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Research Article

Effect of Aflatoxin B_1 on Histopathology and Oxidative Stress Biomarkers in Testis of Rats with Special References to Gene Expression of Antioxidant Enzymes

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Abstract

The stimulation of oxidative stress and lipid peroxidation of liver tissues by AFB1 has been extensively investigated. However, the effect of AFB1 on antioxidant enzyme activities and gene expression in testis has not been completely elucidated. The current study investigated the histopathological picture and activities in addition to gene expression of antioxidant enzymes in testis of rats intoxicated with AFB1. Twenty rats were divided into two equal groups. Rats in the first group served as control whereas, rats in the second group received single i.p. dose of AFB1 (3 mg kg^{-1} b.wt.). Testicular damage and oxidative stress were evident in AFB1-intoxicated rats as indicated by a significant increase in thiobarbituric acid reactive substances (TBARS), reduction of reduced glutathione (GSH) concentration, reduction in the activities of antioxidant enzymes namely catalase (CAT), total superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione-s-transferase (GST) and down-regulation of gene expression of these antioxidant enzymes compare to control. Testicular sections of rats intoxicated with AFB1 showed mild testicular degeneration of some seminiferous tubules manifested by reduced numbers of spermatogenic cells and associated with buckling of the seminiferous basement membranes. In conclusion, testicular injury, high lipid peroxidation, low GSH concentration and inactivation and down-regulation of gene expression of antioxidant enzymes are involved during AFB1 toxicity in rats.

Key words: Aflatoxins, oxidative stress, antioxidants, histopathology, testis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Aflatoxins are produced as metabolites of fungi named *Aspergillus flavus* and *Aspergillus parasiticus* (Goto *et al*., 1996). Among aflatoxins, AFB1 is known the most toxic type (Busby and Wogan, 1984) due to its carcinogenic and teratogenic effects in mammals and fish (Roebuck and Maxuitenko, 1994). After absorption, the concentration of AFB1 was higher in gonads followed by liver, kidneys, spleen, thymus, endocrine gland, lungs and finally brain (Marvan *et al*., 1983). The AFB1 is metabolized by CYP450 with subsequent production of AFB1-8, 9-epoxide and DNA adducts (Smela *et al*., 2001). The oxidative stress is produced when prooxidants level exceeds the antioxidant one (El-Bahr, 2013, 2014; Al-Sultan and El-Bahr, 2015). Therefore, the toxic effect of AFB1 may be attributed to induction of oxidative stress by AFB1-8, 9-epoxide in affected tissues such as liver (Towner *et al*., 2003; El-Bahr, 2015) and testis (Verma and Nair, 2001; Abu El-Saad and Mahmoud, 2009; Tas *et al*., 2010; Ismail, 2012) inducing lipid peroxidation and inactivation or depletion of antioxidant enzymes (El-Bahr, 2015). Biochemical analysis of liver antioxidants revealed a significant reduction in CAT and SOD activities and GSH content in the liver of rats injected with AFB1 compared to control (El-Bahr *et al*., 2015).

The histopathological studies on testicular tissues indicated that, seminiferous tubules of male rats were diminished after oral administration of AFB1 (Bashandy *et al*., 1994; El-Kordy and Abo Gazia, 2015). In addition, sperm motility and count were significantly decreased in AFB1 treated mice (Agnes and Akbarsha, 2003). High dose of AFB1 increased the edematous fluid inside the tumbles and decreased the number of leydig cells, spermatocytes and spermatids (Murad *et al*., 2015). Recently, it has been assumed that, the mechanism of action of AFB1 may attributed to significant down-regulation of gene expression of antioxidant enzymes namely, CAT, SOD, GPX and GST in liver tissues of AFB1 intoxicated rats compared to control (El-Bahr, 2015). However, this mechanism of action has not been checked in testicular tissues of rats intoxicated with the same toxin. Therefore, the current study aimed to investigate the impact of AFB1 toxicity on histopathological picture and activities and gene expression of antioxidant enzymes in testis of rats.

