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The protective effect of follicular fluid against the emerging mycotoxins alternariol and beauvericin

R.R. Santos[1,2*], E.J. Schoevers[3], X. Wu[1], B.A.J. Roelen[3,4] and J. Fink-Gremmels[1]

[1]Division of Veterinary Pharmacology, Pharmacy and Toxicology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM Utrecht, the Netherlands; [2]Laboratory of Wild Animal Biology and Medicine, Federal University of Pará, Rua Augusto Corrêa, Campus Básico, CEP 66075-110 Belém, Pará, Brazil; [3]Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM Utrecht, the Netherlands; [4]Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM Utrecht, the Netherlands; r.rodriguesdossantos@pq.cnpq.br

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RESEARCH ARTICLE

Abstract

Porcine granulosa cells were cultured in the absence or presence of 10% porcine follicular fluid (FF) at different concentrations (0-20 µM) of the mycotoxins alternariol (AOH) and beauvericin (BEA). The analyses were performed after exposure to these mycotoxins in a medium supplemented or not with FF harvested from gilts and sows. Cell enzymatic activity and nuclear membrane integrity were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and ethidium homodimer-1 labelling. Trolox equivalent antioxidant capacity was measured to calculate the capacity of the cells to counteract reactive oxygen species. qRT-PCR was used to determine the relative gene expression of efflux transporters (*ABCG1* and *ABCG2*) as well *CYP11* and *CYP19*. Mycotoxin cytotoxicity was more related to enzymatic activity than to nuclear membrane damage and no direct relationship with oxidative stress was observed, except when cells were exposed to AOH. In this case, medium supplementation with FF from sows increased the antioxidant capacity of the cells. AOH did not regulate gene expression in the present conditions, but 5 µM BEA led to the up-regulation of *ABCG2* gene expression and a down-regulation of *CYP19* expression. In conclusion, follicular fluid from sows is capable to decrease toxicity of AOH and of BEA.

Keywords: efflux transporters, steroidogenesis, cell viability, porcine, granulosa cells

1. Introduction

Fungal species responsible for the production of the emerging mycotoxins alternariol (AOH) and beauvericin (BEA) are ubiquitously present in the environment. AOH produced by the fungal species *Alternaria* has been detected in wheat, fruits and vegetables (EFSA, 2011) as well as in beverages like white wine and apple juice (Ackermann *et al.*, 2011). BEA is produced by *Fusarium* species and is encountered in food and feed, mainly in grains and grain-based products like bread, raw pasta (EFSA, 2014), but also fruits such as banana (Li *et al.*, 2013). However, no regulation on AOH and BEA in food and feed is available as yet (EFSA, 2011, 2014) and studies on the reproductive effects of these mycotoxins are scarce. Little is known about the toxicity of BEA. For instance, it is recognised that BEA has ionophoric properties (Kouri *et al.*, 2005), but no information on reprotoxicity is available. Data indicating toxic levels of AOH in somatic and germ cells are variable. AOH has been attributed clastogenic and oestrogenic potential after *in vitro* exposure of mammalian cell lines (Lehmann *et al.*, 2006). *In vitro* exposure of porcine granulosa cells to 1.6 µM AOH resulted in decreased cell viability and inhibition of progesterone synthesis (0.8 µM AOH) (Tiemann *et al.*, 2009). The viability of HT29 cells was however only decreased after exposure to high concentrations (10 µM) of AOH (Tiessen *et al.*, 2013). Such differences might be explained by the exposed cell type, absence (Tiemann *et al.*, 2009) or presence (Tiessen *et al.*, 2013) of serum in the culture media. Like serum, follicular fluid (FF) is used to supplement cell culture

medium (Bruckova *et al.*, 2011). We suggest, therefore, that medium enrichment with FF may affect the response of granulosa cells to AOH and BEA exposure. Although nothing is known about the effect of BEA on granulosa cells, it is recognised that cytotoxicity of this mycotoxin in somatic cell lines is attenuated by ATP-binding cassette (ABC) transporters like breast cancer resistant protein (BCRP), and to a lesser extent P-glycoprotein (P-gp) (Dornetshuber *et al.*, 2009). Nothing is known regarding AOH and its interaction with ABC transporters. Mycotoxins may act as endocrine active substances affecting steroidogenesis (Santos *et al.*, 2013). To evaluate the effect of mycotoxins on steroidogenesis, two cytochromes 450 (CYP) were selected: CYP11 and CYP19. CYP11 is involved in mitochondrial steroidogenesis, while CYP19 metabolises aromatase and is located in the endoplasmic reticulum. Here we evaluated the protective effect of FF against AOH and BEA, on the viability of granulosa cells and on the expression of the efflux transporters genes *ABCB1*coding for P-gp and *ABCG2* coding for BCRP, and expression of *CYP11* and *CYP19*.

