago, IL). The results were presented as the mean±SE. The difference between groups was considered significant when a probability (P) was <0.05.

Results

Mutations in the D-loop Region of mtDNA

The mtDNA D-loop was sequenced from 12 hepatic tissue samples (four from each of the three groups). The results of the sequence analysis in the control, AFB_1, and AFB_1-Se groups are shown in Fig. 1. Detailed descriptions of the mitochondrial sequence analysis are listed in Table 1.

All samples from the AFB_1 group exhibited at least one single nucleotide polymorphism (SNP). The greatest variation found in the AFB_1 group was a total of nine SNPs. Overall, 14 SNPs were found in ducklings that had received AFB_1. Variations were recorded in two AFB_1-Se ducklings, with a total of four SNPs. The polymorphisms were more frequently encountered in AFB_1 group ducklings than in control or AFB_1-Se group ducklings. The results of molecular analysis were compared with the mitochondrial permeability transition and histopathological changes.

Mitochondrial Permeability

In the present study, AFB_1 is an inducer of mitochondrial swelling (Table 2). The opening of the mitochondrial permeability transition pore was increased in ducklings that had received intragastric AFB_1 for 14 and 21 days ($P<0.05$). However, the ducklings that had also received Se had partial repair of mitochondrial function. The decrease in absorbance of group AFB_1-Se was significantly different from group AFB_1, and this decrease was partially time dependent. The AFB_1-induced swelling was further confirmed using TEM. TEM examination suggested that all mitochondria appeared swollen and vacuolized and displayed loss of the typical cristae structure (Fig. 2b). Se exhibited a protective effect and reduced mitochondrial swelling (Fig. 2c).

Histological Findings

Histological alterations in the hepatic tissue of the AFB_1 group and AFB_1-Se group are showed in Fig. 3. In the control group, the hepatic tissue structures were normal (Fig. 3a). Severe degenerative changes were discovered in the AFB_1 group, including severe hepatic steatosis, necrosis, and formation of lymphoid nodules with infiltrated lymphocytes (Fig. 3b). The incidence of these degenerative changes was reduced by Se treatment in the Se-treated group compared with the AFB_1 group (Fig. 3c).

Discussion

This study documented the effect of Se on AFB_1-induced hepatic tissue damage in ducklings. The results revealed that AFB_1 administration produces mitochondrial DNA D-loop mutation, mitochondrial swelling, and histological damage in hepatic tissue. Although Se treatment did not completely prevent AFB_1-induced impairment, it did reduce the extent of the damage.

Table 1 Summary of polymorphisms found in AFB_1-administrated ducklings

No.	Position	Gene	Sequence change
AFB1	238	D-loop	T→C
AFB1	1093	D-loop	A→G
AFB2	22	D-loop	C→A
AFB2	40	D-loop	T→C
AFB2	42	D-loop	C→T
AFB2	173	D-loop	T→C
AFB2	295	D-loop	G→A
AFB2	642	D-loop	T→C
AFB2	750	D-loop	A→G
AFB2	830	D-loop	C→T
AFB2	1119	D-loop	A→G
AFB3	601	D-loop	G→A
AFB4	295	D-loop	G→A
AFB4	1065	D-loop	A→T
AFB-Se1	276	D-loop	A→G
AFB-Se4	218	D-loop	C→T
AFB-Se4	270	D-loop	C→T
AFB-Se4	1142	D-loop	G→A

Nine SNPs were observed in the AFB_1 group and four mutations in the AFB_1-Se group

Table 2 Permeability transition of liver mitochondria, showing the time-dependent percentage of absorbance decrease

Criteria	The percentage of absorbance decrease (%)			
	1 min	4 min	7 min	10 min
14-day				
Control	0.72±0.02	2.61±0.07	4.46±0.15	6.04±0.07
AFB_1	1.19±0.09*	4.79±0.20*	9.49±0.31*	11.69±0.45*
AFB_1-Se	0.91±0.03	3.93±0.24	6.35±0.27*	8.34±0.24*
21-day				
Control	0.62±0.02	2.88±0.05	4.10±0.18	7.11±0.06
AFB_1	1.32±0.04*	6.06±0.25*	10.64±0.20*	12.90±0.38*
AFB_1-Se	1.08±0.09	4.12±0.06*	6.87±0.32*	8.68±0.16*

The AFB_1 group exhibited a significant decrease in absorbance compared with the control group. The decrease in absorbance in the AFB_1-Se group was significantly attenuated compared to the AFB_1 group. Data is expressed as the means±SE

