

Protection of Selenium on Hepatic Mitochondrial Respiratory Control Ratio and Respiratory Chain Complex Activities in Ducklings Intoxicated with Aflatoxin B₁

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Received: 16 June 2011 / Accepted: 25 August 2011 / Published online: 10 September 2011
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Abstract To investigate the protection of selenium on hepatic mitochondrial functions, 90 7-day-old ducklings were randomly divided into three groups (groups I–III). Group I was used as a blank control. Group II was administered with aflatoxin B₁ (0.1 mg/kg body weight). Group III was administered with aflatoxin B₁ (0.1 mg/kg body weight) plus selenium (sodium selenite, 1 mg/kg body weight). All treatments were given once daily for 21 days. The results showed that the activities of hepatic mitochondrial complexes I–IV in group II ducklings significantly decreased when compared with group I ($P < 0.01$). Furthermore, the activities of hepatic mitochondrial complexes I–IV in group III significantly increased when compared with group II ($P < 0.05$). The hepatic mitochondrial respiratory control ratio (RCR) in group II ducklings significantly decreased when compared with group I ($P < 0.01$). In addition, the hepatic mitochondrial RCR in group III significantly increased when compared with group II ($P < 0.05$). These results revealed that the aflatoxin B₁ significantly induced hepatic mitochondrial dysfunction in the activities of hepatic mitochondrial respiratory chain complexes I–IV and the RCR in ducklings. However, sodium selenite could significantly ameliorate the negative effect induced by aflatoxin B₁.

Keywords Sodium selenite · Hepatic mitochondrial · Respiratory chain complexes · Respiratory control ratio · Aflatoxin B₁

Introduction

Aflatoxins, a group of extremely toxic and biologically active substances, are produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B₁ (AFB₁) is considered to be one of the most potent hepatotoxins and well-known hepatocarcinogens [1]. The liver is especially sensitive to AFB₁. The AFB₁ is firstly a hepatotoxin, causing an excessive buildup of hepatic lipids, with enlargement of the liver, proliferation of the biliary ducts [2], and hepatocellular carcinoma [3]. The carcinogenicity and toxicity of AFB₁ is manifested through its enzymatic activation into a reactive metabolite which interacts with cellular macromolecules like DNA, RNA, and proteins [4, 5].

It is becoming increasingly evident that mitochondrial DNA, which is less protected by associated histones than nuclear DNA, is a major target for carcinogen binding [6]. Administered AFB₁ also caused impairments in hepatic mitochondrial respiration rates and led to alterations in mitochondrial ATPase activity [7]. In a number of studies, the ability of antioxidants to defend chemical carcinogenesis when administered prior to or concomitantly with the carcinogen was demonstrated. Butylated hydroxytoluene and butylated hydroxyanisole were found to inhibit carcinogenesis caused by AFB₁ in rats [8]. The intake of beta-carotene, ascorbic acid, selenium, uric acid, and vitamin E reduced the incidence of AFB₁-induced liver cancer in rats [9]. AFB₁-mediated hepatic lipid peroxidation and serum

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activity of transaminases were reduced by the pretreatment of rats with antioxidants, selenium, and vitamin E [10]. The present study was designed to assess the protective effect of selenium by evaluating the hepatic mitochondrial respiratory control ratio (RCR) and respiratory chain complex activities in ducklings intoxicated with AFB₁.

Materials and Methods

Drugs and Chemicals

D-Mannitol, hydroxyethyl piperazine ethanesulfonic acid (HEPES), 3-(*N*-morpholino)propanesulfonic acid, ethylene glycol tetraacetate (EGTA), bovine serum albumin (BSA), NADH, 2',7'-dichlorofluorescein diacetate, coenzyme Q₁₀, antimycin A, and rotenone were purchased from Sigma. All other chemicals were of analytical grade.

Animals and Treatments

All experimental protocols were approved by the Ethics Committee of South China Agricultural University. This study was carried out on 90 Guangdong white ducklings weighing 180–200 g body weight. Seven-day-old ducklings were obtained from the Research Center of Experimental Animals at South China Agricultural University. The animals were randomly divided into three equal groups containing 30 ducklings with an equal number of male and female ducklings. Group I was used as control and only dimethyl sulfoxide (DMSO) was intragastrically administered. Group II was intragastrically administered with AFB₁ (0.1 mg/kg body weight) dissolved in DMSO. Group III was intragastrically administered with AFB₁ (0.1 mg/kg body weight) plus selenium (sodium selenite, 1 mg/kg body weight). These treatments were administered once daily for a period of 21 days under the same condition. AFB₁ was diluted with DMSO for experimental groups. All ducklings had free access to water and food at room temperature during the study.

