Effect of aflatoxin B1 on bovine spermatozoa's proteome and embryo's transcriptome

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Abstract

This study aims to evaluate the deleterious effect of the mycotoxin aflatoxin B1 (AFB1) on bull spermatozoa and the carryover effect on the developing embryo. Proteomic analysis of AFB1-treated spermatozoa revealed differential expression of proteins associated with biological processes and cellular pathways that involved in spermatozoon function, fertilization competence and embryonic development. Therefore, we assume that factors delivered by the spermatozoa, regardless of DNA fragmentation, are also involved. To confirm this hypothesis, we have used the annexin V (AV) kit to separate the spermatozoa into apoptotic (AV+) and non-apoptotic (AV-) subpopulations which were found to correlate with high- and low DNA fragmentation, respectively. Fertilization with AV+ AFB1-treated spermatozoa, resulted in no blastocyst formation, whereas fertilization with AV- spermatozoa resulted in reduced cleavage rate and formation of genetically altered blastocysts (*POU5F1* and *SOX2*). Microarray analysis of blastocysts derived from 10 µM AFB1-treated spermatozoa revealed differential expression of 345 genes that involved in cellular pathways such as embryo and placenta development, cell cycle, DNA repair and histone modification, and in signaling pathways, especially calcium signaling pathway. This is the first report on deleterious carryover effects of AFB1 from the bovine spermatozoa to the formed embryo. Our findings suggest that aside from the damage caused by AFB1 to spermatozoa's DNA integrity, additional damage mechanisms are involved.

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Introduction

There is growing concern about the effects of environmental toxins on human and domestic animal reproduction. Agricultural crops and food products can potentially be contaminated with mycotoxins produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus* (Dai *et al.* 2017). These fungi can be found on food grains, fruit, nuts and other crops that are improperly stored at high temperature and in high humidity (Zhao *et al.* 2017). Mycotoxin contamination of dairy feed is frequently reported for tropical zones, such as Sub-Saharan Africa (Kemboi *et al.* 2020). In these regions, mycotoxins contamination is extremely high (above 10 µM/3.12 ppm; Kemboi *et al.* 2020) and exceeded the European Union and the American Food and Drug Administration (FDA) allowed threshold levels (2 and 20 ppb, respectively (Klingelhoefer *et al.* 2018). According to the FDA, grains for animal feeding can have up to 0.3 ppm (1 µM) AFB1 (Williams *et al.* 2004). Nevertheless, chronic exposure to relatively low levels, that is, continuous consumption of contaminated food might also have deleterious effects. Aflatoxins are accumulated in edible animal tissues; therefore, can

pass through the food chain to the human costumers. In the body aflatoxins or their metabolites are transferred through the circulation to various physiological systems, including the reproductive tract (Gong *et al.* 2016). In spite of the tight regulation, mycotoxins can be found in the milk and eggs of farm animals fed with aflatoxin-contaminated crops (Yilmaz *et al.* 2017).

Aflatoxin B1 (AFB1) is considered the most toxic mycotoxin for mammals owing to its hepatotoxic, teratogenic, mutagenic and immunosuppressive properties (Macé *et al.* 1997, Meissonnier *et al.* 2008). AFB1 has a detrimental effect on human health and is categorized as a class 1 A human carcinogen (Zhao *et al.* 2017). A deleterious effect of AFB1 on gametes has been reported in male rats exposed to 8.8 µg/g (ppm) for 14 days (Ibeh *et al.* 2000). In vivo study reported that administration of male mice with 50 µg/kg (0.05 ppm) (AFB1 for 45 days, resulted in altered gene expression within the testes (Austin *et al.* 2012). In another study, oxidative stress-related pathways were activated in male mice testes following administration with 0.375–1.5 mg/kg (ppm) body weight AFB1 for 30 days (Huang *et al.* 2019). In vitro exposure to 50 µM AFB1 disrupted porcine oocyte maturation, and induced epigenetic

modifications, oxidative stress and apoptosis (Liu *et al.* 2015). Bovine preimplantation embryos underwent compromised development after *in vitro* exposure to 40 µg/L (0.04 ppm) AFB1 (Jiang *et al.* 2019). AFB1 at 1.660 µg/mL (ppm) was documented in subfertile male semen samples, and was associated with abnormal morphology, and reduced concentration and motility of spermatozoa (Ibeh *et al.* 1994, Uriah *et al.* 2001).

In our previous study (Komsky-Elbaz *et al.* 2018), we showed that exposure to 10 µM AFB1 during 4 h capacitation *in vitro* alters bovine spermatozoa's viability, mitochondrial membrane potential, and acrosome and DNA integrity. Consequently, the proportion of oocytes that were fertilized and cleaved to two- to four-cell-stage embryos was lower after fertilization with spermatozoa exposed to 10 µM AFB1 compared to non-treated spermatozoa. While blastocyst formation rate was not affected, the genetic profile of the formed embryos has not been evaluated. Given that the mechanism by which AFB1-induces damage in spermatozoa is not fully understood, we used a proteomics approach to further explore possible damage-mechanisms. In addition, to estimate a possible carryover effect from the treated spermatozoa to the developing embryo, we evaluated changes in the transcriptome profile of blastocyst stage embryos that derived from AFB1-treated spermatozoa. Graphical abstract summarizing the effect of exposure to AFB1 on spermatozoa and the further carryover effect on the developing embryo is presented in Fig. 1.

Materials and methods

Reagents and materials

All reagents were purchased from Sigma, unless otherwise specified. AFB1 (lot #1162-65-8, ≥98.9% purity) was purchased from Cayman Chemical. A 10 mM stock solution of AFB1 was prepared in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions.

All culture media were prepared in our laboratory, as previously described (Kalo & Roth 2017, Komsky-Elbaz *et al.* 2018). These including NKM buffer (110 mM NaCl, five mM KCl, 20 mM MOPS (3-N-morpholino propanesulfonic acid), pH 7.4), mTALP (modified Tyrode's solution), HEPES-Tyrode's lactate (TL), IVF-TL, oocyte maturation medium and embryo culture medium-potassium simplex optimized medium (KSOM).

Preparation and capacitation of spermatozoa

Bovine spermatozoa were supplied by 'Sion' Artificial Insemination Center (Hafetz-Haim, Israel), from three working bulls. All experiments were performed in accordance with the 1994 Israeli guidelines for animal welfare. The ejaculates were obtained with an artificial vagina, and the 'swim up' technique was applied to obtain motile sperm. The samples were washed three times by centrifugation (600 g for 10 min at 25°C) in NKM buffer and the spermatozoa were allowed to swim up. The washed cells were counted and maintained

at 39°C until use. Only semen that contained at least 80% motile spermatozoa were used in the experiments. *In vitro* capacitation of bovine spermatozoa was induced as described previously (Komsky-Elbaz *et al.* 2018).

