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ORIGINAL ARTICLE

Beauvericin alters the expression of genes coding for key proteins of the mitochondrial chain in ovine cumulus-oocyte complexes

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Abstract

Beauvericin (BEA) is a member of the enniatin family of mycotoxins which has received increasing interest because of frequent occurrence in food and feed. By its ionophoric properties, BEA is able to alter membrane ion permeability uncoupling oxidative phosphorylation. It was also shown to alter oocyte mitochondrial function. In this study, the effects of BEA at 0.5, 1, ,3 and 5 μmol/L on expression of genes coding for key proteins of the mitochondrial chain in ovine oocytes and cumulus cells were evaluated at different time points of in vitro maturation (IVM), germinal vesicle (GV; $t = 0$), metaphase I (MI; $t = 7$ h), and metaphase II (MII; $t = 24$ h). The expression of nuclear (*TFAM*, *NDUFA12*, *UQCRH*, *COX4*, *ATP5O*) and mitochondrial (*ND1*, *COX1*, *COX2*, *ATP6*, *ATP8*) genes coding for proteins of Complexes I, III, IV, and V was analyzed by qRT-PCR. After BEA exposure, perturbed expression of all genes was observed in cumulus cells and in oocytes at the MI stage (7 h IVM). Expression of *ND1*, *UQCRH*, *COX4* and *ATP5O* was downregulated in cumulus cells and upregulated in oocytes starting from 0.5 μmol/L BEA. Expression of *TFAM*, *NDUFA12*, *COX1*, *COX2*, *ATP6*, and *ATP8* was upregulated starting from 1 μmol/L in cumulus cells and from 3 μmol/L in oocytes. Cumulus cells and oocytes displayed different gene expression patterns upon BEA exposure. The downregulation in cumulus cells of four genes coding for proteins of mitochondrial complexes could represent a major toxic event induced by BEA on the cumulus-oocyte complex which may result in mitochondrial functional alteration.

Keywords Beauvericin · Mitochondria · Gene expression · Cumulus cells · Oocyte · Ovine

Abbreviations

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
ATP5O	ATP synthase O subunit
ATP6	ATP synthase 6
ATP8	ATP synthase 8
BEA	Beauvericin
COC	Cumulus-oocyte complex
COX	Cytochrome c oxidase
EFSA	European Food Safety Authority
GV	Germinal vesicle
IVM	In vitro maturation
MI	Metaphase I
MII	Metaphase II
mtDNA	Mitochondrial DNA
ND	NADH dehydrogenase
NDUFA12	NADH: ubiquinone oxidoreductase subunit A12
OXPHOS	Oxidative phosphorylation
TFAM	Mitochondrial transcription factor A
UQCRH	Ubiquinol-cytochrome c reductase hinge protein

Fiorenza Minervini and Maria Elena Dell'Aquila contributed equally to this work.

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Introduction

Beauvericin (BEA) is an is an enniatin-type depsipeptide mycotoxin which is not routinely included in mycotoxin analyses, most likely because no specific regulations for this group of toxins in food and feed exist. Nevertheless, its frequency of analysis is rapidly increasing leading to more frequent reports of its occurrence (Vaclavikova et al. 2013; Gruber-Dorninger et al. 2017). In recent years, BEA has gained interest in the scientific community due to its high frequency of occurrence in food and feed and consequent potential toxicity in humans and animals (Luz et al. 2017). BEA is produced by various fungi, including several *Fusarium* species, in different regions throughout the world and the contamination of BEA has been reported as a serious problem in Southern Europe (Santini et al. 2012; Luz et al. 2017). It has been detected in 80% of analyzed food items and in 79% of feed (Maranghi et al. 2018). In food, it is usually found in cereal grains and cereal-based products as well as in eggs, nuts, dried fruits, coffee, and medicinal herbs (Mallebrera et al. 2018). Concentrations of BEA in grains may range from a few micrograms to over 500 mg/kg (Luz et al. 2017). There is a concern for infants exposed to BEA through the daily consumption of human breast milk, in which BEA has been reported at concentrations up to 0.019 ng/mL (Braun et al. 2018). In feed, it is found in cereals (Gruber-Dorninger et al. 2017; Tolosa et al. 2019) and maize silage (Reisinger et al. 2019), in a concentration ranging from a few micrograms to milligrams per kilograms (Streit et al. 2013). The common use of the above raw materials as ingredients in feed formulations therefore also potentially exposes livestock to BEA.