Mitochondrial Preparation

Mitochondria were isolated from the duckling liver by differential centrifugation as described by Tang et al. [11], with modifications. On 7th, 14th, and 21st day after treatment, five ducklings were randomly taken out to obtain, respectively, about 15 g liver in every experimental group. The liver was trimmed of fat and washed in ice-cold initial liver mitochondria isolation medium A (220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES, 1 mM EGTA, 0.5 mg/mL BSA, adjusted to pH 7.0 with Tris). The liver was then weighed, minced, and transferred to a 50-mL capacity Potter

homogenizer run at 2,000 rpm (0.15 mm radial pestle clearance) for 3 min. The homogenate was diluted to 300 mL using medium A (6×50 mL) and centrifuged for 10 min at 1,000×g. The supernatant was filtered through four-layer cheesecloth and centrifuged again at 1,000×g for 10 min. After the supernatant was centrifuged at 10,000×g for 14 min, the pellet was suspended in 100 mL (2×50 mL) medium B, a second liver mitochondria isolation medium (220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES, 0.5 mg/mL BSA, adjusted to pH 7.0 with Tris), and centrifuged for 10 min at 10,000×g. The resulting pellet was resuspended in liver mitochondria incubation medium C (220 mM D-mannitol, 70 mM sucrose, and 2 mM HEPES, adjusted to pH 7.0 with Tris). All the steps are strictly operated on ice to guarantee the isolation of high-quality mitochondrial preparation. The protein concentration of the final mitochondrial pellet was determined with the Bradford's assay using bovine serum albumin as a protein standard [12].

Assessment of Mitochondrial Respiratory Chain Complex Activities

The activities of respiratory chain complexes were determined according to the methods as described by Zhang et al. [13], with modifications. All assays were performed at 25°C in a final volume of 1 mL using an UV–VIS 8500 spectrophotometer. To establish the optimum conditions for the release of complexes, the mitochondria was freeze-thawed three times 25°C/–25°C in hypotonic media (25 mM potassium phosphate, 5 mM MgCl₂, pH 7.2) before the determination. The enzyme activity was expressed as nanomolars per minute per milligram protein.

Complex I (NADH–ubiquinone oxidoreductase) activity was measured by following the decrease in absorbance due to the oxidation of NADH at 600 nm. NADH (0.1 mM), ubiquinone (0.1 mM), antimycin A (3 μM), and mitochondria (15–30 μg of protein) were added to the assay medium (25 mM potassium phosphate, 5 mM MgCl₂, 2 mM KCN, pH 7.2), and the absorbance change was recorded for 5 min at 600 nm.

Complex II (succinate–ubiquinone oxidoreductase) specific activity was performed at 600 nm using dichlorophenolindophenol (DCPIP; 0.1 mM) as acceptor and succinate (20 mM) as donor. Mitochondria of 15–30 μg were preincubated in the assay medium (25 mM potassium phosphate, 5 mM MgCl₂, 2 mM KCN, pH 7.2) plus 20 mM succinate at 25°C for 10 min. Antimycin A (3 μM), KCN (2 mM), rotenone (2 μg/mL), and DCPIP were added and a baseline rate recorded for 5 min. The reaction was started with ubiquinone (0.1 mM), and then the enzyme-catalyzed reduction of DCPIP was measured for 5 min.

Complex III (ubiquinone–ferricytochrome-c oxidoreductase) specific activity was measured by monitoring the reduction of cytochrome c (15 μ M) at 550 nm (25 mM potassium phosphate, 5 mM $MgCl_2$, 2 mM KCN, 2 μ g/mL rotenone, pH 7.2). Cytochrome c (15 μ M) and ubiquinol (0.1 mM) were added to the assay medium, and the nonenzymatic rate was recorded for 1 min. Then, mitochondria were added, and the increase in absorbance was measured. The increase in absorbance was recorded for 3 min.

Complex IV (cytochrome c oxidase) activity was measured by the oxidation of cytochrome c (II, 40 μ M) at 550 nm in an assay medium containing 20 mM potassium phosphate, pH 7.0. Mitochondria were added to the assay medium, and the reduction of absorbance was recorded for 3 min.