AFB1 treatment and evaluation of spermatozoa

AFB1 was dissolved in DMSO (0.01%, v/v maximal concentration) according to the manufacturer's instructions (Cayman Chemicals). This vehicle was not found to have any deleterious effect on spermatozoa's viability at the final concentrations used in the current study (data not shown). Spermatozoa (10⁸ cell/mL) were incubated in mTALP for 4 h with or without 10 µM AFB1 at 39°C under an atmosphere of 5% CO₂ in air. The AFB1 concentration was chosen based on data from well-known experimental model studies (Adedara *et al.* 2014, Feng *et al.* 2016, Komsky-Elbaz *et al.* 2018). This concentration seems to mimic acute exposure, that is, high dose for short time rather chronic exposure, that is, long term exposure to low doses. It is mostly relevant for tropical zones and cases of feeding with improperly stored crops (Kemboi *et al.* 2020).

Separation of spermatozoa by magnetic-activated cell sorting

Samples were centrifuged at 300 g for 15 min at room temperature (RT). The resultant pellet was washed twice, 5 min each, first using NKM buffer and then 1× binding buffer (Miltenyi Biotec, Bergisch Gladbach, Germany). The pellet was removed and 10⁷ separated spermatozoa were incubated with 100 µL annexin V (AV)-conjugated microbeads (Miltenyi Biotec, Germany) for 15 min at RT. The mixture of spermatozoa and microbeads was loaded into a separation column (MS columns, Miltenyi Biotec), which was placed in a magnetic field (MiniMACS; Miltenyi Biotec). The non-apoptotic, unlabeled spermatozoa (i.e. AV−) passed through the column and were collected in wash media, to perform a final washing. The apoptotic, magnetically labeled spermatozoa (i.e. AV+) were retained in the separation column and were eluted with 1 mL binding buffer after removing the MS column from the separator. The separated AV− and AV+ spermatozoa from each experimental group (control and AFB1) was further used in the *in vitro* fertilization procedure.

Assay for spermatozoa's chromatin structure

The spermatozoa's nuclear integrity was assessed by chromatin structure assay as described previously (Evenson & Jost 2000). Briefly, a sample containing 1 × 10⁶ spermatozoa was diluted in 147 µL Tris/NaCl/EDTA buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) and incubated with 300 µL detergent acid solution (0.17%, w/v Triton X-100, 0.15 M NaCl, 0.08 N HCl, pH 1.2) for 30 s at RT. Then 900 µL of Acridine orange solution at a final concentration of six µg/mL (0.15 M NaCl, one mM EDTA, 0.1 M citric acid, 0.2 M Na₂HPO₄, pH 6.0) was added. The signal from 5000 events was read in a Guava EasyCyte microcapillary flow cytometer with CytoSoft software after 2.5 min at RT. DNA compaction level was evaluated

Figure 1 Graphical abstract summarizing the effect of exposure to AFB1 on spermatozoa and the further carryover effect on the developing embryo. Created with BioRender.com.

and expressed as DNA fragmentation index (DFI), that is, the percentage of spermatozoa with fragmented DNA.

Analysis of spermatozoa's proteome

Sample preparation

Spermatozoa (10^8 cell/mL) were incubated in mTALP for 4 h with or without 10 μ M AFB1 at 39°C under an atmosphere of 5% CO₂ in air. For the proteomic analysis, pellets of spermatozoa samples were collected after 0 and 4 h of capacitation, and after an additional 20-min incubation with 20 μ M Ca⁺⁺ ionophore A23187, in order to evaluate induced acrosome reaction. The pellets stored at -80°C until use.

Frozen bovine spermatozoa (50 μ L) were lysed by adding 100 μ L lysis solution (8 M urea, 4%, w/v sodium dodecyl sulfate (SDS), 10 mM dithiothreitol, 25 mM Tris-HCl pH 8.0). The samples were sonicated for 15 min in a bath sonicator and centrifuged at 15,000 g for 10 min. The clear supernatant (80 μ L) was alkylated by adding 10 μ L of 0.55 M iodoacetamide and incubating for 30 min at RT in the dark. Sample cleanup and digestion with sequencing-grade modified trypsin (Promega) were performed using the S-Trap microspin column kit as specified by the manufacturer (Protifi, LLC, Huntington, NY, USA). The tryptic peptides were desalted on C18 spin tips (Rappsilber *et al.* 2007). A total of 0.8 μ g peptides (determined by absorbance at 280 nm) from each sample was injected into the mass spectrometer.

LC-MS/MS analysis

MS analysis was performed in a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) coupled online to a nanoflow UHPLC instrument (Ultimate 3000 Dionex, Thermo Fisher Scientific). Eluted peptides were separated over a 150-min gradient run at a flow rate of 0.15 $\mu\text{L}/\text{min}$ (during the separation phase) on a reverse-phase 25-cm, C18 column (75 μm ID, 2 μm , 100 $\text{\AA}$, Thermo PepMap®RSLC from Thermo Scientific). The survey scans (380–2000 m/z , target value 3E6 charges, maximum ion injection time 50 ms) were acquired and followed by higher-energy collisional dissociation-based fragmentation (normalized collision energy set at 25). A resolution of 70,000 was used for survey scans and up to 15 dynamically chosen most abundant precursor ions were fragmented (isolation window 1.8 m/z). The MS/MS scans were acquired at a resolution of 17,500 (target value 5E4 charges, maximum ion injection time 57 ms). Dynamic exclusion was 60 s.

Analysis of MS data

MS data were processed using the MaxQuant computational platform, version 1.6.6.0 (Cox & Mann 2008). Peak lists were searched against the bovine Uniprot FASTA sequence database containing a total of 8654 reviewed entries. The search included cysteine carbamidomethylation as a fixed modification and oxidation of methionine as a variable one. Peptides that were at least seven amino acids in length were considered, and the required false discovery rate (FDR) was set to 1% at the peptide and protein levels. Protein identification required at least two unique or razor peptides per protein group. Relative protein quantification in MaxQuant was performed using the label-free quantification algorithm (Cox *et al.* 2014). This algorithm uses only common peptides for pairwise ratio determination for each protein and calculates a median ratio to protect against outliers. It then determines all pairwise protein ratios and requires a minimal number of two peptide ratios for a given protein ratio to be considered valid. Statistical analysis (four replicates per experimental group) was performed using the Perseus statistical package (Tyanova *et al.* 2016). Only sample groups with at least two valid values were used.

Immunoblot analysis

Spermatozoa were washed by centrifugation for 10 min at 10,000 g at 4°C and the pellet washed twice with ice cold PBS and centrifuged again to remove any remaining traces of BSA. Lysates were prepared by the addition of RIPA buffer supplemented with protease inhibitor cocktail 1:100 to the pellet, and kept on ice for 15 min. Lysates were then centrifuged for 5 min at 10,000 g , 4°C, and the supernatant was transferred to a new tube. Laemmli sample buffer (5 $\times$) was added to the supernatant, and the sample was boiled for 5 min. The extracts were separated on a 10% SDS–polyacrylamide gel and then electrophoretically transferred to nitrocellulose membranes. The blots were blocked with 1% BSA in Tris-buffered saline (TBS), pH 7.6, containing 0.1% (w/v) Tween 20 (TBST), for 30 min at RT. The membranes were incubated overnight at 4°C with primary antibodies diluted in 1% BSA

in TBST. Next, the membranes were washed three times with TBST and incubated for 1 h at RT with specific horseradish peroxidase-linked secondary antibody (Aviva Systems Biology, San Diego, CA, USA), diluted 1:5000 in TBST, and 1% BSA. The membranes were washed three times with TBST and visualized by chemiluminescent substrate for western blotting (Cyanagen, Bologna, Italy).