MATERIALS AND METHODS

Chemicals: The AFB1, Ethylene Glycol Tetraacetic Acid (EGTA), ethylenediaminetetraacetic acid (EDTA), sucrose, tris, butanol, mannitol, metaphosphoric acid, hydrogen peroxide (H_2O_2) and Dimethyl Sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Radioimmunoprecipitation assay (RIPA) buffer was provided by Cayman Chemical Company, USA. All other chemicals were of analytical grade.

Animals, experimental design and sampling: Twenty rats (100±5 g) were obtained from the laboratory animal house of the College of Veterinary Medicine and Animal Resources, King Faisal University, Saudi Arabia. They were maintained according to the national guidelines and protocols, which were approved by the University Animal Ethics Committee. They were housed in clean and disinfected plastic cages. Commercial basal diet and water were provided *ad libitum.* Rats were subjected to a natural photoperiod of 12 h light: dark cycle throughout the experimental period which lasted for 5 weeks (35 days). The rats were divided into two groups (10 rats per group). Rats in the first group were injected with a single i.p. dose of 1% DMSO at the first day of the experiment and served as control whereas, rats in the second group were administered with a single i.p. dose of AFB1 3 mg kg^{-1} b.wt. (Kamdem *et al*., 1983; El-Bahr, 2015; El-Bahr *et al*., 2015) dissolved in 1% DMSO at the first day of the experiment. After 5 weeks (day 35), the rats were anaesthetised with diethyl ether. The testicular tissues were removed and divided into three portions. The first portion of the testicular tissue was cut into small pieces and immersed in neutral-buffered formalin for 24 h for histopathological examination. The second portion of the testicular tissue was immediately frozen at -30°C for biochemical analysis of antioxidant enzyme activities, and the last portion of the testicular tissue was immediately frozen in liquid nitrogen and stored at -80°C for molecular analysis of antioxidant enzyme gene expression.

Histopathological assessment of testicular damage: Testicular tissues were cut into small pieces and immersed in neutral-buffered formalin for 24 h. The fixed tissues were processed routinely, embedded in paraffin, sectioned, deparaffinised and rehydrated using standard techniques (Bancroft and Gamble, 2002). The extent of AFB1-induced necrosis was evaluated by assessing the morphological changes in the testicular sections stained with hematoxylin and eosin (H and E) using standard techniques.

Determination of testicular antioxidant enzymes, thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) levels: One gram of testicular tissue was homogenised in 5 mL of cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose. After centrifugation (1500 g, 5 min) at 4°C, the supernatant

was removed and stored frozen at -80°C until further analysis of total superoxide dismutase (SOD). One gram of testicular tissue was homogenised in 5 mL of cold 50 mM potassium phosphate buffer, pH 7, containing 1 mM EDTA. After centrifugation (10,000 g, 15 min) at 4°C, the supernatant was removed and stored frozen at -80°C until further analysis of catalase (CAT), glutathione peroxidase (GPX), glutathione-s-transferase (GST) and reduced glutathione (GSH). The extent of lipid peroxidation in terms of TBARS formation was measured by mixing 1 g of testicular tissue with RIPA buffer (catalogue #10010263, Cayman Chemical Company, USA). After homogenisation, sonication and centrifugation (1600 g, 10 min), the supernatant was removed and stored frozen at -80°C until further analysis. The ELISA kits of Cayman Chemical Company, USA were used for determination of the activities of CAT (nmol min^{-1} g^{-1} tissue, catalogue #707002), GPX (nmol min^{-1} g^{-1} tissue, catalogue #703102), total SOD (U g^{-1} tissue, catalogue #706002), GST (nmol min^{-1} g^{-1} tissue, catalogue #703302), concentrations of GSH (μM, catalogue #703002) and TBARS (μM, catalogue #10009055) by using an ELISA reader (Absorbance Microplate Reader ELx 800TM BioTek®, USA). The results were calculated according to the manufacturer's instruction.