Mitochondrial Oxygen Consumption Experiment

Aliquots of mitochondria (3 mg/mL) were used in all of the respiration measurements using a Clark-type oxygen electrode according to Zhang et al. [13]. Mitochondrial suspension was added to a mixture containing 5 mM glutamate–malate. The assay was performed in a buffer consisting of 130 mM KCl, 3 mM HEPES, 2 mM KH_2PO_4 , 2 mM $MgCl_2$, and 1 mM EGTA. State 4 respiration was first measured in the absence of adenosine diphosphate (ADP). Subsequently, state 3 (phosphorylating) respiration was measured in the presence of ADP, by the addition of 6.0 μ L ADP (0.027 M) to determine the maximal rate of coupled ATP synthesis. The RCR was calculated using the ratio of state 3 and state 4 respiratory rates. Respiratory control ratio is expressed as nanomolars O per minute per milligram mitochondrial protein.

Statistical Analysis

The statistical significance of differences between groups in these studies was determined using a one-way analysis of

variance, and the results were presented as the mean \pm SE. The significance level was $P < 0.05$.

Results and Analysis

Respiratory Chain Complexes Activity

As shown in Table 1, the activities of hepatic mitochondrial complexes I–IV were significantly affected after ducklings were intragastrically administered with AFB₁ (0.1 mg/kg body weight) once daily for 7 days. The activities of complexes I–IV decreased by 26.31%, 25.76%, 17.65%, and 26.28%, respectively. The ducklings in group III were intragastrically administered with AFB₁ plus selenium (sodium selenite, 1 mg/kg body weight), and the activities of complexes I–IV increased by 16.56%, 14.70%, 8.01%, and 28.07%, respectively, compared with group II.

As summarized in Table 2, the activities of hepatic mitochondrial complexes I–IV were significantly affected after ducklings were intragastrically administered with AFB₁ (0.1 mg/kg body weight) once daily for 14 days. The activities of complexes I–IV decreased by 29.62%, 34.81%, 31.54%, and 36.01%, respectively. The ducklings in group III were intragastrically administered with AFB₁ plus selenium (sodium selenite, 1 mg/kg body weight), and the activities of complexes I–IV increased by 23.54%, 20.17%, 25.28%, and 45.36%, respectively, compared with group II.

As shown in Table 3, the activities of hepatic mitochondrial complexes I–IV were significantly affected after ducklings were intragastrically administered with AFB₁ (0.1 mg/kg body weight) once daily for 21 days. The activities of complexes I–IV decreased by 30.51%, 42.40%, 39.42%, and 42.24%, respectively. The ducklings in group III were intragastrically administered with AFB₁ plus selenium (sodium selenite, 1 mg/kg body weight), and the activities of complexes I–IV increased by 25.94%, 40.40%, 40.02%, and 48.77%, respectively, compared with group II.

Table 1 The activities of hepatic mitochondrial respiratory chain complexes I–IV for 7 days after ducklings were administrated with AFB₁

Groups	Complex I	Complex II	Complex III	Complex IV
I	73.16 \pm 3.44	50.14 \pm 5.48	45.32 \pm 3.27	30.25 \pm 0.36
II	53.91 \pm 6.63**	37.22 \pm 3.73**	37.32 \pm 2.69**	22.30 \pm 0.39**
III	62.84 \pm 3.18***	42.69 \pm 2.82***	40.31 \pm 2.56***	28.56 \pm 0.46****

It showed that the activities of hepatic mitochondrial complexes I–IV in group II ducklings significantly decreased when compared with group I ($P < 0.01$), and the activities of hepatic mitochondrial complexes I–IV in group III significantly increased when compared with group II ($P < 0.05$). Data were expressed as the means \pm SE. They were expressed as nanomolars per minute per milligram protein

Complex I NADH–ubiquinone oxidoreductase, *Complex II* succinate–ubiquinone oxidoreductase, *Complex III* ubiquinone–ferricytochrome-c oxidoreductase, *Complex IV* cytochrome c oxidase, *I* the control, *II* administered with AFB₁, *III* administered with AFB₁ plus selenium (sodium selenite)

** $P < 0.01$ (significantly different when group II compared with group I); *** $P < 0.05$ (significantly different when group III compared with group II);

**** $P < 0.01$ (significantly different when group III compared with group II)

Table 2 The activities of hepatic mitochondrial respiratory chain complexes I–IV for 14 days after ducklings were administrated with AFB₁

Groups	Complex I	Complex II	Complex III	Complex IV
I	80.65±3.85	55.21±2.07	48.42±2.56	28.60±0.49
II	56.76±5.44**	35.99±2.82**	33.15±2.29**	18.30±0.68**
III	70.12±4.75***	43.25±5.92***	41.53±3.54****	26.60±0.52****

It showed that the activities of hepatic mitochondrial complexes I–IV in group II ducklings significantly decreased when compared with group I ($P<0.01$), and the activities of hepatic mitochondrial complexes I–IV in group III significantly increased when compared with group II ($P<0.05$). Data were expressed as the means±SE. They were expressed as nanomolars per minute per milligram protein

