Aflatoxin compromises development of the preimplantation bovine embryo through mechanisms independent of reactive oxygen production

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ABSTRACT

Aflatoxin is a potent carcinogen often found in animal feedstuffs. Although it reportedly impairs development of the preimplantation pig embryo, it is not known whether it adversely affects development of the preimplantation bovine embryo. We conducted 3 experiments to investigate this possibility and determine whether deleterious effects of aflatoxin were caused by increased production of reactive oxygen species (ROS). Experiments were conducted with embryos produced in vitro and cultured after fertilization with various concentrations of aflatoxin. For experiment 1, embryos were treated with 0 (control), 40, 400, or 4,000 $\mu\text{g/L}$ of aflatoxin B₁ (AFB₁). Treatment at all concentrations of AFB₁ tended to reduce cleavage rate, with the 2 highest concentrations having significant effects. As compared with the control, 40 $\mu\text{g/L}$ AFB₁ reduced the percentage of oocytes becoming blastocysts and the percentage of cleaved embryos becoming blastocysts (19.7 vs. 8.1% and 30.3 vs. 14.3%, respectively). Complete inhibition of blastocyst formation occurred at concentrations of 400 and 4,000 $\mu\text{g/L}$ of AFB₁. Experiments 2 and 3 involved a 2 $\times$ 2 factorial design with effects of AFB₁ (0 and 40 $\mu\text{g/L}$), the antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble analog of vitamin E; 0 and 5 μM), and their interaction on production of ROS in putative zygotes (experiment 2) and development to the blastocyst stage (experiment 3). Production of ROS was increased by AFB₁, and this effect was reversed by Trolox. However, Trolox did not prevent the reduction in development to the blastocyst stage caused by AFB₁. Thus, the anti-developmental effects of AFB₁ are not caused solely by increased ROS production. Rather, other underlying mechanisms exist for the adverse effects of aflatoxin

on embryonic development. Overall, results indicate the potential for feeding aflatoxin-contaminated feed to cause embryonic loss in cattle.

Key words: aflatoxin, antioxidant, reactive oxygen species, embryonic development

INTRODUCTION

Aflatoxins, a group of secondary metabolites mainly produced by the fungal species *Aspergillus flavus* and *Aspergillus parasiticus*, are one of the most carcinogenic substances identified (Frisvad et al., 2005). An intercontinental survey reported that 33% of feedstuff samples were contaminated with aflatoxin, with up to 2,454 $\mu\text{g/kg}$ detected in finished feeds (Rodrigues and Naehrer, 2012). A survey conducted in Tunisia showed that 55% of plasma samples from lactating cows contained aflatoxin B₁ (AFB₁; Abbès et al., 2012). Among other effects, consumption of feedstuffs contaminated with aflatoxins can reduce milk production in dairy cows, decrease milk quality and safety, reduce feed efficiency in beef cattle, and compromise immune and ruminal functions (Ogunade et al., 2018). In addition, AFB₁ can reduce viability and DNA integrity of bull sperm (Komsky-Elbaz et al., 2018), disrupt maturation of the porcine oocyte (Liu et al., 2015), and cause DNA damage to the porcine preimplantation embryo (Shin et al., 2018). However, it is not known whether aflatoxin can compromise female fertility of dairy cows.

Our first objective in the current study was to examine whether development of the preimplantation embryo would be compromised by exposure to AFB₁. We hypothesized that AFB₁ would compromise preimplantation development at concentrations similar to those found in the tissues of animals consuming contaminated feed. Aflatoxin B₁ was tested rather than other aflatoxin metabolites such as aflatoxin M₁ (AFM₁) because AFB₁ is one of the most prevalent aflatoxins present in animal feeds (Han et al., 2013) and data on concentrations of AFM₁ in blood and tissue are limited. The lowest concentration of AFB₁ tested (40 $\mu\text{g/L}$) was chosen based on previous studies that re-