Total RNA isolation and real-time RT-PCR of testicular antioxidant enzymes: Testicular tissues (approximately 1 g of tissue per sample) were immediately added to 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) and homogenised using a homogeniser (Tissue Ruptor, Qiagen GmbH, Germany). One millilitre of the tissue homogenate was transferred to a microfuge tube and total RNA was extracted by the addition of 0.2 mL chloroform. Next, the samples were vigorously vortexed for 15 sec and incubated at room temperature for 3 min. After centrifugation (12,000 g, 15 min) at 4°C, the aqueous phase containing RNA was transferred to new tubes. RNA was precipitated by mixing the aqueous phase with 0.5 mL isopropyl alcohol and incubated at room temperature for 10 min. After centrifugation at 12,000 g for 10 min at 4°C, RNA pellets were washed by mixing and vortexing with 1 mL of 75% ethanol. After centrifugation (7,500 g, 5 min) at 4°C, RNA pellets were resuspended in nuclease-free water (Life Technologies. USA). The purity of RNA at 260/280 OD ratio and the RNA integrity were evaluated using a Multi-Mode Microplate reader (SYNERGY Mx, BIO-TEK. Winooski, Vermont, USA). Only high purity samples (OD260/280>1.8) were subjected to further manipulation. cDNA was obtained from RNA samples using a Reverse Transcription System Kit (Promega, Madison, USA) and Bio-Rad Thermal Cycler (T100™, Foster city, California, USA). Briefly, total RNA was activated at 70°C for 10 min and 20 μL reaction mixtures were prepared with 4 μL $MgCl_2$, 2 μL of reverse transcription 10x buffer, 2 μL of dNTP mixture (10 mM), 0.5 μL of random primers, 0.75 μL of AMV reverse transcriptase enzyme, 1 ng RNA and nuclease-free water to a final volume of 20 μL. Next, the reaction was incubated at 42°C for 60 min, followed by incubation at 94°C for 5 min. The cDNA was diluted up to 100 μL with nuclease-free water for PCR amplification.

The Real-time RT-PCR was performed using the QuantiFast™ SYBR Green PCR Master Mix Kit (QIAGEN, Hilden; Germany). The 25 μL reaction for each examined gene was prepared from 12.5 μL of master mix, 2 μL forward primer (10 pmol), 2 μL reverse primer (10 pmol), 2 μL cDNA of the sample and 6.5 μL of nuclease-free water. The cycling parameters were 50°C for 2 min, 95°C for 15 min, 40 cycles of 95°C for 10 sec, followed by 55°C for 30 sec and 72°C for 10 sec with a final melting temperature at 95°C for 20 sec. For each gene examined, duplicate samples from each cDNA were analysed using real-time RT-PCR and the Bio-Rad CFX Manager 3.0 Software of the C1000 Touch thermal cycler-CFX96 Real-time PCR (BIO-RAD, Foster city, California, USA). The β-actin mRNA fragment was used as a housekeeping gene to normalise the expression data. The primer sequences are described in Table 1.

Statistical analysis: All data are presented as the Mean±SEM using one way analysis of variance (ANOVA). All tests were performed using a statistical analysis system program. The relative gene expression of target genes compared to the β-actin reference gene was calculated using the Bio-Rad CFX Manager 3.0 Software of the C1000 Touch thermal cycler-CFX96 Real-time PCR (BIO-RAD, Foster City, California, USA).

Table 1: Primers sequences for the studied genes amplified by real-time RT-PCR

Gene	Forward primer sequence	Reverse primer sequence
β-actin	5′-AGC CAT GTA CGT AGC CAT CC-3′	5′- CTC TCA GCT GTG GTG GTG AA-3′
Total SOD	5′- AGG ATT AAC TGA AGG CGA GCA T-3′	5′- TCT ACA GTT AGC AGG CCA GCA G-3′
CAT	5′-ACG AGA TGG CAC ACT TTG ACA G-3′	5′-TGG GTT TCT CTT CTG GCT ATG G-3′
GPX	5′-AAG GTG CTG CTC ATT GAG AAT G-3′	5′-CGT CTG GAC CTA CCA GGA ACT T-3′
GST	5′- GCT GGA GTG GAG TTT GAA GAA-3′	5/- GTC CTG ACC ACG TCA ACA TAG-3′

SOD: Superoxide dismutase, CAT: Catalase, GPX; Glutathione peroxidase, GST: Glutathione-s-transferase

