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Renin mRNA is upregulated in testes and testicular cells in response to treatment with aflatoxin B_1

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Abstract

Aflatoxin B_1 (AFB_1) has been shown to affect fertility in many species; however, the exact molecular mechanisms associated with the disruption are not known. Our objectives were to determine changes in testicular gene expression due to exposure to AFB_1 and to investigate which cell types were affected by treatment with AFB_1. Male mice 4 wk of age were administered a daily placebo (control; N = 9) or 50 μg/kg AFB_1 (AFB_1 treated; N = 10) daily for 45 days. Males were then mated to four females each for 8 days. Male mice were characterized as being "Tolerant" (N = 3) or "Intolerant" (N = 3) to the effects of AFB_1 based on positive terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in the testes and the number of pups sired. Tolerant males produced a similar average number of fetuses (mean ± SEM) (12.5 ± 1.2) per male as selected control males (13.4 ± 1.2), but more fetuses (P = 0.01) than Intolerant males (7.6 ± 1.2). The number of terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cells in Intolerant males tended to be (P = 0.10) greater (136.5 ± 27.2) than in Tolerant (55.0 ± 22.2) and selected control (54.3 ± 22.2) males. Affymetrix microarray (Sunnyvale, CA, USA) analysis revealed differential expression (P < 0.05) of 193 extra cellular space and signaling genes, 49 signal transduction genes, 45 immune regulation genes, and 230 cell differentiation genes in the testis. *Renin* was commonly represented amongst many clusters and was chosen for further analyses. Upregulation (P < 0.001) of *Renin* in Tolerant mice (N = 3) compared with Intolerant mice (N = 3) was confirmed by real-time reverse transcription polymerase chain reaction (RT-PCR) (P = 0.05). This upregulation (P = 0.01) was also observed in representative AFB_1 treated males (N = 8) compared with control males (N = 8) selected for real-time reverse transcription polymerase chain reaction analysis. Spermatogonia cultured in vitro and treated with 0, 5, 10, or 20 μg/mL AFB_1 (N = 6 per treatment) resulted in a 10-fold upregulation (P = 0.01) of *Renin* message at the 20 μg/mL level, whereas Leydig tumor cells showed similar differences (P = 0.03) in message for *Renin* in treated (10 and 20 μg/mL) versus control cell cultures. Based on these results, we inferred a role for *Renin* at the molecular level in the response to the adverse effects of AFB_1 in male mice.

Keywords: Aflatoxin; Leydig cell; Ras; *Renin*; Spermatogonia; Testes

1. Introduction

Aflatoxin B_1 (AFB_1) is highly mutagenic, hepatotoxic, teratogenic, and carcinogenic in a variety of species, including livestock and humans [1–7]. Aflatoxin is produced from the molds *Aspergillus flavus* and *Aspergillus parasiticus* and is commonly found in corn, wheat, barley, oats, rice, nuts, copra, cottonseed, and pepper. There are four major forms of aflatoxins characterized by their fluorescence, which is either blue or green, giving rise to the designations B_1, B_2, G_1, and G_2. The most potent of the aflatoxins, AFB_1, causes reproductive complications in numerous species, including

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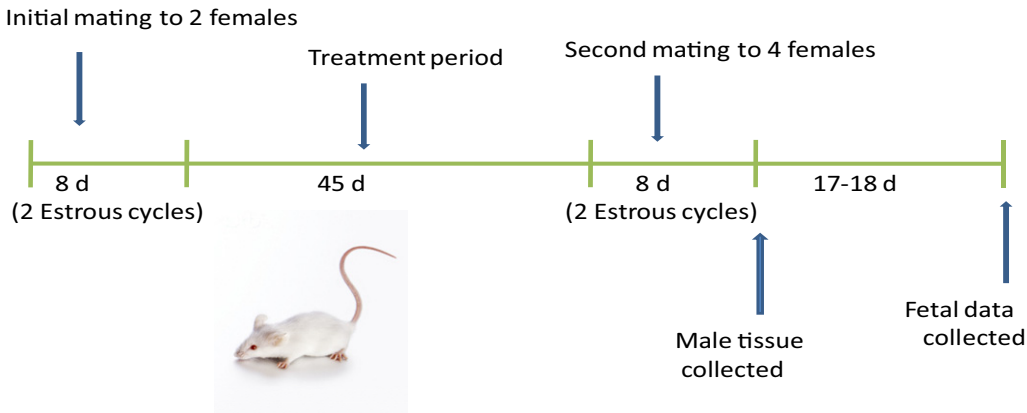