In vitro embryo production

Oocytes were matured in oocyte maturation medium as previously established in our laboratory (Kalo & Roth 2017). Briefly, cumulus oocyte complexes (COCs) were aspirated from 3- to 8-mm follicles. COCs were collected into HEPES–TL supplemented with 0.3% BSA, 0.2 mM sodium pyruvate and 0.75 mg/mL gentamicin (HEPES–TALP) at 38.5°C. At the end of the collection, COCs ($n=30$ –60 per group; six replicates) were *in vitro* matured in humidified air with 5% CO_2 for 22 h at 38.5°C. Then, COCs were transferred in groups of 30 oocytes to four-well plates containing 600 μL IVF–TL supplemented with 0.6% (w/v) essential fatty acid-free BSA, 0.2 mM sodium pyruvate, 0.05 mg/mL gentamicin and 0.01 mg/mL heparin (IVF–TALP). In addition, 25 μL PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% NaCl) was added per well. For IVF, the separated AV– and AV+ spermatozoa collected from the control or AFB1-treated group were co-incubated with COCs for 18 h at 38.5°C in a humidified atmosphere with 5% CO_2 .

After fertilization, putative zygotes were denuded of cumulus cells by gentle vortexing in HEPES–TALP, and placed in groups of 10 in 25- μL droplets of KSOM, overlaid with mineral oil and cultured for 7 days at 38.5°C in an atmosphere of humidified air with 5% CO_2 and 5% O_2 . Oocyte developmental competence was evaluated as the proportion of oocytes that cleaved to two- to four-cell stage embryos 42–44 h postfertilization, and the proportion of embryos that developed to blastocysts seven days postfertilization.

Analysis of blastocyst's transcriptome

Microarray analysis

Total RNA was extracted from blastocysts ($n=4$ embryos per sample; three replicates). RNA was isolated using the PicoPure RNA extraction kit (Arcturus, CA, USA). Prior to the elution, purification was performed using DNase I (Qiagen) treatment at 25°C, for 15 min. Total extracted RNA was stored at -80°C for further RNA labeling. RNA quality was analyzed by determining the RNA integrity number (RIN) using an Agilent 2100 Bioanalyzer (Palo Alto, CA, USA) and only samples with RIN > 8.0 were used for microarray analysis. Total RNA was amplified and labeled using the GeneChip® WT Pico Reagent Kit (Affymetrix), and then hybridized to the Affymetrix GeneChip® Bovine Genome 1.0 ST Array (GPL16500). For the preparation of hybridization probes, 400 pg of total RNA per sample was reverse-transcribed to cDNA using primers containing a T7 promoter sequence, so that the resulting cDNA contained the T7 sequence. Then the cDNA was amplified using low-cycle PCR followed by linear amplification

using T7 *in vitro* transcription technology. The cDNA was converted to biotinylated sense-strand DNA-hybridization targets that were hybridized to the GeneChips in a GeneChip Hybridization Oven 640, using 1 chip per RNA sample, and then visualized using the Affymetrix GeneChip Scanner 3000. The original data were further processed using the Expression Console (V1.3.1.187; Affymetrix). Normalization, background reduction and gene-level summary were performed using the Robust Multichip Average (RMA) procedure with default settings. Raw expression intensity values were determined with Transcriptome Analysis Console 3.0 (TAC3.0, Affymetrix) using ANOVA integrated in the software. The FDR procedure was also implemented in TAC3.0. Levels of significance were set to fold change > 2, $P < 0.05$ and FDR < 0.05. For hierarchical clustering, (unsupervised) default settings of TAC3.0 were used, where the distance is the Euclidean distance, computed by the complete linkage method. The microarray data were deposited in the GEO database under accession no. GPL16500.

Bioinformatics analyses

The differentially expressed genes detected by microarray analysis as well as the differentially expressed proteins were assigned using several functional annotation tools. Gene ontology (GO) and enrichment analysis was performed using the PANTHER Gene ID list (<http://www.pantherdb.org/>) online database. To explore the biological function, we used the DAVID Functional Annotation Bioinformatics Microarray Analysis tool (<https://david.ncifcrf.gov/home.jsp>). Pathway analysis was performed using the Kyoto encyclopedia of genes and genome pathway (KEGG; <https://www.genome.jp/kegg/>).

Quantitative real-time PCR

Blastocysts were collected for qPCR assay on day 7 postfertilization, as previously described (Kalo & Roth 2017). Each sample consisted of four blastocysts for microarray validation and single-embryo samples for evaluation of blastocysts derived from fertilization with AV- separated spermatozoa. Samples were stored at -80°C until RNA extraction. Poly(A) RNA was isolated using the Dynabeads mRNA DIRECT Kit according to the manufacturer's instructions (Life Technologies) as previously described (Kalo & Roth 2017). Briefly, samples were lysed and mixed with oligo (dT)₂₅ Dynabeads. Following mRNA binding, each sample washed twice with buffer A, twice with buffer B and mRNA eluted with

10 mM Tris-HCl. The purified mRNA was used as templates in cDNA synthesis using SuperScript[®] III Reverse Transcriptase (Life Technologies) as was previously described (Kalo & Roth 2017).

The qPCR assay was carried out with primers for seven genes (*POU5F1*, *PTGS2*, *MED6*, *MED21*, *SOX2*, *HNRNPA1* and *ACTA2*). *YWHAZ* and *SDHA* were used as internal reference genes (Kalo & Roth 2017). Validation of the microarray data was performed with primers for five genes (*PTGS2*, *MED6*, *MED21*, *HNRNPA1* and *ACTA2*). The primers were derived from bovine sequences found in Genbank and specific primer pairs were designed using Primer 3.0 software (Table 1). The qPCR was conducted in a LightCycler[®] 96 system (Roche) using the DyNAmo ColorFlash SYBR[®] Green qPCR Kit (Finnzymes, Espoo, Finland) in a final volume of 20 μL containing ultrapure water (Biological Industries), 400 nM of each primer and 3 μL diluted cDNA. A control, without reverse transcriptase, was included to eliminate genomic DNA from RNA preparations. The reaction efficiency ranged between 90 and 110% with $R^2 > 0.995$. The amplification program included preincubation at 95°C for 7 min, followed by 40 amplification cycles of denaturation at 95°C for 10 s and annealing–elongation at 60°C for 15 s. All samples were run in duplicates in 96-well plates. Melting-curve analysis was performed at the end of the amplification to confirm single-gene specificity. Fluorescence was recorded to determine the threshold cycle during the log-linear phase of the reaction in which fluorescence rises above background. Gene expression was quantified and analyzed by LightCycler[®] 96 software ver. 1.1 and the $\Delta\Delta C_t$ method was used to calculate the relative expression of each gene.

Statistical analysis

Data were analyzed by JMP-14 software (SAS Institute Inc., 2004, Cary, NC, USA) using an ANOVA model. Data of the proportion of cleavage to two- to four-cell-stage embryos, blastocyst formation rate and DFI were arcsine-transformed before being subjected to one-way ANOVA followed by Tukey–Kramer test. The qPCR data were analyzed according to the $2^{-\Delta\Delta C_t}$ method, expressing the fold change of each selected gene within experimental groups. Data were normalized against the control (expression set to 1). The fold-change data for each gene were subjected to one-way ANOVA followed by Student's *t*-test. Data are presented as mean $\pm$ S.E.M. For all analyses, $P < 0.05$ was considered significant; *P*-values between 0.05 and 0.1 were also reported as trends that might

Table 1 Primers used for qPCR analysis.