Oocytes and granulosa cells from advanced follicles are in direct contact with FF, and culture medium supplementation with this fluid maintains long-term *in vitro* viability and proliferation of granulosa cells (Bruckova *et al.*, 2011). Interestingly, the concentration of steroid hormones in FF from gilts and sows is different (Grupen *et al.*, 2003; Rátky *et al.*, 2005), and this might be applicable to the other components of the FF. Hence, we hypothesise that the presence of FF, obtained from sows or gilts, in the medium may interfere with the toxicity of AOH or BEA. Cell viability was determined by evaluating their NAD(P)H-dependent enzymatic activity with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and by evaluating the integrity of nuclear membrane with ethidium homodimer-1 labelling.

2. Materials and methods

Unless mentioned otherwise, the culture media and other chemicals including mycotoxins used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ovaries from cyclic gilts were collected in a commercial slaughterhouse. Collection of granulosa cells was carried out as described (Tiemann *et al.*, 2009). The cells were washed with Hank's Balanced Salt Solution, centrifuged at 200×*g* for 5 min and re-suspended in complete phenol red-free Roswell Park Memorial Institute (RPMI) medium containing penicillin (100 IU/ml), streptomycin (100 µg/ml), with or without 10% FF fluid harvested from gilts or sows. As a routine and rapid viability analysis before culturing cells, an aliquot of the harvested granulosa cells was collected for viability analysis, as measured by trypan blue exclusion. The proportion of living cells with intact membranes submitted to *in vitro* culture was >70%. Granulosa cells (1×10^5 per well) were cultured in 24-well plates, each with 1 ml of culture medium, at 38.5 °C in a humidified 95% air – 5% CO_2 environment. After 24 h, cultured granulosa cells were rinsed in RPMI and cultured in RPMI with or without 10% FF for an additional 24 h. *In vitro* culture was performed in the presence of AOH or BEA at concentrations ranging of 0.31-20 µM in 0.01% dimethyl sulfoxide (DMSO). Culture in presence of 0.01% DMSO was used as control. The granulosa cells were then examined for cell viability using an MTT test and trolox equivalent antioxidant capacity was analysed using a kit (nr CS0790, Sigma) to determine the reactive oxygen species clearance capacity of the cells after culture. Four independent experiments were performed, each one in duplicate. Based on the results with MTT, a new set of exposure to mycotoxins was performed in the presence of FF from sows and gilts to detect granulosa cells nuclear membrane integrity using ethidium homodimer-1 (Molecular Probes Europe, Leiden, the Netherlands) staining. Granulosa cells were classified as viable if the chromatin was not labelled with ethidium homodimer-1. Percentages of viable granulosa cells were calculated in relation to the total number of cells.

Total RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was generated with 0.40 µg of total RNA using an iScript RT kit (Bio-Rad, Hercules, CA, USA). Primer sequences designed for this study or selected from literature (Kuijk *et al.*, 2007; Schoevers *et al.*, 2012) were as follows: *PGK1* (forward) 5'-AGA TAA CGA ACA ACC AGA GG-3', (reverse) 5'-TGT CAG GCA TAG GGA TAC C-3'; *UBC* (forward) 5'-TTC GTG AAG ACC TTG ACT G-3', (reverse) 5'-GGA CTC CTT CTG GAT GTT G-3'; *ABCB1* (forward) 5'-CCA CCC CTT CAT CGA GAC AC-3', (reverse) 5'-AGC AAA ATA CGA GGC CGT CT-3'; *ABCG2* (forward) 5'-AAG GAA CAC CAA TGG CCT GC-3', (reverse) 5'-GGT CGA GGT GCT CCA TTG AT-3'; *CYP11* (forward) 5'-GAT CCC CTC TCC TGG TGA CAA T-3', (reverse) 5'-GCT TCT GGT AAT GCT GGT GAT AGG-3'; *CYP19* (forward) 5'-TCA CTG TGT TCT TCA TGC TGT TTC-3', (reverse) 5'-GAT AAT GTT GGT TCC CTT TTT CA-3'. The relative mRNA expression of *ABCB1*, *ABCG2*, *CYP11* and *CYP19* were calculated after normalisation of gene expression using *PGK1* and *UBC* as internal standards.