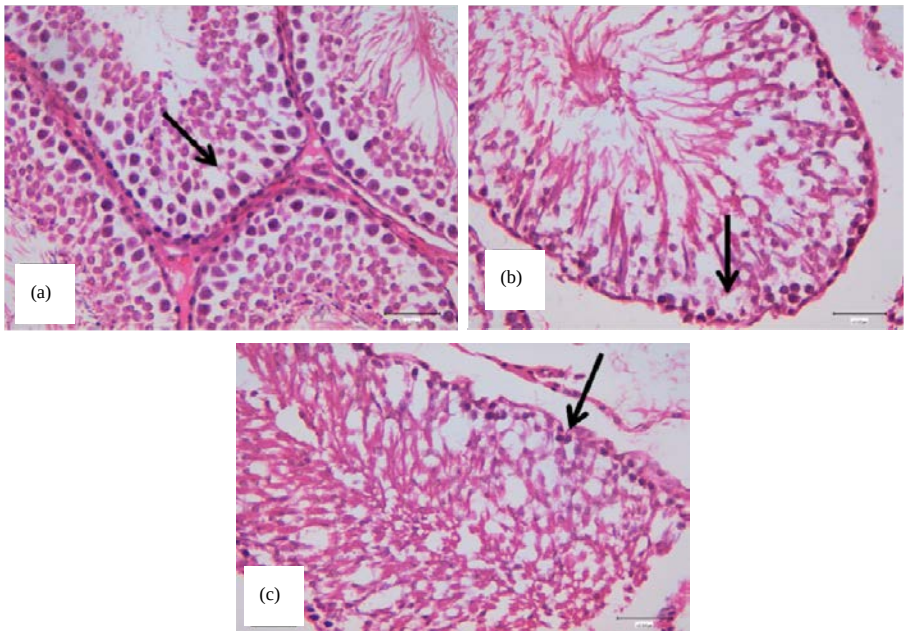

Fig. 1(a-c): Histopathological assessment of testicular damage in (a) Control testis showing normal seminiferous tubules with series of spermatogenic cells (arrow). H.E. Bar = 40.00 μm, (b) Seminiferous tubules of testis in AFB1 treated rats showing reduced numbers of spermatogenic cells (arrow), H.E. bar = 40.00 μm and (c) Seminiferous tubules of testis in aflatoxin treated rats showing buckling of the basement membranes (arrow), H.E. bar = 40.00 μm

RESULTS

Histopathological assessment of testicular damage: Testis of the control rats showed normal seminiferous tubules with normal series of spermatogenic cells associated with sertoli cells (Fig. 1a). Testis of rats intoxicated with AFB1 revealed mild testicular degeneration of some seminiferous tubules manifested by reduced numbers of spermatogenic cells (Fig. 1b). This was associated with buckling of the seminiferous tubules basement membranes (Fig. 1c).

Effect of treatments on testicular lipid peroxidation: The results in Table 2 show that the TBARS level was significantly ($p<0.05$) increased (26.1±2.02 μM) in the liver of AFB1-intoxicated rats compared to the control (14.±2.01 μM).

Effect of treatments on GSH and testicular antioxidant enzyme activities: The GSH concentration and antioxidant enzyme activities (CAT, total SOD, GPX and GST) are shown in Table 2. The concentration of GSH was significantly ($p<0.05$) reduced (2.0±0.20 μM) in AFB1-intoxicated rats compared to the control (4.1±0.20 μM). The activities of CAT (15.0±2.00 nmol min^{-1} g^{-1}), Total SOD (1.1±0.04 U g^{-1} tissue), GPX (95.0±2.30 nmol min^{-1} g^{-1} tissue), GST (61.4±3.22 nmol min^{-1} g^{-1}) were significantly ($p<0.05$) reduced in AFB1-intoxicated rats compared to the control (20.6±2.10, 3.3±0.03, 118.1±3.10 and 93.9±2.66), respectively.