Gene	Accession number	Primer sequences (5' → 3')		Size (bp)
		Forward	Reverse	
<i>POU5F1</i>	NM_174580	GTGAGAGGCAACCTGGAGAG	ACACTCGGACCACGTCTTTC	109
<i>PTGS2</i>	NM_174445	GAAATGATCTACCCGCCTCA	TCTGGAACAACCTGCTCATCG	161
<i>MED6</i>	NM_001034237	TGGTGGCACTTCAAAGATCA	AGCATCCACACGCTGTCTC	102
<i>MED21</i>	NM_001038566	CTGTGAACCTCGCTTGCAGAT	TTAGCCCGCTGATCTTTGTT	115
<i>HNRNPA1</i>	NM_001045911	CACTGTGAATGGCCACAACCT	GACCTCTCTGGCTGGATGAA	83
<i>ACTA2</i>	NM_001034502	CGAGGCTATTCTTCGTGAC	CAGTGGCCATCTCATTCTCA	103
<i>SOX2</i>	NM_001040483	GTCCTATGGTGCTGGATGCT	GTTGATGTTTCATGGCAGAG	113
<i>YWHAZ</i>	NM_00174814	GCATCCACAGACTATTTCC	GCAAAGACAATGACAGACCA	124
<i>SDHA</i>	NM_174178	GGGAGGACTTCAAGGAGAGG	TCAACGTAGGAGAGCGTGTC	112

Table 2 Summary of differentially expressed proteins within biological processes, between control and AFB1-treatment spermatozoa following 4 h capacitation (PANTHER).

Functional annotation	Control		AFB1	
	No. of proteins	Proteins	No. of proteins	Proteins
Biological adhesion	1	CADH1	2	CADH1; ANG1
Cellular process	1	CADH1	15	PARK7; HSP13; PRDX5; DPP4; CADH1; CD9; ANG1; MANBA; 1433Z; A4; SGTA; CETN1; PTPA; MMSA; TM190
Localization	4	COX3; DHSD; NPC2; ACBP	7	HSP13; SCO1; DPP4; DRC4; ATP5H; SGTA; ACBP
Metabolic process	4	COX3; DHSD; NPC2; ACBP	10	PARK7; UCHL3; SCO1; HIBCH; KAD3; ENOB; MANBA; PP1B; MMSA; ACBP
Biological regulation			4	PRDX5, DYL2; SGTA; PP1B
Immune system process			2	DPP4; TM190
Multicellular organismal process			1	PP1B
Reproduction			1	SFP1
Response to stimulus			5	HSP13; PRDX5; SGTA; GPX3; GPX4
Rhythmic process			1	PP1B

be real and worthy of note. For each set of experiments, samples of spermatozoa from at least three bulls were tested.

Results

Proteomic profile in AFB1-treated spermatozoa

During 4 h of capacitation, 753 and 755 proteins were identified in the control and AFB1-treated spermatozoa, respectively. Among them, nine proteins were differentially expressed in the control group compared to 48 in the AFB1-treated group (fold change < 2; $P < 0.05$). Following acrosome reaction induced by Ca^{++} ionophore, 692 and 727 proteins were identified in the control and AFB1-treated spermatozoa, respectively. Among them, 41 proteins were differentially expressed in the control group compared to 25 in the AFB1-treated group (fold change < 2; $P < 0.05$). The distribution of the differentially expressed proteins in control and AFB1-treated spermatozoa within biological processes is presented in Table 2. After 4 h capacitation, the differentially expressed proteins were involved in four biological processes and seven cellular pathways and in nine processes and 16 pathways, in the control group

compared to AFB1-treated spermatozoa, respectively. Some of these proteins were involved in important cellular pathways, such as EGF receptor and PI3K-signaling pathways (1433Z) and the Wnt-signaling pathway (CADH1).

To gain more biological insight, we performed GO enrichment analysis, using PANTHER online database (Table 3). The differentially expressed proteins were classified into three functional groups: cellular component, molecular function and biological process. The most enriched cellular components were TRAPP complex, mitochondrion/ATP synthase complex and actomyosin. Oxidoreductase and ATPase activity were the most enriched for molecular function and actin filament depolymerization and ATP biosynthetic process were the most enriched biological processes.

Following acrosome reaction induced by Ca^{++} ionophore, differentially expressed proteins were involved in seven biological processes in both the control and AFB1-treated spermatozoa (Table 4). In the control, the differentially expressed proteins were involved in 14 cellular pathways compared to five in the AFB1-treated spermatozoa. Some of these proteins were involved in important cellular pathways such as

Table 3 Summary of some of the functional annotation of proteins identified to be altered in spermatozoa due to exposure to AFB1 (enrichment analysis; PANTHER).

Functional annotation	Enrichment score	Protein count	P-value*	Associated proteins (gene names)
Cellular component				
TRAPP complex	>100	1	6.28E-03	TPPC1
Mitochondrion/ATP synthase complex	>100	3	7.42E-03	FTMT; MECR; ATP5H
Actomyosin	91.92	1	1.14E-02	WDR1
Molecular function				
Oxidoreductase activity	11.46	3	2.03E-03	FTMT; MECR; VKOR1
ATPase activity	12.39	2	1.11E-02	CLPP; ATP5H
Biological process				
Actin filament depolymerization	>100	1	7.42E-03	WDR1
ATP biosynthetic process	8.38	3	4.89E-03	MECR; VKOR1; ATP5H

*Bonferroni correction for multiple testing.

Table 4 Summary of differentially expressed proteins within biological processes, between control and AFB1-treatment spermatozoa following Ca^{++} ionophore acrosome reaction induction (PANTHER).

Functional annotation	Control		AFB1	
	No. of proteins	Proteins	No. of proteins	Proteins
Biological regulation	4	PRS8; HXK1; TPPP2; KC1G1	2	DNAL4; TPPP2
Cellular component organization or biogenesis	1	MPI	1	MPI
Cellular process	13	TKFC; PGP; MTNA; TPPP2; KC1G1; SODC; PTGR2; IDHC; NDKA2; HXK1; SAHH; IMPA1; PARK7	6	CDIB; CD9; UBA1; TPPP2; SODC; SRR
Localization	4	KC1G1; CC50A; CYC2; ACPB	2	CDIB; SCO1
Metabolic process	20	TKFC; PGM1; MPI; PGP; MTNA; ENTP1; PTGR2; CYC; PRS8; DHSO; F16P1; IDHC; KAD1; GALM; ACPB; HXK1; SAHH; IMPA1; UCHL5; PARK7	8	SCO1; PGM1; MPI; DHSO; F16P1; KAD1; GALM; SRR
Response to stimulus	1	SODC	1	SODC
Multicellular organismal process			1	GD1B

EGF and EGF receptor signaling pathways (PEBP1), glycolysis (PGAM2), fructose–galactose metabolism (HXK1), and the Wnt-signaling pathway (KC1G1). This was expressed for in the control but not in the AFB1-treated spermatozoa.