BEA is produced by *Fusarium* species (Wu et al. 2018). It is a cyclic hexadepsipeptide, that consists of an alternating sequence of three D-a-hydroxy-isovaleric acid-(2-hydroxy-3-methylbutanoic acid) and three *N*-methyl-L-phenylalanine moieties (Taevernier et al. 2016). It is a lipophilic molecule with ionophoric properties, capable of being incorporated into mammalian cell membranes where it can form a structure acting as a cation-selective channel (Mallebrera et al. 2018). BEA can form a sandwich structure complex with cations and it increases ion permeability in biological membranes, thus affecting ionic homeostasis and cytoplasmic pH (Luz et al. 2017; Mallebrera et al. 2018). By its channel-forming ability, BEA can selectively direct a flux of calcium ions into the cells, thus activating apoptotic signaling pathways (Wu et al. 2018). In different cell systems, BEA led to disturbed mitochondrial function and apoptosis (Jow et al. 2004; Kouri et al. 2005; Tonshin et al. 2010; Prosperini et al. 2013; Mallebrera et al. 2016). A transcriptomic study by Escrivá et al. (2018) reported that BEA alters the expression of several genes related to respiratory chain function in Jurkat cells, a human T lymphocyte cell line. Recently, oxidative phosphorylation (OXPHOS) proteins were reported as the main target for BEA action (Alonso-Garrido et al. 2020).

BEA shows ambivalent biological activities, since it not only exerts cytotoxic effects on many cell lines but also has anti-inflammatory, anticancer, antimicrobial, insecticidal, and nematocidal properties (Mallebrera et al. 2018; Wu et al. 2018; Wu et al. 2019). Due to these beneficial effects, BEA has the potential to be developed as a drug or as a pesticide (Wu et al. 2018; Wu et al. 2019; Caloni et al. 2020). Thus, since its use in medicine and agriculture is foreseeable in the near future, detailed studies on the consequences of its exposure in humans and animals need to be conducted in depth. For these reasons, the European Food Safety Authority (EFSA) paid attention to BEA toxicity. The EFSA panel concluded that acute exposure to BEA should not be a concern to human health, although it solicited to undertake new relevant in vivo toxicity studies necessary to state the effects of BEA chronic exposure (EFSA - European Food Safety Authority, Panel on Contaminants in the Food Chain 2014).

Following exposure, BEA accumulates in biological fluids and tissues in lower concentrations than those in contaminated food and feed (Luz et al. 2017). This could be due to limited absorption in the gastrointestinal tract (Prosperini et al. 2012), and efflux into gut lumen, mainly mediated by ATP-binding cassette (ABC) transporters (Fraeyman et al. 2017). BEA can accumulate in the serum, muscle, colon, fat, brain, kidney and liver in its unmetabolized form (Rodriguez-Carrasco et al. 2016). In 2018, Maranghi et al. reported that, in rodents, BEA targeted the thyroid, kidneys and reproductive systems in both sexes whereas the spleen is affected in male mice only, and adrenals and duodenum in female mice only.

Regarding reproduction, Maranghi et al. (2018) reported that BEA can target specific organs in both sexes and that males are more susceptible than females to repeated oral BEA exposure. In this study, in a repeated-dose oral toxicity test, BEA did not affect ovarian histo-pathological and histomorphometric parameters, but it induced genotoxic effects on ovaries not associated with modifications of serum levels of sexual steroids. In vitro studies reported direct toxic effects of BEA on female reproductive cells in livestock models, such as pigs (Santos et al. 2015; Schoevers et al. 2016), cattle (Albonico et al. 2017), and sheep (Mastrorocco et al. 2019). Exposure of porcine granulosa cells to BEA decreased their proliferation in vitro, inhibited mRNA expression of aromatase and upregulated ABC subfamily G member 2 (*ABCG2*) expression, both playing a role in steroidogenesis (Santos et al. 2015); in vitro exposure to BEA strongly decreased bovine granulosa cell viability by impairing their progesterone and estradiol production capacity by suppressing of cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*) and aromatase mRNA transcription respectively (Albonico et al. 2017); in addition, BEA impaired cumulus cell function leading to abnormal oocyte meiosis (Schoevers et al. 2016). In addition, BEA exposure altered the expression and function of the multidrug resistance protein 1 (*MDR1*) by reducing