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ported a wide range of aflatoxin concentrations present in plasma and tissues of cattle, with up to 56.6 µg/kg being detected (Stubblefield et al., 1983). Our second objective was to determine the role of reactive oxygen species (**ROS**) in the deleterious actions of AFB₁. We hypothesized that adverse effects of AFB₁ involved ROS because aflatoxins can increase ROS production by cells (Shen et al., 1996; Mehrzad et al., 2011; Liu and Wang, 2016; Shin et al., 2018) and because exposure to conditions that increase oxidative stress can compromise the ability of the preimplantation bovine embryo to develop to the blastocyst stage (Moss et al., 2009; Takahashi et al., 2016). To test the hypothesis, we measured ROS production by embryos treated with AFB₁. We also evaluated whether inhibition of ROS with Trolox, a water-soluble version of vitamin E that can react with free radicals and prevent oxidative damage (Nimse and Pal, 2015), could block the deleterious effects of AFB₁ on development. Previously, Trolox was found to prevent overproduction of ROS and the anti-developmental effects of menadione on preimplantation bovine embryos (Moss et al., 2009).

MATERIALS AND METHODS

In Vitro Production of Embryos

Bovine ovaries were collected from a local abattoir and transported in 0.9% (wt/vol) NaCl (saline) to the laboratory at 23°C to arrive within 8 h. Ovaries with greater than 10 follicles on the surface were selected for harvesting of cumulus-oocyte complexes. The ovaries were washed 3 times with saline and, after the follicular fluid from large (>10 mm diameter) follicles was discarded, the surface of the ovary was sliced with a scalpel and the ovary vigorously swirled in a beaker containing 100 mL oocyte washing medium (MOFA Global, Verona, WI). The mixture was then filtered through a 100-µm Falcon cell strainer (Fisher Scientific, Hampton, NH). Subsequently, the material retained on the strainer was washed 3 times with 20 mL of oocyte washing medium using a 10-mL syringe connected to a needle. The cellular material was washed from the strainer into a 100-mm grid plate, and cumulus-oocyte complexes were harvested using a Wiretrol pipette (Drummond Scientific, Broomall, PA) and a dissecting microscope.

Oocytes with ≥3 layers of cumulus cells were selected and cultured in groups of 10 in 50-µL microdrops of in vitro maturation medium (IVF Bioscience, Falmouth, UK) covered with mineral oil (Sigma-Aldrich, St. Louis, MO). Maturation of oocytes proceeded for

22 h at 38.5°C in an atmosphere of 5% (vol/vol) CO₂ in humidified air. Matured oocytes were washed 3 times with HEPES-TALP solution (Tyrode's albumin lactate pyruvate; Ortega et al., 2016) and transferred to a fertilization dish containing 1.7 mL of IVF-TALP fertilization medium (Ortega et al., 2016). Sperm were prepared using a gradient of Puresperm (Nidacon, Gothenburg, Sweden) from 3 straws of frozen semen, as described by Ortega et al. (2016). For each fertilization procedure, semen was used from 3 bulls; a different set of bulls was used for each replicate. After purification, sperm were counted under a microscope and diluted with IVF-TALP medium; 120 µL was transferred to the fertilization plate to achieve final concentration of 1 × 10⁶/mL. Eighty microliters of penicillamine-hypotaurine-epinephrine solution (Ortega et al., 2016) was added to the plate to improve fertilization. Subsequently, the fertilization plate was placed in an incubator set at 38.5°C in a humidified atmosphere of 5% (vol/vol) CO₂ for 15 h.