To validate the results of the proteomic analysis, we performed immunoblots to examine the abundance of the key proteins in spermatozoa before (T0) and after (T4) capacitation *in vitro*. In accordance with the proteomics results, the abundance of COX3 was significantly lower at T0 compared to T4 ($P < 0.04$, Fig. 2).

Annexin V separation of spermatozoa

DNA fragmentation

Separation of spermatozoa into non-apoptotic (AV–) and apoptotic (AV+) subpopulations indicated a significantly higher DFI for the latter. This was true for both the control and AFB1-treated groups (99.5 ± 0.06 vs $20.8 \pm 4.29\%$ and 95.3 ± 1.74 and $22.9 \pm 2.30\%$, respectively; $P < 0.001$; Fig. 3A). The DFI for AV–spermatozoa was significantly higher in AFB1-treated spermatozoa compared to controls (32.2 ± 7.36 vs $20.8 \pm 4.29\%$; $P < 0.05$; Fig. 3A).

Fertilization competence and embryonic development

IVF with AV+ spermatozoa resulted in a reduced proportion of embryos that cleaved to the two- to four-cell stage 42 h postfertilization, compared to AV–spermatozoa. This was true for the control ($31.3 \pm 2.93\%$ vs $78.3 \pm 2.78\%$; $P < 0.001$; Fig. 3B) and AFB-treated ($24.7 \pm 2.67\%$ vs $70.7 \pm 3.38\%$; $P < 0.001$) groups. Among the AV+ subpopulation, the proportion of cleaved embryos in the AFB1-treated group did not differ from that of the control group ($24.7 \pm 2.67\%$ vs $31.3 \pm 2.93\%$; $P < 0.1$). No blastocysts developed after fertilization with AV+ spermatozoa. Among the AV– subpopulation, the

proportion of cleaved embryos in the AFB1-treated group did not differ from that of the control group ($70.7 \pm 3.38\%$ vs $78.3 \pm 2.78\%$; $P < 0.1$). The proportion of developing blastocysts out of cleaved oocytes (20.7 ± 6.69 and $23.5 \pm 4.17\%$; Fig. 3C) or out of total oocytes (14.3 ± 9.24 and $17.5 \pm 7.14\%$; Fig. 3D), did not differ between the AFB1 and control group, respectively.

Blastocysts' genetic profile

To examine whether fertilization with AFB1-treated spermatozoa further affects the transcriptome profile of the formed embryo, a qPCR analysis was performed on embryos developed from AV– spermatozoa. The examination included four blastocysts for each experimental group, collected from four different *in vitro* production runs. The analysis revealed, that out of the examined genes, mRNA expression pattern of two pivotal genes, *POU5F1* and *SOX2*, was altered in the AFB1 blastocysts relative to the control ($P < 0.05$). No effect was observed on *PTGS2*, *MED6*, *MED21*, *HNRNPA1* or *ACTA2* mRNA expression (Fig. 3E).

Figure 2 Western blots for validation of COX3 abundance in the control group spermatozoa before (T0) and after (T4) capacitation *in vitro*. GAPDH was used as a loading control, and the abundance of COX3 protein was corrected relatively to GAPDH. The abundance of COX3 was shown to increase during the 4 h capacitation ($P < 0.04$), which is in agreement with the results of proteomic analysis.

Figure 3 Effect of AFB1 on spermatozoa DNA fragmentation, fertilization competence and the genetic profile of the developing embryos, following separation with annexin V (AV) kit. (A) Spermatozoa were incubated for 4 h with 10 μ M AFB1 dissolved in DMSO and then separated with the AV kit. DNA fragmentation was determined using Acridine orange dye and a spermatozoon-dedicated easyCytometer. DNA fragmentation was evaluated based on 5000 events and is presented as DNA fragmentation index (DFI). (B) Fresh spermatozoa were exposed to 10 μ M AFB1 for 4 h and then separated with the AV separation kit. Bovine oocytes were matured for 22 h and fertilized for 18 h with AFB1-treated or control spermatozoa ($\sim 1 \times 10^6$). Presented is the percentage of oocytes that cleaved into two- to four-cell-stage embryos 42–44 h postfertilization. The experiment included four replicates; $n = 582$ oocytes. (C and D) Percentage of embryos developing to the blastocyst stage on day 7 postfertilization out of cleaved (C) or total (D) oocytes. (E) qPCR analysis of blastocysts derived from fertilization with AV-separated spermatozoa. Samples of single-embryo were collected and further subjected to RNA isolation followed by qPCR. Presented is the fold change of five selected genes (ACTA2, PTGS2, MED6, MED21, SOX2, HNRNPA1 and POU5F1). Fold change was calculated by the $2^{-\Delta\Delta C_t}$ method, normalized against the reference genes YWHAZ and SDHA and relative to the control (expressed as 1). The experiment included four replicates; $n = 582$ oocytes. Data are presented as means $\pm$ S.E.M. $^{**}P < 0.001$; $^{*}P < 0.05$.

In light of the described previous findings and given that embryos are developed only from AV– spermatozoa, a large scale microarray analysis was performed in blastocysts developed from unseparated spermatozoa. A total of 24,415 genes were displayed using a bovine standard array. Differential expression of 345 genes (fold change > 2 ; $P < 0.05$) was observed between blastocysts developed after fertilization with control vs AFB1-treated spermatozoa. Among these genes, 225 were upregulated and 120 were downregulated. Hierarchical clustering

analysis was performed to better demonstrate the genes' striking segregation between control embryos and those developed from AFB1-treated spermatozoa (Fig. 4A). In addition, a volcano plot was generated to visualize the differential expression between the two different conditions (Fig. 4B). The plotted values are the averaged normalized signal values of each group ($\log_2$ scaled). Validation of the microarray data was performed by qPCR assay, using specific primers. Five genes (PTGS2, MED6, MED21, HNRNPA1 and ACTA2) were examined in blastocyst samples ($n = 4$ blastocysts per sample; three replicates) and were found to be correlated with the microarray results ($P < 0.05$; Fig. 4C).

Functional categorization of differentially expressed genes in blastocysts

Ten biological processes were identified using the PANTHER tool. The most prominent ones, with the highest percentages of differentially expressed genes, were cellular (32.1%) and metabolic (26.5%) processes, biological regulation (16.2%) and localization (17.9%) (Fig. 5A). Within each biological process, most of the genes were upregulated in blastocysts derived from AFB1-treated spermatozoa vs controls (Fig. 5B).

Using the DAVID functional annotation tool, 262 records were identified. Some of the functional annotations for the differentially expressed genes between blastocysts developed from fertilization with AFB1-treated vs control spermatozoa are summarized in Table 5. Of these genes, 28 were associated with methylation and acetylation processes (e.g. ACTA2, ANXA1, MED6 and HNRNPA1), 22 with transcription (for example MED21 and MYF5), and 14 with embryo development (POU5F1 and PTGS2). In addition, 15 genes were related to cell differentiation and proliferation, 13 to the cell cycle and 4 to pluripotency.

