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Toxicity of beauvericin on porcine oocyte maturation and preimplantation embryo development

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ABSTRACT

Beauvericin (BEA) is one of many toxins produced by *Fusarium* species that contaminate feed materials. The aim of this study was to assess its effects on porcine oocyte maturation and preimplantation embryo development. Cumulus-oocyte-complexes and developing embryos were exposed to BEA and cultured until the blastocyst stage. Cumulus cells, oocytes and embryos were examined for viability, progesterone synthesis, multidrug resistance protein (MDR1), ATP content and gene expression related to MDR1 function, oxidative phosphorylation, steroidogenesis and apoptosis. BEA was toxic in embryos, oocytes and cumulus cells at concentrations exceeding 0.5 μM, and embryos were most vulnerable after the four-cell stage. Since BEA exerted different effects in embryos, oocytes and cumulus cells, the toxic mechanism is suggested to involve different pathways. Currently there are no consistent data on adverse effects of BEA in pig farms.

1. Introduction

Mycotoxins are secondary metabolites produced worldwide by fungi under certain environmental conditions. These toxins are common contaminants in raw food materials, have diverse chemical properties and toxicity, and may present a risk for animal and human health [1]. Beauvericin (BEA) has been increasingly detected as contaminant in cereal products in the past years. The prevalence of feed samples contaminated with beauvericine varies considerably (for detail see EFSA, 2014) and in a recent survey of multi-mycotoxin analysis the contamination rate reached 98% with maximum concentration amounting to 2326 μg/kg [2]. In consideration of these findings, BEA is classified as an emerging mycotoxin [3–5].

BEA is a hexadepsipeptide with ionophoric properties. Even at low concentrations (1–10 μM), this lipophilic molecule can be incorporated into membranes of cells and forms dimeric structures that transport monovalent ions across cellular membranes. Particularly in the mitochondrial membrane this can disturb the ion balance and the cytoplasmic pH [6,7]. In turn, BEA exposure can lead to accumulation of calcium ions in mitochondria, which initiates mitochondrial degeneration and cellular apoptosis [8–10]. Moreover, increased levels of intracellular reactive oxygen species and reduced intracellular glutathione levels have been observed after BEA exposure, as signs of oxidative stress [11–13]. Exposure of human lymphocytes to BEA caused chromosomal aberrations during mitosis [14]. In all mammalian cells, the toxicity of BEA is dose and time dependent [11,12,15–17].

Little is known about reproductive or embryo toxicity of ionophores such as BEA. The ionophoric drug Monensin, which is applied in veterinary medicine in the prevention of coccidiosis in poultry and to improve performance in cattle, did not exert toxic effects in reproduction or development *in vivo* in the recommended doses, but *in vitro* studies revealed that Monensin disturbed the Na-Ca exchange in oocytes and inhibited gap junction assembly in preimplantation embryos [18,19]. However, no functional studies have been done to determine the toxicity of ionophores in maturing oocytes and developing preimplantation embryos. There are indications that BEA may affect reproductive performance in pig, since this toxin reduced granulosa cell function *in vitro*, thereby affecting the quality of the oocyte enclosed by these cells [20]. Moreover, by its ionophoric properties BEA may give rise to high calcium levels in the ooplasm during maturation and fertilization, possibly leading to mitochondrial dysfunction, disturbed calcium regulated pathways, and even abnormal embryo development [21,22].

Abbreviations: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; BEA, beauvericin; COC, cumulus oocyte complex; DAPI, 4,6-diamino-2-phenyl-indole; EFSA, European Food Safety Authority; EtHD-1, ethidium homodimer-1; IVM, in vitro maturation; M2, metaphase II; MDR1, multidrug resistance protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; R123, rhodamine 123; SOF, synthetic oviductal fluid.