After 15 h of fertilization, up to 300 putative zygotes per group were transferred to a microcentrifuge tube containing 600 µL of HEPES-TALP and 10,000 U/mL of hyaluronidase. Subsequently, putative zygotes (i.e., oocytes exposed to spermatozoa) were denuded by vortexing the tube for 5 min to remove cumulus cells. Denuded zygotes were then transferred in groups of 25 to 30 to a 45-µL drop of synthetic oviduct fluid-bovine embryo 2 (**SOF-BE2**) covered in mineral oil (Ortega et al., 2016). Treatments were added to drops in a volume of 5 µL at 10 times the final concentration. For experiment 1, treatments (final concentration) were vehicle control (i.e., 0 µg/L AFB₁) and 40, 400, and 4,000 µg/L AFB₁. For experiments 2 and 3, treatments were arranged in a 2 × 2 factorial design with main effects of 0 or 40 µg/L AFB₁ and 0 or 5 µM Trolox (all final concentrations). Embryos were cultured for up to 7.5 d at 38.5°C in a humidified atmosphere of 5% (vol/vol) O₂, 5% (vol/vol) CO₂, and the balance N₂ in benchtop incubators (WTA, Carvinhos, SP, Brazil).

Preparation of AFB₁

Aflatoxin B₁ in powder form (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (**DMSO**; Sigma-Aldrich) to make 5 mg/mL stock solutions that were stored at −20°C. For each replicate, the treatment solutions were prepared fresh from stock solutions on the day of use by diluting the stock with SOF-BE2 culture medium. The concentration of DMSO was adjusted for each treatment so that the final concentration in the culture drop was the same for all treatments including the control

(0.08% vol/vol for experiment 1 and 0.1% vol/vol for experiments 2 and 3).

Experiment 1: Concentration-Dependent Actions of AFB₁ on Development to the Blastocyst Stage

After fertilization, putative zygotes were transferred to 45- μ L culture drops covered with mineral oil in groups of 30, and 5 μ L of the respective treatment was added to achieve the desired concentration of AFB₁. Treatments were vehicle control and 40, 400, and 4,000 μ g/L AFB₁. Embryos were cultured until d 7.5 after fertilization. Cleavage rate and the proportion of putative zygotes that developed to the blastocyst stage were evaluated at d 3.5 and 7.5 after fertilization, respectively, using a stereomicroscope. In total, 4 replicates were performed. The total numbers of putative zygotes used were 96, 97, 95, and 95, for control and 40, 400, and 4,000 μ g/L AFB₁, respectively.

Experiment 2: Induction of ROS by AFB₁

Putative zygotes were treated after fertilization when placed in SOF-BE2. Treatments were arranged in a 2×2 factorial design with main effects of AFB₁ concentration (0 and 40 μ g/L) and Trolox (0 and 5 μ M). The concentration of Trolox was chosen because a previous study found that a 5 μ M solution reduced production of ROS by preimplantation bovine embryos (Moss et al., 2009). In addition to the 4 treatments, a fifth group of embryos cultured with 5 μ M menadione was included as a positive control. The concentration of menadione used was reported to increase ROS production in bovine embryos (Moss et al., 2009). To measure acute production of ROS by embryos after exposure to AFB₁, putative zygotes were cultured in SOF-BE2 for 3 h at 38.5°C in a humidified atmosphere of 5% (vol/vol) O₂, 5% (vol/vol) CO₂, and the balance nitrogen. After incubation, ROS production was determined as described by Ortega et al. (2016). CellRox reagent (ThermoFisher, Waltham, MA) was added to drops of putative zygotes to a final concentration of 5 μ M. Putative zygotes were then cultured for an additional 30 min at 38.5°C as before. Embryos were then washed 3 times in 50- μ L drops of Dulbecco's PBS containing 2% (wt/vol) polyvinylpyrrolidone, placed on microscope slides in groups of up to 10 embryos, and covered with coverslips using Prolong Gold antifade reagent (Invitrogen, Carlsbad, CA). Subsequently, embryos were observed by using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany) with a 490/525-nm excitation/emission filter to visualize fluorescence and by

digital interference contrast microscopy to visualize the structure of the embryo. Digital images of each embryo were captured using AxioVision software (version 4.8.2, Zeiss, Peabody, MA) at a magnification of 40 $\times$. Quantification of fluorescent intensity was determined by manually tracing the perimeter of the embryo on the computer screen and using the software to calculate average pixel intensity within the boundaries of the embryo. In total, 2 replicates were performed. The total number of putative zygotes per treatment ranged from 13 to 19. In addition, 12 putative zygotes were treated with menadione.