adenosine triphosphate (ATP) levels (Schoevers et al. 2016). Mastrorocco et al. (2019) demonstrated that ovine oocytes exposed to BEA during in vitro maturation (IVM) underwent abnormal meiotic progression related to cumulus cell apoptosis and damage of mitochondrial arrangement and functionality with carry-over effects on sperm-induced fertilization, embryo development, and blastocyst quality. Considering exposure potential of livestock to BEA, investigations in these species are of double utility, allowing to obtain data useful for animal production industry and with translational relevance for human reproductive medicine. In particular, the sheep is a suitable animal model as it shares many aspects of reproductive physiology with humans, including basic mechanisms controlling ovarian follicle development (Baird 1983; Ledda et al. 1997; Noakes et al. 2001), oocyte structure, size and maturation (Campbell et al. 2003; Leoni et al. 2007; Leoni et al. 2015), and bioenergetic status during oogenesis (Cotterill et al. 2013).

Even though previous studies have identified the mitochondria as a major cell target of BEA-induced toxicity (Schoevers et al. 2016; Mastrorocco et al. 2019), no information is available to date on the effects of this mycotoxin on the expression of genes encoding for key proteins of the mitochondrial respiratory chain. Therefore, the aim of this study was to determine whether BEA affects the expression profiles of genes coding for key proteins of the mitochondrial chain, both in oocytes and their surrounding cumulus cells, at different time points of IVM culture corresponding to major meiotic stages. Genes that code for subunits of the major mitochondrial chain complexes I, III, IV, and V were analyzed. Whenever possible, per each analyzed complex, the expression of a nuclear and a mitochondrial gene was examined in order to determine whether BEA may act differently on transcription levels of genes from nuclear or mitochondrial DNA.

Materials and methods

Chemicals

All chemicals for in vitro cultures and analyses were purchased from SIGMA-ALDRICH (Milan, Italy) unless otherwise indicated. BEA (SIGMA cod. B7510—5 mg—purity > 97%) was dissolved in Dimethyl sulfoxide (DMSO, SIGMA cod. 276855; 99.9% purity). In order to check the toxin quantity in the vial, BEA has been weighed twice, solubilized in DMSO and vortexed thoroughly. A 50 mmol/L stock solution was obtained. Aliquots of this stock solution were stored at − 20 °C. On the day of experiments, the stock solution was diluted to the final concentrations in a culture medium (Schoevers et al. 2016; Mastrorocco et al. 2019). All experiments were conducted using a single vial.

Collection of ovaries

Ovaries were recovered at a local slaughterhouse from adult ewes (< 1 year) subjected to routine veterinary inspection in accordance with the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications. Ovaries were transported to the laboratory at room temperature within 4 h from slaughtering.

Oocyte retrieval

For cumulus-oocyte complex (COC) retrieval, ovaries underwent follicular fluid aspiration using an 18 G needle followed by slicing (Martino et al. 2012). Follicular contents were released in sterile Petri dishes containing phosphate-buffered saline (PBS). Only COCs with several intact cumulus cell layers and homogeneous cytoplasm were selected for culture. For each experimental condition (a specific BEA concentration or control and a specific IVM time point), 3 replicates (= wells) were performed. In each well, 25 COCs were cultured and analyzed. After culture, COCs were processed as described below. In order to obtain the $t = 0$ samples, part of COCs were denuded immediately after collection, as described below, and oocytes and their related cumulus cells were stored at − 80 °C in lysis buffer until molecular analysis.