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Multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP), the products of the *ABCB1* and *ABCG2* genes, respectively, are energy-dependent efflux pumps that belong to the superfamily of ATP-binding cassette (ABC) transporter [23]. The major physiological role of these efflux transporters is the excretion of intracellular metabolites and xenobiotics. For example, MDR1 is involved in the transport of steroids and their metabolites over the cell membrane [24,25]. Steroid hormone producing cells, such as granulosa cells, express higher levels of *ABCB1* mRNA when they originate from follicles with a large diameter, and MDR1 activity in these cells was increased after exposure to gonadotropins or steroid hormones [26,27]. During maturation, porcine oocytes express more MDR1 activity reaching a maximum at the Metaphase II stage [27,28]. Also in murine 2–8 cell embryos, MDR1 is located in the cell membrane and functionally active [29,30]. These findings suggest that the quality of matured oocytes or developing embryos will partly depend on MDR1 activity in the maturing oocyte and surrounding cumulus cells [31]. BEA was shown to inhibit the MDR1 and BCRP-mediated efflux of fluorescent model substrates *in vitro* and BEA exposure might alter the expression and function of these efflux pumps [32]. It was therefore hypothesized that the quality of oocytes and preimplantation embryos is affected by the presence of BEA.

Hence, the aim of the study was to determine the reproductive and developmental toxicity of BEA by investigating its effect on porcine cumulus cells, oocyte maturation and embryo development. In addition, the effects of BEA on mitochondria and ABC efflux transporters in cumulus cells, oocytes and embryos were investigated.

2. Materials and methods

2.1. Chemicals and culture media

Chemicals were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. BEA (purity >97%) was dissolved in DMSO at a concentration of 50 mM. 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 culture medium and sonicated for 15 min. Medium with 0.02% DMSO was used as a control. Culture media were equilibrated at 5% CO_2 and 38.5 °C for at least two hours before use.

2.2. Selection and culture of cumulus oocyte complexes

Collection and selection of porcine cumulus oocyte complexes (COCs) were as described before [33]. Briefly, ovaries from prepubertal gilts collected from a local slaughterhouse were transported to the laboratory and maintained at 30○C. Antral follicles (2–6 mm in diameter) were aspirated and COCs with a compact cumulus mass were selected, transferred to HEPES-buffered M199 (Gibco BRL, Paisley, UK) supplemented with penicillin and streptomycin, and washed 3 times in oocyte maturation medium (OMM) consisting of M199 supplemented with 2.2 mg/ml $NaHCO_3$, 10% (v/v) sow follicular fluid and 200 μM cysteamine. COCs were cultured for 20–22 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air in groups of 35–50 in 500 μl OMM with 0.05 IU/ml recombinant human FSH (Organon, Oss, The Netherlands), followed by a 20–22 h culture period in OMM without FSH.

2.3. Electrical activation of oocytes and parthenogenetic embryo development

At the end of 40–44 h COC culture, cumulus cells were removed by repeated pipetting and denuded oocytes were transferred to modified Tris-buffered medium [34] and kept for 1–2 h at 5% CO_2, 38.5 °C. The oocytes were washed three times in activation medium consisting of 0.3 M Mannitol, 1 mg/ml PVP, 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 0.52 M HEPES. Oocytes were transferred between electrodes connected to an electrical pulsing device (FC-150, BLS Ltd, Budapest, Hungary) covered by a 0.2-ml drop of the activation medium. Oocytes were activated by 2 × 80 μsec consecutive pulses of 1.0 kV/cm DC. After activation, oocytes were directly transferred to synthetic oviductal fluid (SOF) [108.5 mM NaCl, 7.2 mM KCl, 1.2 mM KH_2P0_4, 0.74 mM $MgS0_4$. $7H_20$, 25 mM $NaHC0_3$, 1.8 mM $CaCl_2.{}_2H2O$, 3.2 mM NaLactate, 0.33 mM NaPyruvate, essential and non-essential amino acids (Gibco, Life Technologies, Breda, The Netherlands)] with 4 mg/ml BSA (Probumin; Celliance, Kankakee, IL, USA) as protein source [35]. To induce formation of diploid embryos, activated oocytes were cultured for 3 h in SOF with 5 μg/ml cytochalasin B, followed by a culture period of 7 days in SOF at 5% CO_2 and 7% O_2 in a humidified atmosphere at 38.5○C [36]. After 7 days, collected embryos were stored at −80 °C until further analysis.