Complex I NADH–ubiquinone oxidoreductase, *Complex II* succinate–ubiquinone oxidoreductase, *Complex III* ubiquinone–ferricytochrome-c oxidoreductase, *Complex IV* cytochrome c oxidase, *I* the control, *II* administered with AFB₁, *III* administered with AFB₁ plus selenium (sodium selenite)

** $P<0.01$ (significantly different when group II compared with group I); *** $P<0.05$ (significantly different when group III compared with group II); **** $P<0.01$ (significantly different when group III compared with group II)

Mitochondria Respiration Control Ratio

As shown in Table 4, the hepatic mitochondrial RCR were significantly affected after ducklings were intragastrically administered with AFB₁ (0.1 mg/kg body weight) once daily for a period of 21 days. The RCR decreased by 28.62%, 37.63%, and 42.25%, respectively, for 7, 14, and 21 days after treatment. The ducklings in group III were intragastrically administered with AFB₁ plus selenium (sodium selenite, 1 mg/kg body weight), and the RCR increased by 18.5%, 22.73%, and 46.69%, respectively, compared with group II.

Discussion

It is well known that aflatoxins have hepatotoxic and hepatocarcinogenic effects [14]. The cytotoxic effect of AFB₁ has been studied in vivo or in vitro using different cellular models, mostly the liver. It has been observed that AFB₁ is activated by hepatic cytochrome P450 enzyme system to produce a highly reactive intermediate, AFB₁-8,9-epoxide, which subsequently binds to nucleophilic

sites in DNA, and the major adduct 8,9-dihydro-8-(*N*⁷-guanyl)-9-hydroxy-AFB₁ is formed [15, 16]. The formation of AFB₁-DNA adducts is regarded as a critical step in the initiation of AFB₁-induced hepatocarcinogenesis [17]. In the organism, mitochondria consume about 90% of the cell's oxygen and carry out the most important function of oxidative phosphorylation. So in mitochondria, energy derived from oxidation of fatty acids, carbohydrates, and amino acids is conserved as ATP, which is transported to the energy requiring reactions of the cell. According to current concepts, cancer is assumed to arise because a lack of oxygen, or of respiratory enzymes, produces fermentation in the body cells and leads to a destruction of the differentiation of these cells. The impairment of the respiratory system is considered an early event in carcinogenesis [18].

Although adduct formation with nuclear DNA initiates events that lead to cancerous cell formation, recent evidences have recorded certain associated changes in mitochondria that might be considered important. Moreover, damaged and swollen mitochondria are commonly observed in tumor tissues as well as after carcinogen administration. Metabolites of many carcinogens and in

Table 3 The activities of hepatic mitochondrial respiratory chain complexes I–IV for 21 days after ducklings were administrated with AFB₁

Groups	Complex I	Complex II	Complex III	Complex IV
I	92.04±4.46	60.47±2.18	50.28±1.35	28.86±0.62
II	63.96±8.05**	34.83±2.59**	30.46±3.36**	16.67±1.10**
III	80.55±3.77****	48.90±1.92****	42.65±4.69****	24.80±0.82****

It showed that the activities of hepatic mitochondrial complexes I–IV in group II ducklings significantly decreased when compared with group I ($P<0.01$), and the activities of hepatic mitochondrial complexes I–IV in group III significantly increased when compared with group II ($P<0.05$). Data were expressed as the means±SE. They were expressed as nanomolars per minute per milligram protein

Complex I NADH–ubiquinone oxidoreductase, *Complex II* succinate–ubiquinone oxidoreductase, *Complex III* ubiquinone–ferricytochrome-c oxidoreductase, *Complex IV* cytochrome c oxidase, *I* the control, *II* administered with AFB₁, *III* administered with AFB₁ plus selenium (sodium selenite)

** $P<0.01$ (significantly different when group II compared with group I); **** $P<0.01$ (significantly different when group III compared with group II)

Table 4 Changes of the hepatic mitochondrial RCR in 21 days after ducklings were administrated with AFB₁

Groups	7 days	14 days	21 days
I	3.18±0.06	3.88±0.14	4.71±0.27
II	2.27±0.15**	2.42±0.31**	2.72±0.26**
III	2.69±0.19***	2.97±0.21***	3.99±0.25****

It showed that the hepatic mitochondrial RCR in group II ducklings significantly decreased when compared with group I ($P<0.01$), and the hepatic mitochondrial RCR in group III significantly increased when compared with group II ($P<0.05$). Data were expressed as the means±SE. They were expressed as nanomolars O per minute per milligram mitochondrial protein