In vitro maturation

In vitro maturation (IVM) was performed as previously reported (Martino et al. 2012). Briefly, TCM-199 medium with Earle's salts was used, buffered with 5.87 mmol/L-(2-hydroxyethyl)-1piperazineethanesulphonic acid (HEPES) and 33.09 mmol/L sodium bicarbonate and supplemented with 0.1 g/L L-glutamine, 2.27 mmol/L sodium pyruvate, calcium lactate pentahydrate (1.62 mmol/L Ca^{2+} and 3.9 mmol/L lactate), 50 μg/mL gentamicin, 20% (v/v) fetal calf serum (FCS), gonadotropins (10 μg/mL ovine follicle-stimulating hormone (FSH), and 20 μg/mL ovine luteinizing hormone (LH) and 1 μg/mL 17β estradiol. COCs were placed in 400 μL of IVM culture medium/well of a four-well dish (Nunc Intermed, Roskilde, Denmark), covered with pre-equilibrated lightweight paraffin oil and cultured in vitro for 24 h at 38.5 °C under 5% CO_2 in air. On the day of experiments, BEA stock solution was diluted to the final concentrations of 0.5, 1, 3, and 5 μmol/L in IVM medium. These concentrations were selected on the basis of previous studies reporting the in vitro effect of BEA in several cell lines (Mallebrera et al. 2018; Maranghi et al. 2018), porcine, and ovine oocytes (Schoevers et al. 2016; Mastrorocco et al. 2019) and in the study from Escrivá et al. (2018) on effects of BEA on expression of genes involved in the OXPHOS and electron transport chain in Jurkat cells. Moreover, an in vivo study (Mei et al. 2009) reported the same concentrations of BEA in rat plasma

after oral ingestion of BEA-contaminated feed. Seven and 24 h after IVM, COCs underwent cumulus cell and oocyte separation by incubation in TCM-199 with 20% FCS containing 80 IU hyaluronidase/mL and aspiration in and out of finely drawn glass pipettes. Oocytes denuded at 24 h were evaluated under a stereomicroscope and only those showing the first polar body extruded (MII oocytes) and their corresponding cumulus cells were selected and analyzed separately. Samples were stored at − 80 °C in a lysis buffer for subsequent use.

RNA extraction

Total RNAs from control and BEA-exposed oocytes and cumulus cells were isolated using the RNAeasy Mini Kit (Qiagen, Valencia CA, USA) as per manufacturer's instructions. Total RNA (18 μL from each sample) was kept at 70 °C for 5 min and then chilled on ice. Ten microliters of RNA were used for reverse transcription-polymerase chain reaction (RT-PCR). Reverse transcription was performed in a volume of 20 μL, consisting of 10 μL of samples and 10 μL of a mastermix containing 4 μL 5 × 1st strand buffer, 0.4 μL random primers (0.09 IU/ml), 0.2 μL RNAse (40 IU/ml), 0.75 μL Superscript III (200 IU/ml) (Invitrogen, Groningen, The Netherlands), 2 μL dithiothreitol (0.1 mol/L), 1 μL dNTP mix (10 mmol/L), and 1.65 μL H_2O. The mixture was incubated at 50 °C for 1 h. As a negative control, reverse transcriptase was replaced by H_2O (-RT blanks). Samples were subsequently kept at 80 °C for 15 min and stored at − 20 °C.

Quantitative real-time PCR assay

The list of genes selected for the study, with an indication of their nuclear or mitochondrial DNA localization, and their coded protein, is indicated in Table 1. Gene-specific primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) using software default criteria with predicted products ranging from 100 to 400 bp. Primer sequences used in the qRT-PCR analyses are presented in Online Resource 1. Primer amplification efficiency was determined from standard curves generated by serial dilutions of cDNA (5-fold each) for each gene in triplicate. Real-time amplification reactions were performed in 96 well plates using SYBR Green detection chemistry and run in triplicate using the real-time PCR detection system (MyiQ Single-color, Real-Time Detection System; Bio-Rad). Reactions were prepared in a total volume of 25 μL containing the following: 1 μL cDNA, 0.125 μL forward and reverse primers (0.5 μmol/L each; Isogen, Maarssen, The Netherlands), 12.25 μL iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA), and 11.25 μL H_2O. Non-template controls were also included for each primer pair, replacing the template by H_2O. The cyclic conditions were set as follows: initial denaturation step of 95 °C for 3 min to activate the DNA