Experiment 3: Effectiveness of Trolox in Alleviating Developmental Impacts of AFB₁

The experiment used a 2×2 factorial design with AFB₁ concentration (0 and 40 μ g/L) and Trolox concentration (0 and 5 μ M) as main effects. After fertilization, putative zygotes were placed in SOF-BE2 containing appropriate treatments and incubated at 38.5°C in a humidified atmosphere of 5% (vol/vol) O₂, 5% (vol/vol) CO₂, and the balance nitrogen for 7.5 d. Cleavage and the cell number of each embryo were estimated by microscopic evaluation at d 4 after fertilization, and blastocyst formation was determined at d 7. A total of 6 experimental replicates were performed. The total number of putative zygotes per treatment ranged from 160 to 195.

Statistical Analysis

The percentages of oocytes undergoing cleavage; cleaved embryos developing to the ≥ 3 -cell, ≥ 5 -cell, and ≥ 9 -cell stages; and oocytes and cleaved embryos becoming blastocysts were calculated for each replicate. Treatment effects were analyzed using the GLIMMIX procedure of SAS v 9.4 (SAS Institute, Cary, NC). For experiment 1, the statistical model included the fixed effect of treatment and random effect of replicate. Pre-planned orthogonal contrasts were conducted to compare the differences between various levels of treatment as follows: control vs. 40 μ g/L AFB₁, 400 vs. 4,000 μ g/L AFB₁, and low AFB₁ vs. high AFB₁ (control and 40 μ g/L AFB₁ vs. 400 and 4,000 μ g/L AFB₁). For experiments 2 and 3, the model included fixed effects of AFB₁ and Trolox and their interaction as well as the random effect of replicate. Data on ROS production in experiment 2 were also analyzed by GLIMMIX except that embryo was the experimental unit. In all cases, significance was declared at $P \leq 0.05$, while a tendency was regarded as $0.05 < P \leq 0.10$.

RESULTS

Experiment 1: Concentration-Dependent Actions of AFB₁ on Development to the Blastocyst Stage

Results are shown in Figure 1. Overall, the effect of treatment on cleavage tended to be significant ($P < 0.10$). Analysis using orthogonal contrasts revealed that

Figure 1. Effects of culturing putative zygotes after fertilization with various concentrations of aflatoxin B₁ (AFB₁) on cleavage (A) and development to the blastocyst stage (B, C) in experiment 1. Data are LSM ± SEM.

40 µg/L AFB₁ tended to reduce ($P = 0.09$) cleavage compared with the control and that 400 and 4,000 µg/L AFB₁ reduced cleavage compared with lower concentrations ($P = 0.02$). We observed no difference between 400 and 4,000 µg/L AFB₁. An overall effect of treatment was found for the percentage of putative zygotes and cleaved embryos that developed to the blastocyst stage ($P = 0.01$). Using orthogonal contrasts, 40 µg/L AFB₁ reduced the percentage of putative zygotes ($P = 0.07$) and percentage of cleaved embryos ($P = 0.03$) that became blastocysts. Similarly, blastocyst development, whether expressed as percentage of putative zygotes ($P = 0.01$) or cleaved embryos ($P = 0.03$), was lower for 400 and 4,000 µg/L AFB₁ than either control or 40 µg/L AFB₁. In fact, no embryos cultured in 400 or 4,000 µg/L AFB₁ became a blastocyst. As a result, no significant difference existed between 400 and 4,000 µg/L AFB₁.