*$P<0.05$, significantly different, AFB_1 group compared with the control; significantly different, AFB_1-Se group compared with the AFB_1 group

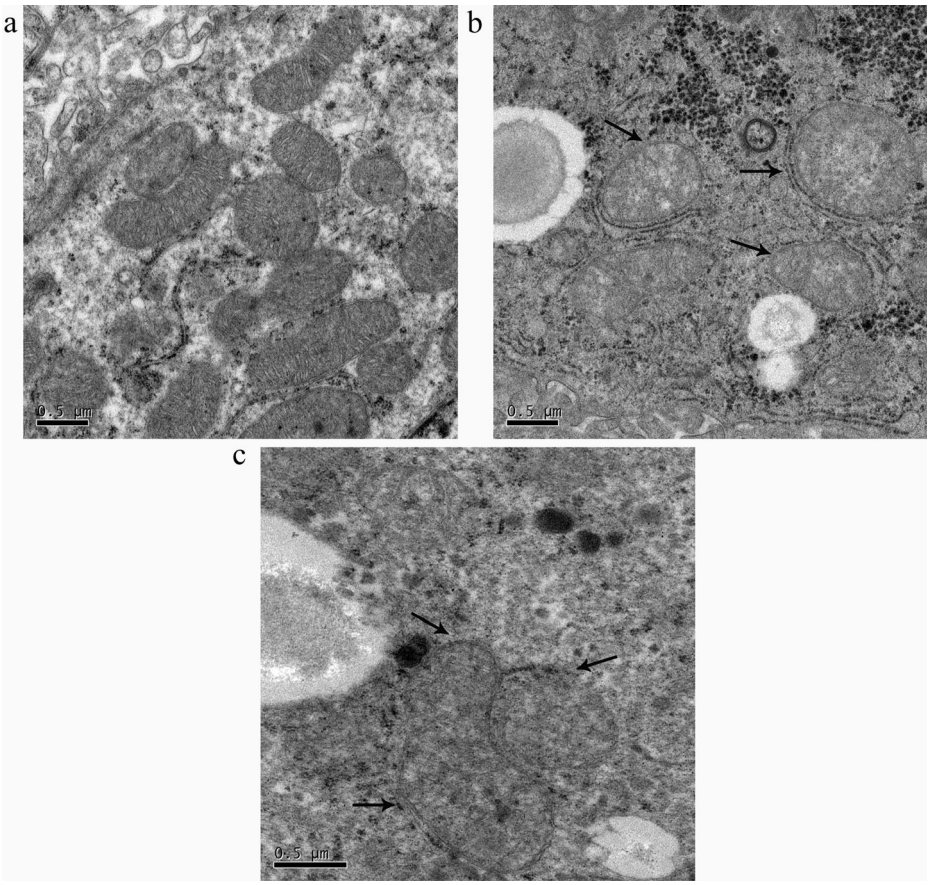

Fig. 2 TEM detection of liver mitochondria in control (**a**), AFB_1 (**b**), and AFB_1-Se (**c**) groups. Treatment of Se significantly reduced the liver mitochondria damage. Mitochondrial swelling is indicated by *arrows* (TEM; *scale bar*, 0.5 μm)

Aflatoxins are naturally occurring mycotoxins produced as secondary metabolites by the fungi *Aspergillus flavus*, *A. parasiticus*, and *A. nominus* [1]. The risk of developing hepatic disease due to AFB_1 exposure is highest in developing countries. The liver is the main target organ for AFB_1, which exerts its toxicity through oxidative damage. In a prior study, we demonstrated that AFB_1 at a dose of 0.1 mg/kg body weight adversely affected the activities of superoxide