Table 1 Mitochondrial activity–related genes: gene symbol, description, corresponding protein site and role and chromosomal location on ovine genome

Gene Symbol	Gene name	Protein site and role	Location
TFAM	Mitochondrial transcription factor A	Maintenance and stability of the mitochondrial genome, initiation, and regulation of mTDNA transcription	Chr. 25
MT-ND1	Mitochondrially encoded NADH dehydrogenase 1	Subunit of the hydrophobic membrane arm of the complex I. Participation in proton translocation from the mitochondrial matrix to the inter-membrane space	mTDNA
NDUFA12	NADH: ubiquinone oxidoreductase subunit A12	Accessory subunit of complex I. Required either as a late step in the assembly of complex I or in the stability of complex I itself	Chr. 3
UQCRH	Ubiquinol-cytochrome c reductase hinge protein	Subunit 6 of the complex III, also known as mitochondrial hinge protein. Essential for cytochrome c1 and cytochrome c complex formation	Chr. 1
MT-COX1	Mitochondrially encoded cytochrome c oxidase I	Subunits forming the catalytic core of the complex IV together with COX3. Containing the redox metal active centers of the enzyme.	mTDNA
MT-COX2	Mitochondrially encoded cytochrome c oxidase II	Subunits forming the catalytic core of the complex IV together with COX3. Containing the redox metal active centers of the enzyme.	mTDNA
COX4	Cytochrome c oxidase subunit 4	Subunit of the complex IV. Involved in the optimization of the electron transfer chain under different conditions	Chr. 14
MT-ATP6	Mitochondrially encoded ATP synthase 6	Subunit within the non-catalytic transmembrane F_o portion of the complex V. Key role in the coupling of the proton flow across the membrane with the rotation	mTDNA
MT-ATP8	Mitochondrially encoded ATP synthase 8	Core subunit of the F_o component of the complex V.	mTDNA
ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	Subunit of complex V. Transmission of conformational changes and proton conductance.	Chr. 1

Chr, chromosome; *mT*, mitochondrial

polymerase, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing at a specific temperature for each primer for 10 s and 72 °C for 20s. Melting curves were generated by temperature increments of 0.5 °C from 65 to 95 °C at steps of 5 s. Standard curves made on cDNA dilutions were used to calculate the relative starting quantity of each experimental sample. Data normalization was performed by using the ratio of the relative starting quantity of the target gene with the reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Statistical analysis

Gene expression data were compared by ANOVA one-way using a threshold of $p < 0.05$. Per each gene, data obtained in control conditions (absence of BEA) were compared among the three time points (GV vs MI vs MII). To evaluate the effects of BEA, per each meiotic stage (MI and MII), data were compared between BEA-exposed and controls.

Results

In the absence of BEA (controls), cumulus cells and oocytes showed a different pattern of gene expression during meiosis. In cumulus cells, the expression of almost all genes dramatically increased from the GV stage to the MI stage but subsequently decreased at the MII stage (Fig. 1a). This was observed for all genes except *NDUFA12* that showed a similar pattern of expression but with no statistically significant difference. Instead, in oocytes, the expression of the same genes increased continuously from the GV through MI to the MII stage (Fig. 1b). This was observed for all genes except *ATP8* which did not show statically significance. In detail, the highest increases of mRNA relative abundance ($p < 0.001$) were recognized for *TFAM*, *ND1*, *ATP6*, and *ATP5O* in cumulus cells and for *TFAM*, *NDUFA12*, *ATP6*, and *ATP5O* in oocytes. Intermediate upregulation ($p < 0.01$) was observed for *UQCRH* and *COX1* in cumulus cells and for *ND1*, *UQCRH*, *COX1*, and *COX2* in oocytes. Finally, a mild increase ($p < 0.05$) was observed for *COX2*, *COX4*, and *ATP8* in cumulus cells and for *COX4* in oocytes.