Experiment 2: Induction of ROS by Aflatoxin

Representative images of ROS production are shown in Figure 2. As expected, the positive control menadione increased production of ROS. In addition, ROS production was increased by 40 µg/L aflatoxin. Treatment with Trolox alone did not affect ROS production, but Trolox reduced ROS production in embryos cultured with AFB₁.

Results of quantification of ROS production are presented in Figure 3. Production of ROS was affected by AFB₁ ($P = 0.004$) and the interaction between AFB₁ and Trolox ($P = 0.03$). These effects reflect increased ROS production caused by 40 µg/L AFB₁ and inhibition of this action of AFB₁ by Trolox.

Experiment 3: Effectiveness of Trolox in Alleviating Developmental Impacts of AFB₁

Treatment with 40 µg/L AFB₁ did not affect cleavage but reduced the percentage of putative zygotes and cleaved embryos becoming blastocysts ($P < 0.05$ for both variables) (Figure 4). The main effect of Trolox and the effect of the interaction between aflatoxin and Trolox were not significant ($P > 0.10$) for any variable.

To better understand the stage of development at which AFB₁ blocked development, we evaluated the percentage of cleaved embryos at d 4 that had advanced to the >3-cell stage, 5-cell stage, or 9-cell stage (Figure 5). The percentage of cleaved embryos that developed to ≥3- or ≥5-cell stages was not affected by AFB₁, Trolox, or their interaction. In contrast, the percentage of cleaved embryos that developed to ≥9-cell stage tended to be reduced ($P = 0.09$) by AFB₁.

Figure 2. Representative images of effects of aflatoxin B₁ (AFB₁) and 5 μ M Trolox on reactive oxygen species production by putative zygotes. Reactive oxygen species were detected using the fluorescent dye CellRox (ThermoFisher, Waltham, MA; experiment 2). The images were prepared by overlaying the image from the epifluorescence channel (490-nm excitation filter) on the image from the digital interference contrast channel.

DISCUSSION

Results of the present experiment indicate that AFB₁ can reduce competence of embryos to cleave and become blastocysts. Negative effects of AFB₁ were seen at concentrations as low as 40 μ g/L. Although higher concentrations of 400 and 4,000 μ g/L completely blocked development of embryo, these concentrations are not likely to occur in animals. An experiment in which levels of ROS were measured indicated that one cause for the reduction in development caused by AFB₁ is increased production of ROS. However, other mechanisms are also likely to be involved because treatment with the antioxidant Trolox, although reducing ROS production, did not reverse the negative effects of aflatoxin on embryonic development.

It is well known that excessive production of ROS can compromise development of the preimplantation embryo (Soto et al., 2003; Moss et al., 2009; Yazaki et al., 2013) and that AFB₁ can induce ROS production in several cell types (Shen et al., 1996; Mehrzad et al., 2011; Liu and Wang, 2016) including the pre-

implantation embryo (Shin et al., 2018). The fact that Trolox did not prevent the negative effect of AFB₁ on development to the blastocyst stage despite reducing ROS production indicates that AFB₁ causes more than one insult to the developing embryo. In addition, AFB₁ possibly interferes with embryonic genome activation, which occurs at the 8-cell stage in cattle (Graf et al., 2014). This possibility is surmised because AFB₁ did not affect the percentage of cleaved embryos developing to the ≥ 5 -cell stage but tended to reduce the number that became 9 cells or more. Moreover, exo- and endo-epoxides of AFB₁ can react with DNA and lead to DNA mutations (McCullough and Lloyd, 2019). In the pig embryo, exposure to AFB₁ increased DNA damage and decreased DNA repair (Shin et al., 2018).