Culture medium was submitted to high performance liquid chromatography (HPLC) analysis to quantify the presence of free mycotoxins in the FF using a Luna C18 HPLC column (150×4.6 mm; Phenomenex, Utrecht, the Netherlands) and a mobile phase composed of water:acetonitrile (60:40, v/v). UV detection was performed at a wavelength of 220 nm. Quantification was conducted by comparing peak areas of samples with standard curves obtained from the control samples (AOH and BEA).

For each test, at least four independent experiments were performed, each one in duplicate. Data were evaluated using two-way ANOVA with Tukey as post hoc test. Differences were considered significant when $P<0.001$.

3. Results and discussion

AOH and BEA were not detected in the FF used in the present study. The cytotoxic effect of AOH and BEA on granulosa cells was evaluated by a MTT assay and with ethidium homodimer-1 staining. In the absence of FF, a significant decrease in cell viability occurred after exposure to 1.25 µM AOH as evaluated with MTT assays, while in the presence of FF, only 20 µM AOH was toxic to granulosa cells (Figure 1A) These results are in line with other studies, where Tiemann *et al.* (2009) observed that 1.6 µM AOH was toxic to granulosa cells cultured in a serum-free medium, and Tiessen *et al.* (2013) determined that 10 µM AOH was toxic to HT29 cells cultured in the presence of serum. Studies on the composition of FF generally consider