2.4. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

For concentration range finding, HT-29 cells (human colon carcinoma; ATCC HTB-38^T) were used as surrogates. Cells were cultured in McCoys 5A with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in a 96 well plate until confluency. The cells were subsequently cultured in the same medium containing 0–10 μM BEA for 24–168 h in 5% CO_2 at 37 °C. Media were refreshed every 48 h. After culture, 50 μg MTT was added per well and incubated for 4 h. Cells were subsequently lysed with isopropanol and light absorption was measured at 595 nm using a microplate reader (Zyneth DTX880; Molecular Devices, Wokingham, Berkshire, UK).

2.5. Alamar blue assay

COCs or developing embryos were exposed for 44 h to 0–10 μM BEA. Subsequently, cumulus cells and oocytes were separated and allowed to metabolize Resazurin for 4 h in the presence of BEA. Developing embryos were cultured for 44 h after the onset of activation in the presence of 0–2.5 μM BEA and allowed to metabolize Resazurin within the following 4 h. Fluorescence was subsequently measured with a microplate reader (Fluostar Optima; BMG labtechnologies, Offenburg, Germany) at 545 nm excitation and 590 nm emission wavelength. Sample signals were corrected for background and expressed as percentage of the signal from the control sample.

2.6. Assessment of cumulus morphology and viability

The diameters of COCs were determined at 0 and 22 h after culture from digitized images using Image J and converted to mm^2 [37]. Per group of cultured COCs, the average of projected surface areas at 22 h was divided by those at the start of culture, resulting in the fold increase of COC surface area. At 44 h of culture, COCs were incubated with 2 μM Ethidium Homodimer-1 (EtHD-1; Invitrogen-Molecular Probes, Eugene, OR, USA) in OMM and cultured for another 4 h. COCs were washed 3 times in PBS and fixed in 2% (w/v) formaldehyde. Subsequently, COCs were incubated for 5 min in 0.1 μg/ml 4,6-diamino-2-phenyl-indole (DAPI; Invitrogen-Molecular Probes), mounted under a coverslip with anti-fade mounting medium (Vectashield; Vectorlab, Burlingame, CA, USA), and imaged by confocal laser scanning microscopy (CLSM; Leica SPE-II, Heidelberg, Germany). Imaging was performed using a 368- and 543-nm laser to excite DAPI and EthD-1, respectively. Nuclei of cumulus cells were considered degenerated when stained

for both DAPI and EthD-1, or when nuclei had a condensed or fragmented appearance.

2.7. Assessment of nuclear development in oocytes

Oocytes were fixed in PBS with 2% (w/v) formaldehyde, washed in PBS, stained with 0.1 μg/ml DAPI, mounted in Vectashield on slides and examined with a fluorescence microscope. Oocytes containing a metaphase spindle together with a polar body were considered to be at the Metaphase II (M2) stage. After 24 h electrical activation, oocytes having one polar body together with two separate or fused pronuclei, or embryos with two blastomeres were considered as activated.

2.8. Progesterone assay

Concentrations of progesterone in COC-conditioned maturation medium collected from five replicates were determined using commercially available diagnostic radioimmunoassay kits (IM1188; Beckman Coulter, Brea, CA, USA). Following storage at −20 °C, samples were thawed immediately and diluted ten times before the assay was conducted. To avoid inter-assay variation all samples were analyzed in a single kit. To exclude possible interactions of BEA with the progesterone receptor, unconditioned medium containing 0–10 μM BEA was analyzed. Before performing statistical analysis, progesterone levels found in conditioned media were corrected for levels determined in unconditioned media.