Fig. 1. Timeline showing initial mating of male mice with fertile females, treatment with aflatoxin B_1 (AFB_1), and mating with new females, resulting in tissue collection from males and fetal data collection from females.

mice, rats, pigs, poultry, sheep, and cattle [6–9]. Aflatoxicosis in humans occurs through inhalation of contaminated feedstuffs in the workplace, leading to respiratory tumors, colon, and liver disease [4,10,11]. Aflatoxicosis in men has been associated with decreased sperm counts and increased sperm abnormalities [3,12]. In mice, exposure to AFB_1 caused histological changes in the testes, including germ cell loss, hypertrophied and vacuolated Leydig cells, and sperm abnormalities (i.e., two heads). Decreased sperm counts, sperm motility, and litter size after mating were also reported [8,12]. Although there has been much research, little is known regarding the molecular mechanisms associated with decreased fertility in AFB_1-exposed males. In addition, there exists a known phenomenon of tolerance to aflatoxins which is highly variable among subjects [13]. The research described herein aimed to: (1) examine molecular mechanisms associated with the disruption in male fertility caused by administration of AFB_1; (2) confirm gene expression differences associated with tolerance and intolerance to AFB_1; and (3) delineate which cell types in the testes are most affected by treatment with AFB_1 in culture.

2. Materials and methods

2.1. Mice and tissue collection

All mice experiments were approved by the University of Wyoming Institutional Animal Care and Use Committee. Male ICR mice (Harlan Laboratories, Indianapolis, IN, USA) 4 wk of age (N = 32) were initially mated with two females each for a period of two estrous cycles (mean estrous cycle of 4 to 5 days) to confirm that they were fertile at the onset of these experiments. Males (N = 19) that successfully mated at least one female were considered fertile. After fertility was established, male mice (N = 19) were treated with AFB_1 (50 μg/kg in 80% corn oil/20% ethanol; N = 10; "AFB_1 treated") daily for 45 days or placebo (80% corn oil/20% ethanol; N = 9; "Control") via intraperitoneal injection for 45 days (Fig. 1). Mice were weighed weekly and the amount of AFB_1 adjusted accordingly to ensure a constant dosage on a body weight basis. Control mice received the same volume of placebo as that of treated mice with equivalent weights. Male mice were killed after pairing with four new multiparous females for two estrus cycles. One testis from each male was snap-frozen for RNA extraction, whereas the other testis was preserved in 4% paraformaldehyde and paraffin-embedded for morphological analysis. Females were killed on Day 17 of gestation; fetuses were counted and examined for abnormalities.

2.2. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Numbers of cells undergoing apoptosis were determined by TUNEL assay of testes using the colorimetric assay (Promega Corp., Madison, WI, USA). Preserved paraffin-embedded testes were sectioned at 6 μm, mounted on slides, and air dried for 2 days. Sections were then deparaffinized in xylene (3 times for 5 min) and rehydrated through graded ethanol washes followed by a final wash in PBS. Sections were fixed in 4% paraformaldehyde for 15 min at room temperature and then washed twice in PBS. Tissue sections were incubated in 20 μg/mL proteinase K solution for 20 min at room temperature. Slides were washed once with PBS and then fixed again with 4% paraformalde-