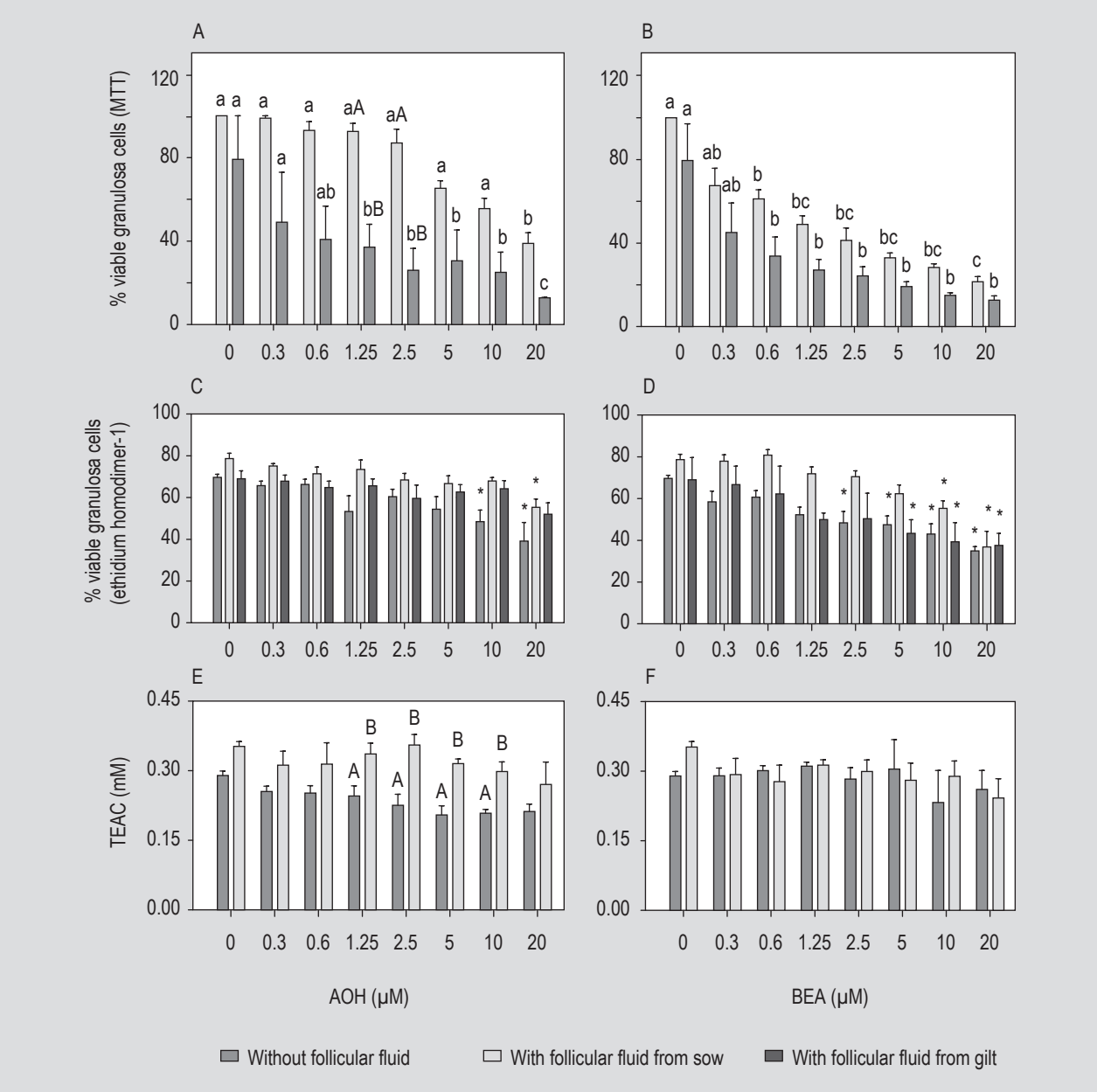

Figure 1. Mean (± standard error of the mean) percentages of viable granulosa cells after exposure to alternariol (AOH) (A, C) or beauvericin (BEA) (B, D) according MTT assay (A, B) and ethidium homodimer labelling (C, D). Trolox equivalent of antioxidant capacity (TEAC) is show in panels E (AOH) and F (BEA).* Indicates significant differences when compared to control; [a,b] Different lower-case letters indicate significant differences between concentrations within each mycotoxin group; [A,B] Different upper-case letters indicate significant differences between medium with and without follicular fluid within the same mycotoxin concentration.

the levels of hormones (Grupen *et al.*, 2003; Rátky *et al.*, 2005) and growth factors (Oberlender *et al.*, 2013). The content of this fluid, however, also includes interleukins, antioxidant and anti-apoptotic factors, proteins, peptides, amino acids and sugars (Revelli *et al.*, 2009). Regrettably, comparisons of the levels of such compounds have been made only considering follicular size, but not related to donor age. It seems that the FF was not sufficient to protect granulosa cells from depletion of cell enzymatic activity after exposure to BEA, as cell viability was already significantly decreased at a concentration of 0.6 µM (Figure 1B). BEA induces mitochondrial damage via reactive oxygen species (Prosperini *et al.*, 2013), which may explain the MTT assay results. We also determined nuclear membrane integrity with ethidium homodimer-1 labelling to evaluate the presumptive protective effect of FF from gilts and sows against toxicity of AOH and BEA. Medium supplemented with sow FF decreased