Table 2: Effect of AFB1 for five weeks on levels of TBARS (μM) and GSH (μM) and activities of CAT (nmol min^{-1} g^{-1} tissue), total SOD (U g^{-1} tissue), GPX (nmol min^{-1} g^{-1} tissue) and GST (nmol min^{-1} g^{-1} tissue) in rats testicular tissues

	Experimental groups	
Parameters	Control	AFB1
TBARS	14.7±2.01	26.1±2.02*
CAT	20.6±2.10	15.0±2.00*
Total SOD	3.3±0.03	1.1±0.04*
GPX	118.1±3.10	95.0±2.30*
GST	93.9±2.66	61.4±3.22*
GSH	4.1±0.20	2.0±0.20*

Values are expressed as Mean±SEM, n = 7 for each group, Significance was calculated at $p<0.05$, TBARS: Thiobarbituric acid reactive substances, CAT: Catalase, SOD: Superoxide dismutase, GPX: Glutathione peroxidase, GST: Glutathione-s-transferase, GSH: Reduced glutathione and *Significant as compared to control animals

Effect of treatments on the gene expression of antioxidant enzymes: The mRNA expression of total SOD (Fig. 2a), CAT (Fig. 2b), GPX (Fig. 2c) and GST (Fig. 2d) was measured using

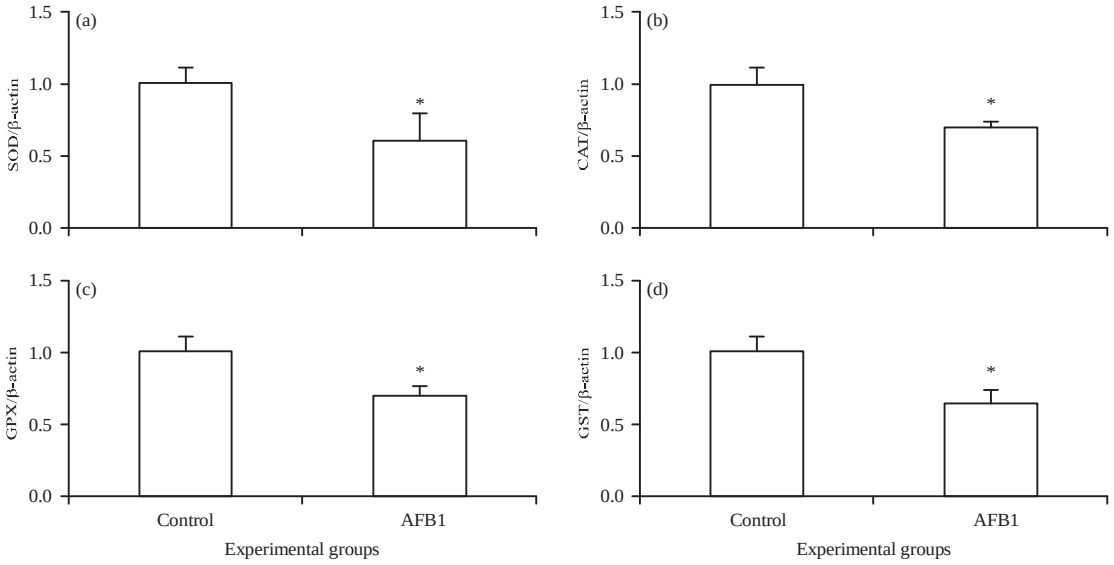

Fig. 2(a-d): Real time RT-PCR analysis of (a) SOD, (b) CAT, (c) GPX and (d) GST in testicular tissues of control and aflatoxin B1 (AFB1) treated rats. Values are expressed as Mean±SEM, *Values are significantly different ($p<0.5$) compared to control

real-time RT-PCR. These results showed that the expression of all of the antioxidant enzymes was significantly down-regulated ($p<0.05$) in AFB1-intoxicated rats compared to the control.