2.9. Quantitative RT-PCR

Total RNA was extracted with an RNAeasy Mini Kit (Qiagen, Valencia CA, USA) as per manufacturer's instructions. Per experimental group, in five replicates, RNA samples consisted of pooled cumulus cells derived from 50 COCs, 50 oocytes and 4–18 pooled blastocysts. Total RNA (10 μl) was kept at 70 °C for 5 min and then chilled on ice. Ten μl of a mastermix containing 4 μl 5 × 1st strand buffer, 0.4 μl random primers (0.09 IU/ml), 0.2 μl RNAsin (40 IU/ml), 0.75 μl SuperscriptIII (200 IU/ml) (Invitrogen, Groningen, The Netherlands), 2 μl dithiothreitol (0.1 M), 1 μl dNTP mix (10 mM) and 1.65 μl H_2O were added and 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 70 °C for 15 min and stored at −20 °C.

The PCR mixture contained 0.1 μl cDNA, 0.1 μl forward and reverse primers (100 μM each) (Isogen, Maarssen, The Netherlands), 10 μl iQ SYBR Green supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 9.8 μl H_2O. The oligonucleotide primers (Supplemental Table 1) were designed using Primer Select software (DNAstar, Madison, WI, USA); After an initial denaturation step at 95 °C for 3 min, 40 cycles were carried out each consisting of 95 °C for 5 s, the primer specific annealing temperature (Supplemental Table 1) for 5 s, and 72 °C for 20 s. Quantitative RT-PCR was performed in duplicate on two replicates of cDNA and singular on the −RT blanks. For each target gene all the samples were quantified simultaneously in one run in a 96-well plate using a real-time PCR detection system (MyiQ Single-color, Real-Time Detection System; Bio-Rad). Melting curves were plotted to verify single product amplification. 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 geometric mean of two reference genes (*BACT* and *B2M*, cumulus cells; *GAPDH* and *PGK1*, oocytes and embryos).

2.10. MDR1 function: Rhodamine 123 accumulation-efflux assay

At the onset of activation oocytes were exposed to 0–2.5 μM BEA for 3 h, rinsed in Hanks buffered salt solution (HBSS) and incubated with 20 μM Rhodamine 123 (Rh123; Invitrogen) for 30 min. Afterwards oocytes were rinsed 2 times in HBSS and allowed to release Rh123 for 30 min in HBSS. Uptake and release of Rh123 was performed in presence of 0–2.5 μM BEA, whereas 10 μM of Verapamil was used as positive control [38]. Fluorescence was determined with an inverted fluorescent microscope and quantified from digitized images using Image J v1.47 image analysis software (NIH, Bethesda, MD, USA). Efflux of Rh123 was calculated as: % efflux = 100% − ((fluorescence at 30 min efflux/fluorescence at 0 min efflux)*100%).

2.11. ATP analysis

Adenosine triphosphate (ATP) content was measured by luminescence generated from the ATP-dependent luciferin-luciferase bioluminescence assay (ATP Determination kit, A22066; Invitrogen-Molecular Probes) in the line with the manufacturer's instructions: serial dilutions of ATP were prepared ranging from 0.1 to 1000 nM. The reaction solution contained 0.5 mM D-luciferin, 1.25 μg/ml firefly luciferase, 25 mM Tricine buffer pH 7.8, 5 mg $MgSO_4$, 100 μM EDTA and 1 mM dithiothreitol. Oocytes were lysed in 50 μl 0.1 M Tris/HCl pH 7.4, 0.5% (w/v) Triton X-100 and 5 mM β-mercaptoethanol. Serial ATP dilutions, reaction solution and lysates were kept on ice. In a 96 well Plate 20 μl ATP solution was added to 180 μl reaction mixture, mixed and equilibrated for 5 min at 28 °C. Luminescence was measured using a luminometer (Zyneth DTX880; Molecular Devices) for 1 min. A standard curve was generated from the relative light intensity of the serial dilutions and used to determine ATP content from the samples. ATP content per oocyte was calculated by dividing the total concentration by the number of oocytes in the sample. Experiments were repeated five times, with 30–40 oocytes per sample.