Upon BEA exposure, the expression of all examined genes was altered, and this occurred only at the MI stage. The most serious damage was recorded in cumulus cells, in which the expression of four genes (*ND1*, *UQCRH*, *COX4*, and *ATP5O)* was significantly downregulated (Fig. 2a) and the expression of six genes (*TFAM*, *NDUFA12*, *COX1*, *COX2*, *ATP8*, and *ATP6*) was significantly upregulated compared with the expressions of the control at the MI stage (Fig. 2a). Interestingly, for downregulated genes, the described scenario was observed starting from the lowest tested concentration (0.5 μM) whereas, for upregulated genes, starting from the concentration of 1 μM. In cumulus cells, the highest overexpression levels occurred at 3 μM.

In oocytes, the expression of all genes was significantly upregulated in exposed MI oocytes versus controls. In detail, *ND1*, *UQCRH*, *COX4*, and *ATP5O* were upregulated even at the lowest tested BEA concentration (Fig. 2b). Interestingly, these genes were the same which were found downregulated in cumulus cells. In addition, in oocytes, the expression of *TFAM*, *NDUFA12*, *COX1*, *COX2*, *ATP8*, and *ATP6* was upregulated starting from 3 μM BEA (Fig. 2b). Noteworthy, *TFAM* was found to be the most significantly upregulated gene, either in cumulus cells (three times) and in oocytes (four times). The expression of all the tested genes did not significantly vary, both in oocytes and cumulus cells, between exposed MII COCs versus controls (data not shown).

Discussion

Recent studies have highlighted the increased scientific interest in BEA which resulted in more analyses of its presence in food and feed, in terms of variety of contaminated food and feed ingredients or in terms of concentrations. Moreover, due to its dual nature, BEA is considered a potential drug in medicine and agriculture. Therefore, humans and animals could become exposed to high levels of BEA underlining the need to fill the gap in knowledge relative to its reproductive toxicology. In particular, concerning female reproduction, it is important to clarify BEA effects on COC mitochondrial functionality since mitochondria are main targets of BEA. Functional changes in mitochondria, induced by mycotoxins, can disturb metabolic programming during oocyte maturation. In the present study, we demonstrated that BEA-induced impaired expression of genes encoding for key proteins of the mitochondrial respiratory chain in the two components, somatic (cumulus cell) and germinal (oocyte), of the COC. In control conditions, during meiosis, in cumulus cells, the highest expression levels of such genes were recorded at the MI stage and, subsequently, their expression levels regressed to lower values at the MII stage. These data are in line with the well-known role of cumulus cells which, at the MI stage, provide support to the oocyte with the supply of energetic nutrients, ATP and regulatory factors required during the maturation. Conversely, in oocytes, the expression of the same genes increased progressively, from the GV to the MII stage. This different transcriptional behavior is in line with the concept that, at this stage, the female gamete needs energy to face fertilization and embryo development. To the best of our knowledge, this is the first study examining the expression of genes coding for proteins of the mitochondrial chain in