GO enrichment analysis was performed using PANTHER online database (Supplement 1, see section on supplementary materials given at the end of this article). The differentially expressed genes were classified into four functional groups: protein class, cellular component, molecular function and biological process. The most enriched protein classes were G-protein and calcium-binding protein. The most enriched biological processes were tissue remodeling and development, reproductive structure and system development, regulation of G1/S transition of mitotic cell cycle, actin cytoskeleton reorganization, mitochondrial translation and gene expression and positive regulation of apoptotic process.

Discussion

This study is the first to explore the deleterious effects of AFB1 on mammalian (bovine) spermatozoa which carryover to the formed blastocyst. Exposure of spermatozoa to low concentrations of the mycotoxin

Figure 4 Analysis of differentially expressed genes of blastocyst stage embryos identified by microarray analysis. (a) Hierarchical clustering (unsupervised) analysis of differentially expressed genes for control vs AFB1-treated spermatozoa. Columns and rows represent samples and genes, respectively. Each horizontal line and column represents a single gene and sample (control or AFB1-treated), respectively. Left branches indicate the relationships among the different genes and upper branches indicate differences among samples. (b) Volcano plot for visualizing differential expression patterns between groups. P -values were plotted against the fold change in gene expression for all genes. The vertical bars in the plot represent the statistical significance and the horizontal lines represent the genes that were upregulated (red) or downregulated (green) in blastocysts developed the following fertilization with control vs AFB1-treated spermatozoa. (c) qPCR validation for microarray analysis. COCs were in vitro matured for 22 h and then were co-incubated with spermatozoa capacitated in the presence or absence of 10 μ M AFB1. Putative zygotes were then in vitro cultured for additional 7–8 days to allow blastocyst formation. Samples of four blastocysts were collected from each group (control or AFB1) and further subjected to RNA isolation followed by qPCR. Presented is the fold change of five selected genes (ACTA2, PTGS2, MED6, MED21 and HNRNPA1). Fold change was calculated by the $2^{-\Delta\Delta C_t}$ method, normalized against the reference genes YWHAZ and SDHA and relative to the control (expressed as 1). Data are presented as means $\pm$ S.E.M. * $P < 0.05$.

AFB1 impaired the genetic profile of embryos derived from treated spermatozoa. We suggest that alterations in the proteomic profile of AFB1-treated spermatozoa may be involved in the mechanism underlying these effects.

Effect of AFB1 on the spermatozoa

We have recently shown that AFB1 increases the proportion of spermatozoa with DNA fragmentation and reduced the proportion of oocytes that fertilized and developed to two- and four-cell stage embryos, but not the proportion of the formed blastocysts (Komsky-Elbaz *et al.* 2018). DNA damage in spermatozoa is strongly related to reduced fertilization and blastocyst formation (Egozcue *et al.* 2000) and linked to delayed chromosomal instability in blastocyst stage embryos and abnormalities after implantation (Kumar *et al.* 2013). Nonetheless, spermatozoa with fragmented DNA can successfully fertilize the oocyte, expressed by first embryonic

cleavages (Simon *et al.* 2014). In bovine, the embryonic genome is activated after the 8- to 16-cell stage (Viuff *et al.* 1996) therefore, alterations in the paternal genome are less likely to affect the developing embryo through the first two cleavages. If so, the question is what is the mechanism underlies the reduced cleavage reported for AFB1-spermatozoa (Komsky-Elbaz *et al.* 2018). One possibility is that AFB1 impairs the acrosome integrity and function, which in turn lead to fertilization failure. In support of this, a high proportion of pseudo acrosome reaction was reported in bovine spermatozoa exposed to 100 μ M AFB1 in vitro (Komsky-Elbaz *et al.* 2018) or in rams were fed with 250 μ g/day AFB1 for three weeks (Ataman *et al.* 2014).

AFB1-induced alteration in a wide range of proteins is also suggested to be involved. Spermatozoa are transcriptionally and translationally silent cells thus, an aberrant expression of proteins may occur due to post-translational modifications (Panner Selvam & Agarwal

Figure 5 Distribution of differentially expressed genes into biological processes, in blastocysts developed following fertilization with control vs AFB1-treated spermatozoa. (A) Distribution of genes in a total of ten identified biological processes. (B) Distribution of upregulated (red) and downregulated (green) differentially expressed genes within each of these processes.

2018). In human, altered spermatozoa proteomic profile is associated with poor blastocyst formation and IVF outcome in donor oocytes (McReynolds *et al.* 2014). Alteration in the expression of proteins that are involved in the fertilization process and early embryonic development was associated with impairment of the morula stage and blastocyst formation (Castillo *et al.* 2018). Here we report a differential expression of proteins in AFB1-treated spermatozoa which related to various processes, as discussed in detail subsequently.

One of the most enriched biological processes was the actin filament depolymerization, which involves actomyosin, one of the most enriched cellular components. *In vitro* capacitation of bull spermatozoa is accompanied by a time-dependent increase in actin polymerization (Brener *et al.* 2003). Induction of the acrosome reaction in capacitated cells initiated by F-actin breakdown. Thus, prevention of actin polymerization may interfere capacitation and spermatozoa's ability to undergo acrosome reaction, leading to reduced fertilization rates.

In our previous study, exposure of bovine spermatozoa to AFB1 induced alterations in mitochondrial membrane potential and reduced fertilization/cleavage rates (Komsky-Elbaz *et al.* 2018). Among the differentially expressed proteins reported here is the PARK7, a multifunctional oxidative stress response protein that

Table 5 Summary of some of the functional annotations for the differentially expressed genes in blastocysts developed from fertilization with AFB1-treated vs control spermatozoa (DAVID).