2.12. Mitochondrion-actin-chromatin analysis

Two days after oocyte activation, embryos were washed in 80 mM PIPES, 5 mM EGTA, 2 mM $MgCl_2$, pH 6.8, supplemented with 0.3% (w/v) PVP (PEM-PVP). After washing, embryos were fixed in PEM-PVP containing 4% (v/v) formaldehyde at room temperature (RT) for 1 h and stored at 4 °C in PEM-PVP with 1% formaldehyde. Embryos were rinsed two times in PBS with 0.3% (w/v) PVP (PBS-PVP) and thereafter permeabilized in PBS-PVP with 0.1% (v/v) TritonX-100 for 10 min. To block non-specific binding and to reduce levels of free aldehydes, embryos were kept in PBS with 2% (v/v) normal goat serum (PBS-NGS) and 100 mM glycine for 2 h RT. To label mitochondria, embryos were incubated for 1 h with mouse monoclonal antibodies anti-COX IV (ab 14744; Abcam) diluted 1:100 in PBS-NGS followed by rinsing 3 x in PBS-PVP with 0.1% (v/v) Tween-20. The cells were subsequently incubated for 1 h with Alexa Fluor 488-labelled goat anti-mouse antibodies (Invitrogen) 1:100 diluted in PBS-NGS and rinsed 3x in PBS-PVP with 0.1% (v/v) Tween-20. To label F-Actin, embryos were incubated with Alexa Fluor 568-labelled phalloidin (8 IU/ml) in PBS-PVP for 20 min at RT. Chromatin was labelled by incubation with 1 μM DAPI (Invitrogen-Molecular Probes) in PBS-PVP for 5 min at RT. After staining, embryos were mounted under a coverslip with Vectashield (Vector Lab). Fluorescence microscopy was performed on a SPE-2/DMI 4000 Leica confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with 405, 488 and 561 nm diode lasers. Images were acquired sequentially using standard settings for Alexa Fluor488, Alexa Fluor568 and DAPI.