An important question is whether embryos from cows fed aflatoxin-contaminated feeds are exposed to AFB₁ at concentrations high enough to compromise embryonic development. Concentrations of AFB₁ in uterine fluid have not been measured, and large differences in AFB₁ accumulation exist between tissues. For example, concentrations of AFB₁ in one cow fed aflatoxin-con-

Figure 3. Production of reactive oxygen species (CellRox net intensity; ThermoFisher, Waltham, MA) of putative zygotes as affected by incubation with 40 $\mu\text{g/L}$ aflatoxin B₁ (AFB₁) and 5 μM Trolox (experiment 2). Data are LSM $\pm$ SEM.

taminated feed ranged from 7.1 $\mu\text{g/kg}$ in liver, 2.0 $\mu\text{g/kg}$ in gallbladder, 1.3 $\mu\text{g/kg}$ in kidney, and 0.3 $\mu\text{g/kg}$ in skeletal muscle. Further, AFM₁, which is also a potent hepatotoxin and carcinogen (Green et al., 1982), ranged from 56.6 $\mu\text{g/kg}$ in the kidney, 6.1 $\mu\text{g/kg}$ in the liver, and 24.5 $\mu\text{g/kg}$ in the mammary gland (Stubblefield et al., 1983). Concentrations of AFB₁ in the blood of a steer treated with 0.8 mg AFB₁/kg of BW peaked at 21 $\mu\text{g/L}$ (Cook et al., 1986), whereas peak concentrations in the blood of cows fed aflatoxin-contaminated corn was 0.034 $\mu\text{g/L}$ (Gallo et al., 2008). A survey of lactating cows in Tunisia found that 84% of 58 feed samples and 55% of 56 plasma samples contained detectable concentrations of AFB₁ (Abbès et al., 2012). For the positive samples, the average concentrations of AFB₁ were 18.7 $\mu\text{g/kg}$ in feed and 7.0 $\mu\text{g/kg}$ in blood plasma. Only 10% of positive plasma samples had concentrations of AFB₁ > 10 $\mu\text{g/kg}$. Taken together, the evidence suggests that the lowest concentration of AFB₁ tested here, 40 $\mu\text{g/L}$, likely represents a concentration of AFB₁ that is higher than that typically experienced by cows fed aflatoxin-contaminated feed. However, embryos in vivo are likely to be exposed to a mixture of aflatoxin metabolites including AFB₁ and AFM₁. Additional experiments to

measure concentrations of AFB₁ and AFM₁ in uterine fluid of cows fed aflatoxin-contaminated feed can provide useful information for future experiments to evaluate direct effects of aflatoxins on embryonic development. It should be noted, however, that AFB₁ has been reported (Liu et al., 2015) to reduce development of pig embryos at a concentration of 1 nM (i.e., 0.3 $\mu\text{g/L}$), so even low amounts of aflatoxin contamination may lead to embryotoxicity.

In conclusion, our results indicate that AFB₁ can reduce competence of oocytes to cleave and become blastocysts. The mechanism of action of AFB₁ involves ROS formation as well as other effects because antioxidant treatment was not sufficient to block the antidevelopmental effects of aflatoxin. Future experiments should be performed to determine concentrations of AFB₁ in uterine fluid of cows fed aflatoxin-contaminated feeds to determine concentrations of AFB₁ that are physiologically relevant to the embryo.

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Figure 4. Effects of culturing putative zygotes after fertilization with 40 $\mu\text{g/L}$ aflatoxin B₁ (AFB₁) in the presence or absence of 5 μM Trolox on oocyte cleavage (A) and subsequent development to the blastocyst stage (B, C) in experiment 3. Data are LSM $\pm$ SEM.

Figure 5. Effects of culturing putative zygotes after fertilization with 40 $\mu\text{g/L}$ aflatoxin B₁ (AFB₁) in the presence or absence of 5 μM Trolox on the proportion of cleaved embryos reaching specific developmental stages at d 4 (experiment 3). Treatment with 40 $\mu\text{g/L}$ AFB₁ did not reduce the percentage of cleaved embryos developing to the 3-cell (A) or 5-cell (B) stage but reduced ($P = 0.09$) the percentage of cleaved embryos that developed to the 9-cell stage (C). Neither Trolox nor the interaction had a significant effect. Data are LSM $\pm$ SEM.

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