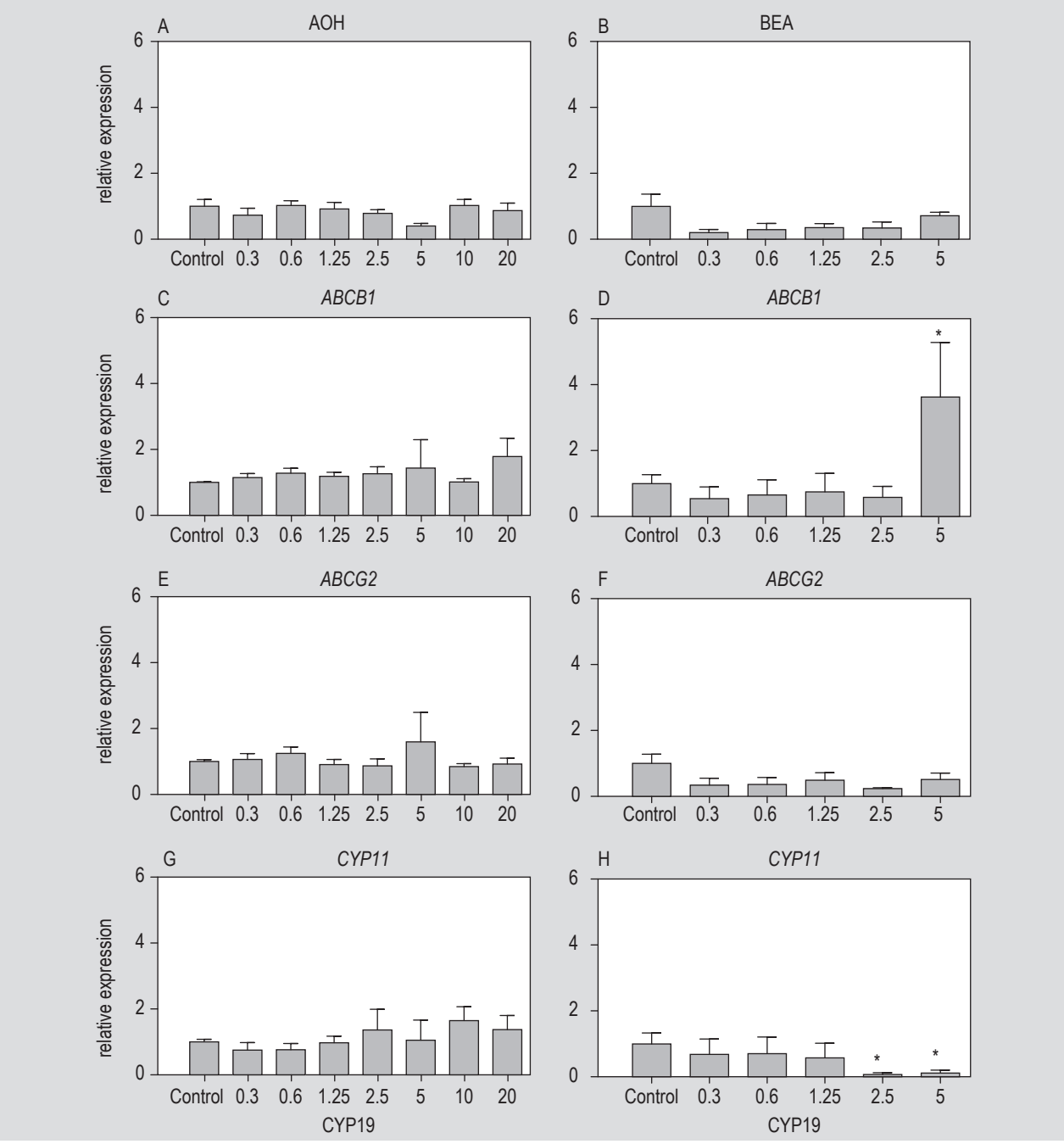

Figure 2. Mean (± standard error of the mean) mRNA relative expression of *ABCB1, ABCG2, CYP11* and *CYP19* in porcine granulosa cells exposed *in vitro* to alternariol (AOH) and beauvericin (BEA) in a follicular fluid-supplemented medium.* Indicates significant differences (*P*<0.001) when compared to control.

the toxic concentration of AOH from 10 µM to 20 µM (Figure 1C). The viability of granulosa cells was significantly decreased after exposure to 2.5 µM BEA in FF-free medium. Interestingly, sow FF offered a better protection to mycotoxins than gilt FF. Medium supplementation with gilts FF decreased the toxic concentration of BEA to 5 µM, and with sow FF to 10 µM. The difference in cell viability observed as determined with MTT and ethidium labelling of nuclear damage is not surprising, once both assays are used to evaluate different parameters. Most probably anti-apoptotic factors present in the FF (Revelli *et al.*, 2009) played a role by counteracting cell apoptosis. Possibly, the level/activity of such factors is higher in sows than in gilts. Presence of FF fluid in the culture medium increased antioxidant capacity in cells exposed to AOH (1.25-10 µM) when compared to culture medium without FF (Figure 1 E,F). Similar to the findings from Bruckova *et al.* (2011), mRNA expression of *ABCB1* and *ABCBG2* in granulosa cells cultured in a FF-free medium was too low to generate reliable data. Exposure to AOH in presence of sow FF did not affect the mRNA expression of the efflux transporter genes *ABCB1* and *ABCG2* nor the expression of *CYP11* and *CYP19* (Figure 2). Exposure to 5 µM BEA, however, resulted in the significant up-regulation of *ABCG2* expression and down-regulation of *CYP19* expression. It has been shown that *ABCG2* expression was a response of the cells to counteract BEA toxicity (Dornetshuber *et al.*, 2009). BEA did not affect *CYP11* expression, but inhibited *CYP19* expression (aromatase), which might be related to *ABCG2* up-regulation and its role in the steroidogenesis (Huuskonen *et al.*, 2013) (Figure 2).

4. Conclusions

In conclusion, 0.6 µM BEA and 1.25 µM AOH impair granulosa cell enzymatic activity, and damage in nuclear membrane is observed only after exposure to higher concentrations of BEA (2.5 µM) and AOH (10 µM) in a FF-free medium. FF, especially from sows, plays a protective role against the toxic effects caused by AOH, by increasing the antioxidant capacity in granulosa cells exposed to this mycotoxin. Regarding BEA, FF does not protect granulosa cells from its harmful effect on enzymatic activity, but appears as a source of anti-apoptotic factors protecting cells from nuclear damage. Also, BEA inhibits mRNA expression of aromatase together with an up regulation of *ABCG2* expression.

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