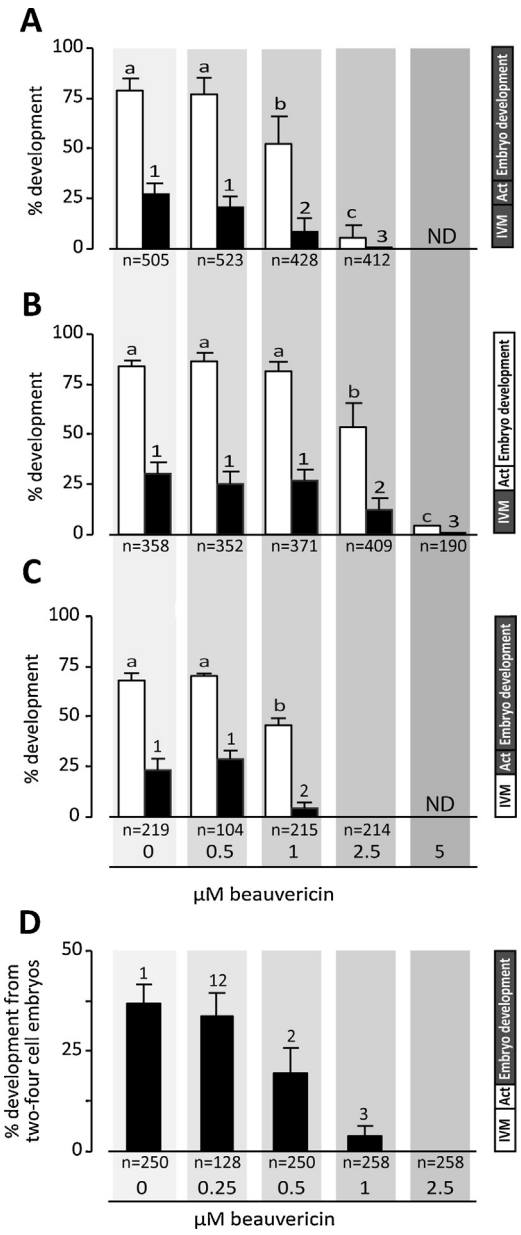

Fig. 1. Development of parthenogenetic embryos after exposure to beauvericine (BEA). Oocytes and embryos were exposed to BEA during oocyte maturation, activation and embryo development. Bars aside graphs represent exposure schemes where black indicates presence and white absence of toxins. Embryo development was expressed by proportions of two-four cell stage (white bars) and blastocysts (black bars) developing from activated oocytes (A,B,C) or two-four cell stage embryos (D). Numbers under bars represent the total number of activated oocytes or two-four cell embryos from 5 replicates. Bars represent means and standard error of the means. Bars with different letters (abc) or numbers (123) are significantly different ($P < 0.05$).

2.13. Statistical analysis

Statistical analysis was conducted with SPSS software (SPSS Inc., Chicago, IL, USA). Following a normal distribution, cumulus projected surface areas, reduction of Resazurin, progesterone quantity in culture medium, normalized RNA quantities and Rh123 fluorescence in zygotes were analyzed by ANOVA with the model $y = \alpha + [BEA]$, where α = intercept and [BEA] was used as independent categorical variable. Following a binomial distribution, embryo development, oocyte meiosis, fractions of EtHD-1 stained cells were analyzed by a generalized linear model: log $\pi/(1-\pi) = \alpha +$ toxin-exposure, where π=frequency of positive outcome, α = intercept and toxin-exposure was an independent categorical variable. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity of BEA on HT29 cells

To establish working concentrations and hence differentiate specific reproductive toxicity from unspecified cytotoxicity of BEA in maturing oocytes and developing embryos, HT29 cells were exposed to BEA at concentrations ranging from 0.31 to 10 µM for 24–168 h. HT29 cells were selected as surrogates as they also express different efflux transporters, including MDR-1. A time and concentration-dependent toxicity was observed, as cytotoxicity increased at concentrations exceeding 5 µM and at concentrations between 2.5 and 5 µM a time dependent increase in cytotoxicity was observed (Fig. S1). From these data the working concentration for the following experiments were deduced.

3.2. Exposure to BEA impairs developmental competence of oocytes and embryos.

To determine the dose-response of BEA exposure during oocyte maturation and early embryo development, oocytes and embryos were exposed to various concentrations of BEA during different time periods. The oocytes were matured *in vitro*, activated using an electrical pulse and cultured for six days to the blastocyst stage. Activation of oocytes to commence embryo development was induced with an electrical pulse rather than fertilization with a sperm cell to exclude a possible sperm effect. Exposure to BEA occurred during three periods: oocyte maturation, activation, and embryo development, in different combinations. When maturing oocytes and developing embryos were exposed continuously to BEA at concentrations of >1 µM, significantly lower percentages of embryos at the 2–4 cell stage (day 2 of embryo culture) and blastocyst stage (day 6 of embryo culture) were observed (Fig. 1A). When exposure to BEA was restricted to oocyte maturation only, oocytes exhibited reduced embryo developmental capacity at concentrations exceeding 2.5 µM (Fig. 1B). When matured oocytes were exposed to BEA for six days from the onset of activation, similar percentages of embryo development were observed as after exposure during both maturation and embryo development; the percentages of developing embryos decreased when BEA concentrations exceeded 1 µM (Fig. 1C). When embryos were exposed only from the two-four cell stage onwards (exposure during day 1–6 of embryo culture), blastocyst formation was reduced in the presence of 0.5–1.0 µM BEA and no blastocysts developed at a concentration of 2.5 µM BEA (Fig. 1D). The blastocysts that did develop during BEA exposure exhibited no differences in embryo diameter or fraction of apoptotic cells (Fig. S2AB). Moreover, during blastocyst development expression levels of genes related with ABC efflux (*ABCB1*, *ABCG2*), mitochondrial function (*MT-CO1*), apoptosis (*BCl2*, *BAX*, *CASP3*) or autophagy (*BECN*) remained constant (Fig. S2C-I). These results show that BEA reduces the developmental competence of both the maturing oocyte and the two-four cell stage embryo, and that BEA only affected the rate of developing embryos, but not their quality (size, fraction of apoptotic cells). In addition, the obtained results clearly show that maturing oocytes are less sensitive to BEA than activated oocytes or two-four cell stage embryos.