DISCUSSION

The histopathological findings reported in the current study come in concurrent with current biochemical results and indicated degenerative changes in the testis of rats intoxicated with AFB1. Similar histopathological changes of aflatoxin-induced testicular damage have been previously reported in rats (Mathuria and Verma, 2007; Abu El-Saad and Mahmoud, 2009; Tas *et al*., 2010; Ismail 2012; Murad *et al*., 2015) and mice (Faridha *et al*., 2006). The significant increase in TBARS in the testis of AFB1-intoxicated rats indicated oxidative stress to these tissues. Similar results (Abu El-Saad and Mahmoud, 2009; Tas *et al*., 2010) have been obtained in the testis of rats intoxicated with AFB1. The significant increase in testicular TBARS in AFB1-intoxicated rats was potentially attributed to the action of cytochrome P450 enzyme on AFB1 giving rise AFB1-8, 9-epoxide reactive intermediate which induced lipid peroxidation of biomolecules and cellular damage (Stresser *et al*., 1994). As a result of lipid peroxidation, GSH content and the activities of antioxidant enzymes (total SOD, GPX, CAT and GST) have been decreased in testis of rats of the current study to attenuate the deleterious lipid peroxidation effect of AFB1. The GSH maintains the normal structure and function of the cells via a redox and detoxification reaction. In the current work, a significant reduction in GSH concentration was observed in AFB1-intoxicated rats. The evacuation of testicular GSH in examined tissues perhaps attributed to the conjugation of GSH with electrophilic metabolites of AFB1, a reaction that is catalysed by GST. Similar results were reported in rats (Abu El-Saad and Mahmoud, 2009) and mice (Verma and Nair, 2001) intoxicated with AFB1. The imbalance between pro-oxidants and antioxidants in biological systems defined as oxidative stress. Thus, the significant increase in lipid peroxidation could be due to a significant reduction in the activities of enzymatic antioxidants, such as CAT, total SOD, GPX and GST as well as non-enzymatic antioxidants concentration such as GSH, in the testis of AFB1-intoxicated rats compared to the control. The SOD converts superoxide radicals into H_2O_2 which is converted to oxygen and water by CAT or GPX. Therefore, SOD, CAT, GPX and GST constitute essential components of the antioxidant system, and their deficiencies result in oxidative stress (Ahmad *et al*., 2012). Furtherly, the significant reduction of the activities of these enzymes in the testicular tissue of AFB1-intoxicated rats as presented in Table 2 may responsible for the observed high lipid peroxidation and low GSH concentration in these animals compare to control. Significant reductions in GPX, SOD (Abdel-Wahhab *et al*., 2007) and CAT (Verma and Nair, 2001; Abu El-Saad and Mahmoud, 2009) have been reported in the testis of AFB1 intoxicated rats. It has been documented that,

the activities of GST in mammalian testis is very high to facilitate the inhibition of DNA damage of spermatogenic cells (Sahu *et al*., 2000). However in the current study, the activity of this enzyme has been reduced significantly in the testicular tissues of AFB1 intoxicated rats compare to control. This perhaps indicated a consumption of enzyme in the conjugation process of GSH with AFB1 epioxide to attenuate the induced AFB1 toxicity. Conversely, intermittent exposure of AFB1 increased the activity of GST in liver and tissues of rats in dose and time dependant manner (Sahu *et al*., 2000). At the gene expression level, the current findings indicated that, AFB1 induced a down-regulation of all antioxidant enzymes gene expression in AFB1-intoxicated rats. Gene expression studies of antioxidant enzymes in testis of rats intoxicated with AFB1 are lack. However, down regulation of gene expression of SOD, CAT, GPX and GST has been documented in liver of rats intoxicated with the same dose of AFB1 (El-Bahr, 2015). In contrast to the current findings, Yarru *et al.* (2009) demonstrated that the expression of GPX and CAT genes was not significantly decreased in birds fed with AFB1. The conjugation between reactive xenobiotic metabolites and GSH is an important step in the detoxification mechanism. The GST is responsible for this conjugation. An overload of xenobiotics (AFB1) depleted the GSH via the conjugation process (Table 2). Down-regulation of GST gene expression in AFB1-intoxicated rats compared to the control as demonstrated in the current study (Fig. 2c) could limit the ability of testicular tissues to conjugate the reactive metabolites.

CONCLUSION

The present study concluded that AFB1-induced testicular damage in rats. Testicular injury, high lipid peroxidation; low GSH concentration and inactivation and down-regulation of gene expression of antioxidant enzymes are involved during AFB1 toxicity in rats.

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