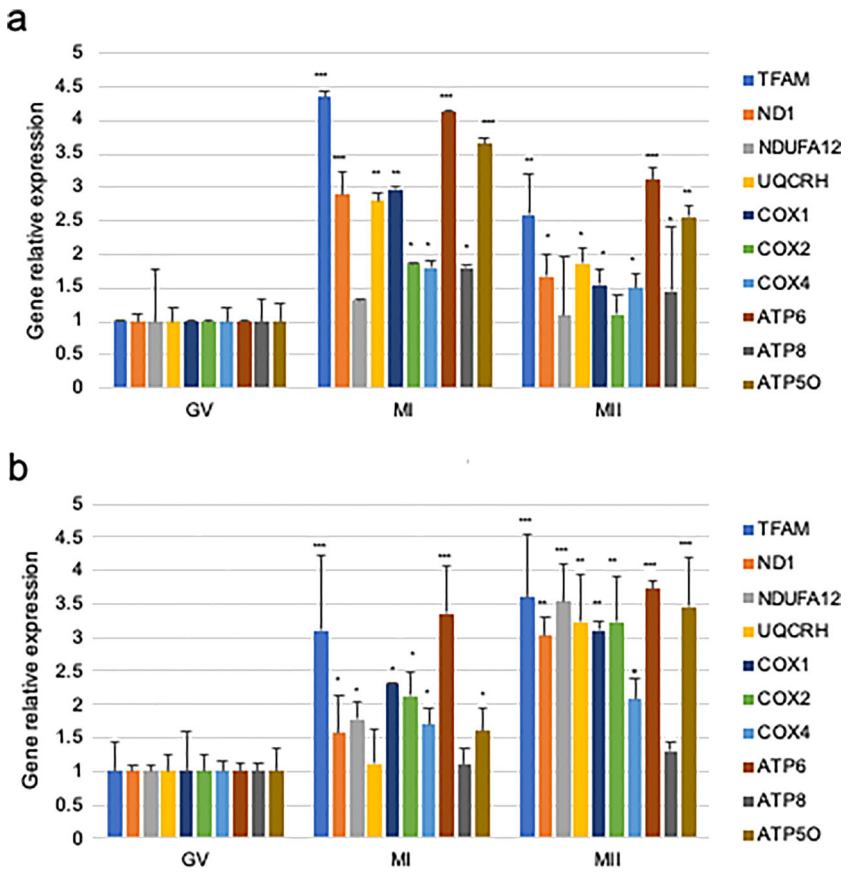

Fig. 1 Relative expression of genes of the respiratory chain in control cumulus cells (**a**) and oocytes (**b**) at different time points ($t = 0$, GV stage, $t = 7$ h MI stage and $t = 24$ h MII stage) of IVM culture. In each histogram, data are derived from 3 replicates and, in each replicate, 25 cumuli (**a**) and 25 oocytes (**b**) were analyzed. One-way ANOVA: per each gene, data were compared between GV and the other stages (MI and MII); $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

ovine oocytes and cumulus cells at different time points of IVM culture.

Upon BEA exposure, the expression of genes coding for proteins of complexes I, III, IV, and V was altered, both in oocytes and in cumulus cells. This evidence brings greater relevance to mitochondria as a target site for BEA-induced cytotoxicity in the female reproductive cell (Schoevers et al. 2016; Mastrorocco et al. 2019). In particular, in cumulus cells, the expression of four genes (*ND1*, *UQCRH*, *COX4*, *ATP5O*) was downregulated at the MI stage when cumulus cells are characterized by physiologically high mitochondrial activity, necessary to sustain oocyte meiosis resumption. These results are consistent with those by Escrivá et al. (2018) who reported that BEA downregulates several genes related to respiratory chain functionality in Jurkat cells including those of the ATP synthase subunits. *ND1* expression is essential in complex I formation (Lim et al. 2016). The failure of complex I can lead to a block of the electron and proton transfer (Duchen 2004). Protons that leak back across the mitochondrial inner membrane and into the matrix reduce the inner membrane potential (Lemarie and Grimm 2011). Downregulation of the *UQCRH* gene, essential for cytochrome c1 and cytochrome c complex formation, may lead to a reduction of electron and proton transfer (Park et al. 2017). Interestingly, apart from the four downregulated genes in cumulus cells, upregulation was observed for all other genes both in oocytes and in cumulus cells. Particularly, in oocytes, all examined genes were found as upregulated. This may be interpreted as a protective response of the COC against a damage occurring at a crucial step of oocyte maturation, the MI stage, when the oocyte must gather all its energy to perform the crucial task of genome haploidization. Given the ionophoric BEA properties and given its known ability to create micropores in the inner mitochondrial membrane (Mallebrera et al. 2018), BEA can possibly cause direct damage to respiratory complex structures and thus the COC may respond with an overexpression of the genes coding for such complexes. The expression of *TFAM* was found to be upregulated at the MI stage, both in cumulus cells and oocytes. These observations suggest that the upregulation of this gene during the MI stage is necessary, at least for increasing the production of OXPHOS proteins, to stabilize and protect mtDNA from oxidative damage (Xu et al. 2009; Chakrabarty et al. 2014). It is important to note that the increased expression of *TFAM* had no compensatory effect on the expression of the mitochondrial gene *ND1* probably because this gene undergoes additional mechanisms of transcription regulation or because damage directly to mtDNA occurred at the expense of this gene.