3.3. Beauvericin compromises meiosis and cytoplasmic maturation in oocytes

To determine the dose-response of BEA exposure to oocyte viability and oocyte maturation, COCs were cultured for 44 h in the

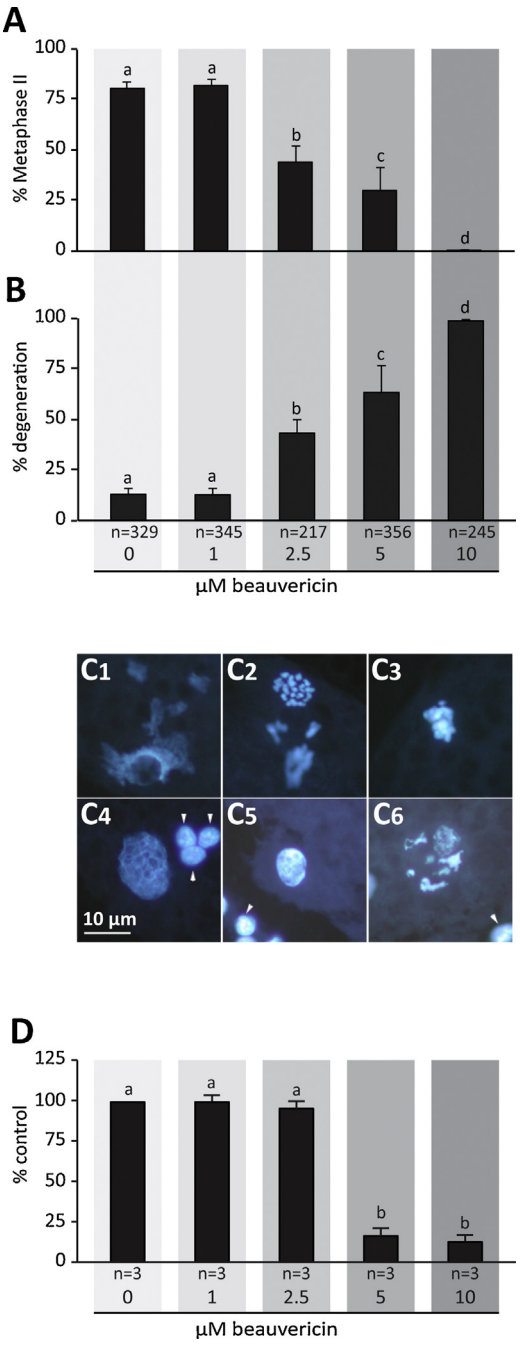

Fig. 2. Progression through meiosis (A), degeneration (B), chromatin structure (C) and mitochondrial activity (D) in porcine oocytes after exposure to beauvericin for 44 h. A and B, numbers under bars represent the total number of oocytes from eight replicates. C, chromatin structures in control oocytes (C1-C3) represent Germinal Vesicle (C1), Metaphase II (C2) and degeneration by clumped chromatin (C3), whereas in BEA exposed oocytes (C4-6) nuclear degeneration is visible. D, reduction of resazurin by beauvericin exposed oocytes as percentage of that of control oocytes. Identical bars with different letters (abcd) are significantly different ($P < 0.05$).