Functional annotation	No. of genes	Downregulated genes	Upregulated genes
Metabolic pathway	29	<i>POLR3G; GALT</i>	<i>GBE1; BDH2; ALG8; ATP5G1; ATP6V1C2; UCGC; B4GALT6; BCAT1; DPM3; ENPP1; FDFT1; FABP5; FPGT; GCNT1; GSTO1; LOC613966; HSD3B1; HSD17B7; IDI1; LCLAT1; LOC781164; MSM01; PIGP; PCK2; PGP; PTGS2; PRKAA1</i>
Methylation and acetylation	28	<i>RAB8A; CSTF2; DPYSL2; HNRNPA1; H4 (LOC527388; LOC527645); COPZ1; FAM53C; GLTP; STK38; SLC25A11</i>	<i>GNGT1; RAB30; ACTA2; MTRF1; ABRACL; NAA20; WDR83OS; ANXA1; CNN2; CAMK2D; FABP5; FABP7; IDI1; MED6; MPC1; PNO1; ZUFSP</i>
Transcription	22	<i>ZBTB34; LOC100336984; ETV3; POU5F1; PPARGC1B; POLR3G; CSTF2; HLTFF; HDAC5; RNF4</i>	<i>ZBTB11; E2F5; HBP1; SATB2; GTF2B; HMBOX1; MED18; MED21; MED6; MYF5; NGG1; PEX2</i>
Cell differentiation and proliferation	15	<i>ETV3; CDK6; DPYSL2; EDA; POLR3G; FGFR4; HILPDA</i>	<i>MYF5; NRG1; SDC2; USP42; CNN2; FABP7; PTGS2; SDCBP</i>
Embryo and placenta development	14	<i>RTCB; POU5F1; DPYSL2; EDA</i>	<i>POLG2; HUS1; RBBP6; DSC3; NRG1; PTGS2; SLC34A2; CYSTM1; MED21; MYF5</i>
Cytoskeleton	14	<i>BBS2; KITLG; RAB8A; DPYSL2; FGFR4; KPTN</i>	<i>SDCBP; ERCC6L2; GABARAP; RAP2A; ACTA2; ANXA1; CNN2; RAC3</i>
Cell cycle	13	<i>CTDSP2; UHMK1; CDK6; PMP22; PRKACA</i>	<i>DCLRE1B; E2F5; HUS1; RAD1; ANXA1; CAMK2D; PTGS2; SDCBP</i>
Apoptosis	11	<i>KITLG; NAA16; MAP3K8; SHISA5</i>	<i>GABARAP; THOC6; AIFM2; LGALS3; PDCC6; PRKAA1; P2RX4</i>
Calcium signaling	11	<i>ITPR2; PRKACA</i>	<i>S100A3; ANXA1; ANXA8L1; CAMK2D; DSC3; MCU; P2RX4; TACR1; TRPC1</i>
WNT signaling	6	<i>EDA; PRKACA</i>	<i>ATP6V1C2; HBP1; CAMK2D; RAC3</i>
cAMP signaling	6	<i>PRKACA; ITPR2</i>	<i>RIIAD1; CAMK2D; HHIP; RAC3</i>
PI3K-Akt signaling	6	<i>KITLG; CDK6; FGFR4</i>	<i>GNGT1; PCK2; PRKAA1</i>
Oxidative phosphorylation	5		<i>ATP5G1; ATP6V1C2; ATP8A1; AAED1; POLG2</i>
MAPK signaling	5	<i>FGFR4; MAP3K8; PRKACA</i>	<i>RAC3; NRG1</i>
Pluripotency	4	<i>POU5F1; FGFR4</i>	<i>LOC615792; MYF5</i>

involved in cell protection against mitochondrial damage and high levels of ROS (Sun *et al.* 2014). In addition, GO enrichment analysis revealed that the mitochondria, and specifically ATP synthase complex are of the most enriched cellular components. Out of the enriched molecular functions were the oxidoreductase and ATPase activity. Among ATP synthase related proteins, ATP5H which related to complex IV of the mitochondrial respiratory chain, is pointing out. Increased level of ATP5H was shown to be involved in maintenance of pig spermatozoa motility (Zhao *et al.* 2019). Taken together, AFB1-reduced expression of PARK7 and ATP5H is suggested to be involved in the reduced fertilization rate in treated spermatozoa.

The ubiquitin-proteasome system (UPS) is an ATP-dependent enzymatic machinery that targets substrates for degradation by tagging proteins with a small chaperone protein (Sutovsky 2011). The UPS is involved in capacitation, acrosome reaction and zona pellucida penetration (Zimmerman & Sutovsky 2009). Here we report that the E1-type ubiquitin-activating enzyme (UBA1) and the ubiquitin C-terminal hydrolase L3 (UCHL3) are downregulated in AFB1-treated spermatozoa. UBA1 is present in the acrosome and is responsible for ubiquitin activation – the first step of ubiquitin–protein ligation; its involvement is essential in spermatozoon capacitation and acrosome reaction during fertilization in porcine (Yi *et al.* 2012). UCHL3 belongs to the group of deubiquitinating enzymes, regulating the UPS (Wilkinson 2000). This protein is located mainly in the acrosome and the flagella, including the mitochondrial sheath, and shows reduced expression in male infertility (Wang *et al.* 2016). Sperm-borne UCHL3 has been shown to be a primary antipolyspermic agent during human and porcine fertilization (Yi *et al.* 2007). Thus, AFB1-induced downregulation of UCHL3 and UBA1 observed in the current study might further interfere with acrosome reaction and normal fertilization. This alteration can explain, at least in part, the reduced fertility competence reported for AFB1-treated spermatozoa (Komsky-Elbaz *et al.* 2018).

Among the differentially expressed proteins recorded here are the members of the glutathione peroxidase (GPX) and peroxiredoxin (PRDX) families. GPXs are important for chromatin remodeling in spermatozoa during epididymal maturation (O’Flaherty & de Souza 2011). In humans, impaired GPX expression in spermatozoa has been correlated with infertility (Drevet 2006). In mice, GPX4 is involved in spermatozoon chromatin stability (Puglisi *et al.* 2012). PRDXs are the major enzymatic regulators of redox homeostasis in human somatic cells (Rhee *et al.* 2005). In porcine spermatozoa, PRDX5 is associated with plasma membrane integrity and involved in zona pellucida binding (van Gestel *et al.* 2007). In human spermatozoa, PRDX5 is expressed in the acrosome, postacrosomal region and midpiece (Kichine *et al.* 2013), and involved

in regulating ROS activity in the mitochondria and in egg–sperm fusion events (O’Flaherty & de Souza 2011). Therefore, downregulation of PRDX5 and GPX3 upon exposure to AFB1 reported here, is also involved in reducing fertilization competence.

Taken together, the proteomic analysis provides various evidences that AFB1 has a deleterious effect on the spermatozoa proteome, which in turn might lead to reduced fertilization competence. Surprisingly, while AFB1 reduced the fertilization rate, the proportion of embryos that developed to the blastocyst stage following fertilization with AFB1-treated spermatozoa was relatively fair (Komsky-Elbaz *et al.* 2018). One possible explanation is that although the spermatozoa were exposed to AFB1 through capacitation, fertilization was performed by those that had not been impaired. To confirm this, we separated spermatozoa into two populations: non-apoptotic (AV–) and apoptotic (AV+). The proportion of spermatozoa with DNA fragmentation was significantly higher in the AV+ population. Moreover, IVF with AV+ spermatozoa resulted in relatively low cleavage rates and no blastocyst formation, indicating that AV+ spermatozoa are of low fertilization competence, thus fertilization was most likely performed by AFB1-treated spermatozoa with intact DNA (i.e. AV– spermatozoa). Interestingly, within the AV– subpopulation AFB1-treated spermatozoa expressed a lower fertilization rate than untreated ones. A possible explanation is that the spermatozoa’s contribution to the embryo is not limited to the paternal DNA and might also include other paternal components such epigenetic marks on the DNA, as well as RNAs and proteins that are transferred through fertilization (Castillo *et al.* 2018). As many as 108 sperm-originated proteins have been identified in the human blastocyst (Castillo *et al.* 2018), and suggested be crucial for embryonic development and health (Castillo *et al.* 2015). Paternal exposure to endocrine-disrupting compounds can cause alterations in the spermatozoa’s RNA, which persist in the offspring (Uppangala *et al.* 2016).

Carryover effect of AFB1 – from the exposed spermatozoa to the developing embryo

In the current study, qPCR analysis of blastocysts revealed alterations in two important genes, *POU5F1* and *SOX2*, when fertilization was performed with AFB1-treated spermatozoa AV– subpopulation. Both genes are expressed in the blastocyst’s inner cell mass, and in bovine, *POU5F1* is also expressed in the trophectoderm (Ozawa *et al.* 2012) and have a major role in early embryonic development (Avilion *et al.* 2003). In light of these findings, it was reasonable to assume that blastocysts developed from AFB1 spermatozoa might express an impaired genome. This assumption was further confirmed by microarray analysis.