I the control, *II* administered with AFB₁, *III* administered with AFB₁ plus selenium (sodium selenite)

** $P<0.01$ (significantly different when group II compared with group I); *** $P<0.05$ (significantly different when group III compared with group II); **** $P<0.01$ (significantly different when group III compared with group II)

some instances the carcinogen itself interfere with the mitochondrial process of oxidative phosphorylation [19]. The deterioration in the capacity of oxidative metabolism and the fall in the activity of mitochondrial enzymes are some of the mitochondrial abnormalities observed in cancer cells [20]. It is also apparent from the present study [21, 22] that mitochondrial functional changes precipitate because of the extensive interaction of AFB₁ metabolite with mitochondrial proteins and nucleic acids. It is concluded from the foregoing observations that AFB₁ metabolite is capable of imparting damage to mitochondrial membrane resulting in altered respiratory capacity. Although recovery is achieved in animals exposed to a single dose of AFB₁, continued exposure might bring a sustained disturbance in mitochondrial oxygen consumption that could possibly lead to effects more related to carcinogenesis.

In continuation of these studies, we investigated the effect of AFB₁ administration on ducklings' hepatic mitochondrial energy-linked functions in this research. It showed that the hepatic mitochondrial RCR was significantly affected after ducklings were intragastrically administered with AFB₁ (0.1 mg/kg body weight) once daily for a period of 21 days. The RCR decreased by 28.62%, 37.63%, and 42.25%, respectively, for 7, 14, and 21 days after treatment. It was observed that the activities of hepatic mitochondrial complexes I–IV were significantly affected after ducklings were intragastrically administered with AFB₁ (0.1 mg/kg body weight) once daily for a period of 21 days. The activities of complexes I–IV decreased by 26.31%, 25.76%, 17.65%, and 26.28%, respectively, after AFB₁ administration for

7 days. The activities of complexes I–IV decreased by 29.62%, 34.81%, 31.54%, and 36.01%, respectively, after AFB₁ administration for 14 days. The activities of complexes I–IV decreased by 30.51%, 42.40%, 39.42%, and 42.24%, respectively, after AFB₁ administration for 21 days.

Although the mechanism underlying the hepatotoxicity of aflatoxins is not fully understood, several reports suggest that toxicity may ensue through the generation of intracellular reactive oxygen species like superoxide anion, hydroxyl radical, and hydrogen peroxide during the metabolic processing of AFB₁ by cytochrome P450 in the liver [23, 24]. These species may attack soluble cell compounds as well as membranes, eventually leading to the impairment of cell functioning and cytolysis [25]. But peroxidative damages induced in the cell are encountered by elaborate defense mechanisms, including enzymic and nonenzymic antioxidants [26]. AFB₁-mediated hepatic lipid peroxidation and serum activity of transaminases were reduced by the pretreatment of rats with antioxidants, selenium, and vitamin E.

In this study, we assessed the protective effect of selenium on hepatic mitochondrial energy-linked functions in ducklings administrated with aflatoxin B₁. The hepatic mitochondrial RCR in the ducklings administered with AFB₁ plus selenium (sodium selenite, 1 mg/kg body weight) increased by 18.5%, 22.73%, and 46.69%, respectively, compared with the ducklings administrated with AFB₁ only. It was showed that the activities of hepatic mitochondrial complexes I–IV in the ducklings administered with AFB₁ plus selenium (sodium selenite, 1 mg/kg body weight) for 7 days increased by 16.56%, 14.70%, 8.01%, and 28.07%, respectively, compared with the ducklings administrated with AFB₁ only for 7 days. The activities of hepatic mitochondrial complexes I–IV in the ducklings administered with AFB₁ plus selenium (sodium selenite, 1 mg/kg body weight) for 14 days increased by 23.54%, 20.17%, 25.28%, and 45.36%, respectively, compared with the ducklings administrated with AFB₁ only for 14 days. The activities of hepatic mitochondrial complexes I–IV in the ducklings administered with AFB₁ plus selenium (sodium selenite, 1 mg/kg body weight) for 21 days increased by 25.94%, 40.40%, 40.02%, and 48.77%, respectively, compared with the ducklings administrated with AFB₁ only for 21 days. The results of the present study demonstrate that sodium selenite ameliorates the negative effects of aflatoxin B₁ on the hepatic mitochondrial RCR and on the activities of the respiratory chain complexes I–IV in ducklings.

Acknowledgments This work was supported by the National Natural Science Foundation of China (grant no. 38071900).

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