In conclusion, BEA indistinctly changed the expression of nuclear and mitochondrial genes and coding for all the four

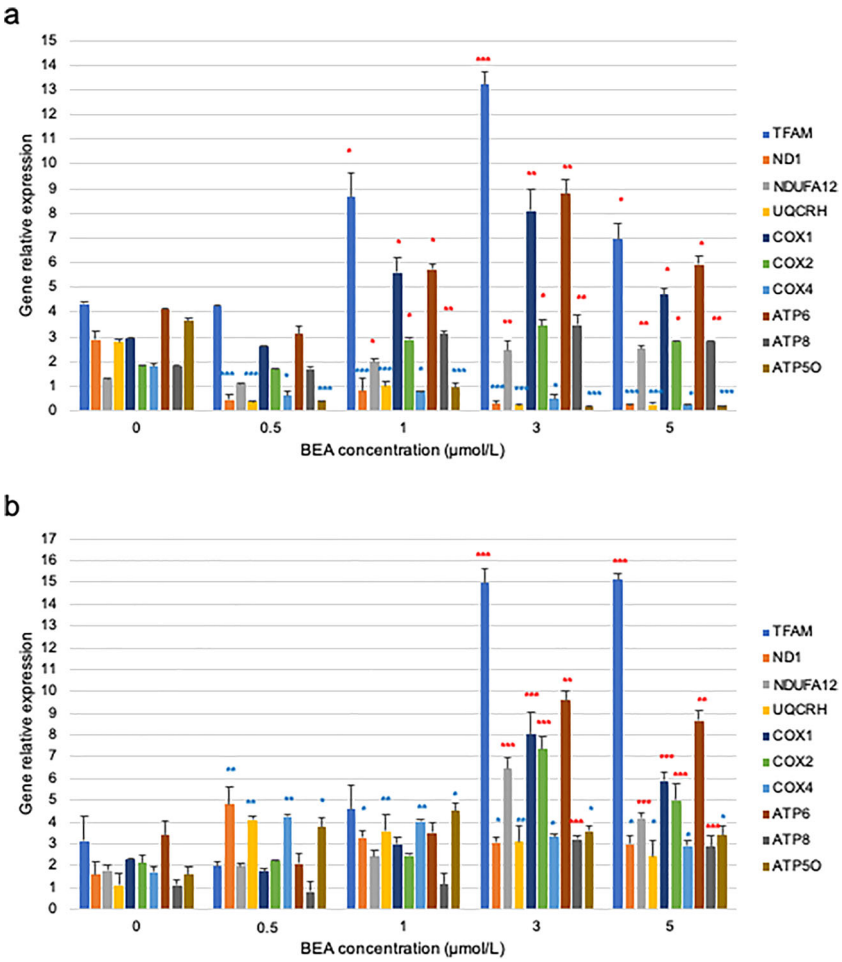

Fig. 2 Relative expression of genes *TFAM*, *ND1*, *NDUFA12*, *UQCRH*,*COX1*, *COX2*, *COX4*, *ATP8*, *ATP6*, and *ATP5O* in ovine cumulus cells (**a**) and oocytes (**b**) at $t = 7$ h of IVM culture (MI stage) in presence of BEA at different concentrations (0, 0.5, 1, 3, and 5 μmol/L). In each histogram, data are derived from 3 replicates and, in each replicate, 25 cumuli (**a**) and 25 oocytes (**b**) were analyzed. One-way ANOVA: per each gene, data of each concentration were compared between BEA-exposed and control; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Blue stars indicate significances of genes which behaved differently in cumulus cells (downregulated) and in oocytes (upregulated), after BEA exposure. Red stars indicate significances of genes which behaved similarly (upregulated) in cumulus cells and oocytes

mitochondrial chain complexes. A major toxic event induced by BEA on the COC is the downregulation of the expression of genes *ND1*, *UQCRH*, *COX4*, and *ATP5O* in cumulus cells which may result in an alteration of the electron transport chain. The other genes were found to be upregulated possibly as an effort of the COC to counteract the damage, avoid autophagy, and keep constant ATP levels. The results of the present study provide a contribution to the knowledge of the mechanisms of BEA effects in the COC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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