Here we report that the transcriptome profiles of blastocysts derived from fertilization with AFB1-treated

Figure 6 Hypothetical pathway by which AFB1-treated spermatozoa induces impairment in the formed blastocysts. Red color represents differentially expressed genes; arrows beside represent upregulation or downregulation of the gene. Black color represents genes without a difference in RNA levels, that is, not directly affected.

spermatozoa differed significantly from that of the control spermatozoa, suggesting a carryover effect of AFB1 from the spermatozoa to the developing embryo.

Embryonic development is regulated by various signaling pathways that are related to different biological processes, such as pluripotency, cell differentiation and proliferation, cell cycle, apoptosis and cell division and growth (Zhang *et al.* 2007). Thus, any alteration in these signaling networks may lead to abnormal embryo development. Our microarray analysis indicates that most of the differentially expressed genes belong to the calcium signaling pathway. In the blastocyst, calcium waves are associated with two essential developmental functions, that is, induction of cell-cycle progression and establishment of an embryonic axis following the remodeling of the pre-gastrula embryo (Whitaker 2008). In addition, Ca^{2+} fluxes are essential for trophoblast projections before hatching from the zona pellucida and involved in embryo implantation regulation (Miao & Williams 2012). Based on these understanding, we suggest a hypothetical pathway by which AFB1-treated spermatozoa induces impairment in the formed blastocysts (Fig. 6). It involves downregulation of the transmembrane receptor *GPCR*, which in turn leads to an eventual downregulation of *PKA*. *PKA* is involved in embryonic development and is critical for normal morphogenesis (Ungar & Moon 1996). The cAMP/*PKA* pathway is one of the major signaling pathways involved in human trophoblast fusion (Dubey *et al.* 2018). In mice, fetal growth and placental development were altered by a

paternal low-protein diet, which was reflected by reduced expression of *PRKACA*, encodes the catalytic alpha subunit of (Watkins *et al.* 2017). Downregulation of *PKA* via cAMP pathway, resulting in reduced intracellular Ca^{++} levels, which in turn lead to: (1) *BAD* activation, involved in apoptosis; (2) *NFκB* activation, involved in cell survival; (3) upregulation of mitochondrial transmembrane gene, *MCU*, resulting in Ca^{++} overload in the mitochondria and increased ROS. In addition, alteration in the expression of *GPCR* leads to *GNGT1* overexpression, involved with *RAS* and *ERK* signaling pathways, associated with cell proliferation, DNA repair and angiogenesis.

In parallel, downregulation of *KITLG* and *FGFR4* leads to: (1) *IP3R* downregulation via *PLC*, and reduced intracellular Ca^{++} release, which in turn leads to *CAMK2D* upregulation and possible *PKC* downregulation; (2) reduced activation of *PI3K*, leads to upregulation of *RAC3* gene, involved in actin reorganization, and downregulation of *MAP3K8*; (3) reduced activation of *Akt* signaling pathway leads to downregulation of *CDK6* gene, involved in cell cycle and upregulation of *PCK* gene, involved in metabolism.

With respect to actin reorganization, 14 genes related to the cytoskeleton were differentially expressed in blastocysts derived from AFB1-treated spermatozoa. These included *GABARAP*, *PRARG*, *RAP2A*, *ACTA2*, which are directly associated with actin filament organization. The blastocyst's ability to hatch from the zona pellucida depends on, among other factors, changes in the actin filament network (Gallicano 2001).

Although not examined here, such alterations might have a negative impact on the blastocyst's ability to hatch and implant. The paternal centrioles are inherited by the embryo (Ntostis *et al.* 2017, Ortiz-Rodriguez *et al.* 2019); thus, it is possible that damage induced by environmental toxins in spermatozoa might lead to further alterations in the cytoskeleton of the developing embryo. In the marine invertebrate *Galeolaria caespitosa*, exposure of spermatozoa to 0.2 mg/L of the endocrine-disrupting compound, dibutyl-phthalate, resulted in alkylation of their centrioles, which in turn led to defective cytoskeletal organization and chromosome segregation during early embryonic cleavages (Lu *et al.* 2017). It therefore seems that the effect of AFB1-treated spermatozoa is not limited to the blastocyst and might also be expressed in as early as the zygote stage.

Some other genes related to embryonic and placental development had altered expression in blastocysts derived from AFB1-treated spermatozoa. One of these was the mitochondria polymerase gamma two accessory subunit (*POLG2*), which encodes factors essential for the transcription and replication of mitochondrial DNA (McGeehan *et al.* 2009). Disrupted expression in *POLG*-knockout mouse embryos resulted in embryonic lethality soon after implantation (McGeehan *et al.* 2009). In addition, downregulation of *POU5F1* was observed. This gene plays a central role in the pluripotency-regulation network and is crucial for mammalian embryogenesis (Simmet *et al.* 2018). In light of these, it is possible that fertilization with AFB1-treated spermatozoa impairs embryonic development, placental formation and further implantation.

Among the differentially expressed genes observed in our microarray analysis is a large number of genes related to epigenetic methylation and acetylation processes; for instance, transcripts encoding histone 4. Epigenetic marks are known to be affected by environmental factors (Diamanti-Kandarakis *et al.* 2009) such as toxicants, which may be transmitted through the gametes from parents to their offspring (Wei *et al.* 2015, Ly *et al.* 2017). In particular, the epigenome of spermatozoa is known to contribute to, and have potential implications for the developing embryo (Jenkins & Carrell 2012). Non-genetic modifications in the spermatozoa include DNA methylation, histone modifications, targeted histone retention, and protamine incorporation into the chromatin (Kumar *et al.* 2013). Supporting our findings, environmentally induced alterations in spermatozoon DNA methylation can be potentially transmitted to the embryo (Wei *et al.* 2015). Equine cryopreserved spermatozoa exhibit higher levels of DNA methylation than fresh spermatozoa, resulting in downregulation of genes involved in early embryo development, including histones (Ortiz-Rodriguez *et al.* 2019). Taken together, it is suggested that AFB1-induced nongenetic modification in the spermatozoa might be further expressed in the formed blastocyst.

In summary, proteomic analysis of AFB1-treated spermatozoa revealed the differential expression of a number of proteins involved in spermatozoon function and fertilization competence. Exposure of spermatozoa to AFB1 prefertilization did not affect blastocyst formation rate, but found to alter the transcriptomic profile of the developing blastocysts. These findings suggest a deleterious carryover effect of AFB1 from the spermatozoa to the developing embryo. With respect to the AFB1-induced DNA damage, fertilization with the AV- subpopulation of spermatozoa (i.e. low proportion of spermatozoa with DNA-damaged), led to the formation of genetically altered embryos. Taken together it is suggested that the effect AFB1 on spermatozoa involves multiple damage mechanisms. An evaluation of implantation and clinical pregnancy rates following transfer of embryos formed from spermatozoa exposed to AFB1 is thus warranted.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-20-0286>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

A K-E and Z R developed the concept, designed the experiments and prepared the manuscript. A K-E carried out the experiments, data organization and statistical analyses. D K analyzed the microarray and proteomics results. All authors read and approved the final manuscript.

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