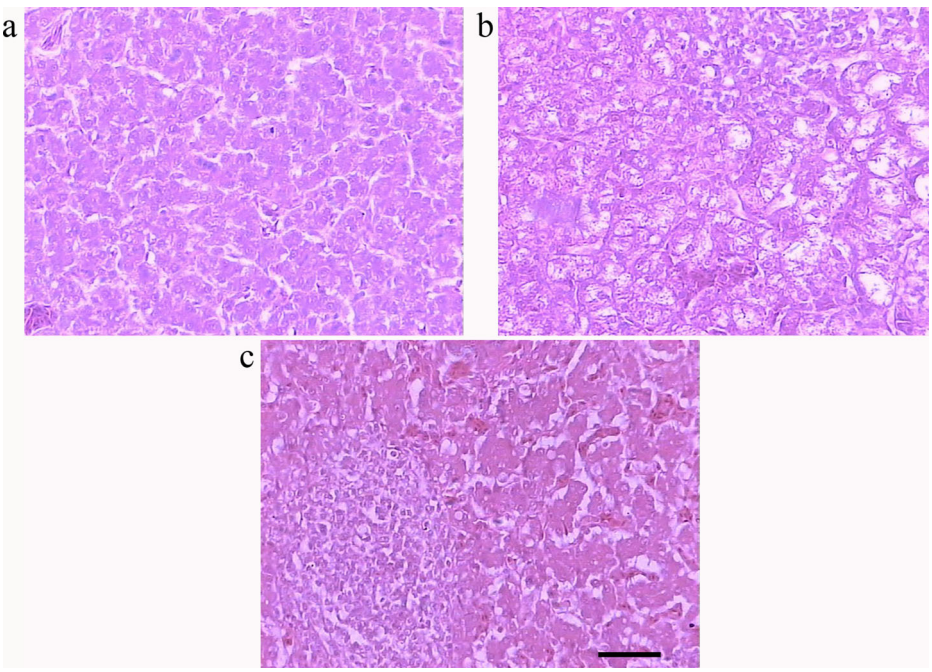

Fig. 3 Histological section of control (**a**), AFB_1 (**b**), and AFB_1-Se (**c**) groups liver tissue. The AFB_1 group shows lesions including severe hepatic steatosis, necrosis, and formation of lymphoid nodules with infiltrated lymphocytes. The AFB_1-Se group shows hepatic steatosis and formation of lymphoid nodules (H&E; *scale bar*, 50 μm)

dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione reductase (GR) in liver mitochondria [12]. The results suggested that AFB_1 is a significant inducer of hepatic mitochondrial antioxidant dysfunction.

In this study, we further investigated the development of oxidative DNA damage induced by AFB_1. Sequence analysis of the mtDNA D-loop region indicated that AFB_1 does induce mitochondrial DNA damage. Nine SNPs (C22A, T40C, C42T, T173C, G295A (twice), T238C, G601A, T642C, A750G, C830T, A1065T, A1093G, A1119G) were more frequently found in the AFB_1 group than in the control and AFB_1-Se groups. Mutations in the D-loop region, a non-encoding region of mtDNA, interfere with transcription of the entire mtDNA genome, possibly causing severe alterations in mitochondrial function. Mutations in the D-loop region have also been characterized in breast cancer, Barrett's esophagus, and pancreatic cancer [16–18]. Oxidative damage to mtDNA followed by mtDNA mutations has been verified as a critical step in carcinogenesis [19, 20]. Our work suggests that Se is a potential antioxidative agent to attenuate AFB_1-induced oxidative damage. The results are in line with the conclusion described by Xu [21]. In the study, Xu indicated that Se deficiency induced oxidative damage and disbalance of Ca^{2+} homeostasis in the brain of a chicken. Se plays an important role in antioxidativity and Ca^{2+} modulation.

Previous studies reported that AFB_1 is a potent compound leading to liver damage and changes in hepatic function [22]. Previous histopathological studies indicated that exposure to AFB_1 led to a granular appearance of hepatocyte cytoplasm, together with severe hydrophilic and vacuolar degeneration [23]. In our study, histopathological studies documented fat necrosis, steatosis, and formation of lymphoid nodules with infiltrated lymphocytes in subjects that had received AFB_1. Se could afford partial protection to reduce liver damage following AFB_1 treatment. It was found that Se supplementation ameliorated Cd-induced hepatotoxicity to birds in previous reports [24].

The mitochondrial swelling assay indicated that opening of the liver mitochondrial permeability transition pore appeared to be increased in AFB_1-treated ducklings. This result was further verified by TEM. Previous research has demonstrated that animals exposed to high levels of mitochondrial reactive oxygen species exhibit severe mitochondrial dysfunction and a marked propensity to undergo the permeability transition [25]. It has also been suggested that the cells accumulate oxidative damage, cross the mitochondrial permeability transition pore threshold, and are destroyed by cellular apoptosis [26]. Whether mitochondrial oxidative stress-induced apoptosis plays a critical role in mitochondrial damage will be characterized in our future studies.

In summary, our study indicates that the liver is an important target organ of AFB_1 toxicity. AFB_1 exposure induced morphological changes, mitochondrial swelling, and mtDNA damage in the liver tissue of ducklings. Se treatment ameliorated AFB_1-induced liver oxidative damage and may contribute to reduce the accumulation of free radicals.

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