presence of 0–10 μM BEA and oocytes were subsequently examined for progression through meiosis by chromatin distribution with DAPI staining and for viability by reduction of Resazurin. Reduced percentages of M2 oocytes and increased percentages of degenerated oocytes were observed after culture with BEA (Fig. 2AB). Different types of nuclear aberration were observed between oocytes from control COCs and BEA-exposed COCs: in control oocytes condensed chromatin illustrated oocyte degeneration, whereas oval-shaped chromatin with a condensed or disintegrated appearance was observed in BEA-exposed oocytes (Fig. 2C). Reduction of Resazurin by oocytes was diminished after culture with 5 and 10 μM BEA (18% and 14% respectively of control) suggesting a damaged electron transport chain in the mitochondria, whereas at lower BEA concentrations Resazurin reduction was similar to that in control oocytes (Fig. 2D).

The observation that the chromatin morphology of degenerated oocytes that had been exposed to BEA was comparable to that of Germinal vesicle stage oocytes suggests that abnormal progression through meiosis was the result of damage caused by BEA at the start of meiosis. However, M2 oocytes exposed as COCs to 5 μM BEA during maturation did not cleave after electrical stimulation, whereas >95% of M2 oocytes were activated after maturation in the presence of 1 and 2.5 μM BEA, indicating compromised cytoplasmic maturation.

3.4. Beauvericin alters cumulus cell function

To determine the toxicity of BEA to cumulus cells, intact COCs were cultured for 22 and 44 h in the presence of BEA, and cumulus expansion, cell viability, cell membrane integrity and progesterone synthesis were determined. At the start of culture, the mean of the projected surface areas of COCs was $0.036 \pm 0.013\ mm^2$. After 22 h culture cumulus cells exposed to 1–5 μM BEA had expanded their area approximately 2.5-fold, similar to that of control COCs, whereas significantly reduced cumulus expansion was observed in the presence of 10 μM BEA (Fig. 3A). Cumulus viability, as determined by metabolization of Resazurin, compared with non BEA exposed cumulus cells, was significantly reduced to 57% and 37% after 44 h exposure to 5 and 10 μM BEA (Fig. 3B). Cell membrane integrity was determined by EtHD-1 stain and nuclear morphology. COCs exposed to 10 μM BEA revealed a significantly higher percentage of dead cumulus cells (control $3 \pm 3\%$ vs 10 μM BEA $14 \pm 12\%$; $P < 0.05$), and degenerated cells were detected throughout the cumulus cell mass (Fig. 3C). Progesterone synthesis was determined in maturation media after 44 h of BEA exposure. In line with the decreased viability, 10 μM BEA significantly reduced progesterone synthesis after a 44 h exposure period (Fig. 3D).

3.5. Preimplantation embryos become more vulnerable for beauvericin at the four cell stage

To analyze time dependent effects of BEA during embryo development, activated oocytes were exposed to 1 μM BEA for two and four days of culture, or alternatively, two day old embryos were exposed to 1 μM BEA for two days and further cultured towards the blastocyst stage. After two days culture in the presence of BEA, significantly reduced numbers of embryos at the two- and four cell stage were observed (Fig. 4A). When BEA- or non-exposed cleaved embryos were further cultured without BEA 27% and 31% of these embryos (groups NE and 0–2) developed to the blastocyst stage, whereas those further cultured for two days in presence of BEA (groups 2–4 and 0–4) exhibited only 14% and 8% blastocyst development (Fig. 4B). No differences in the numbers of nuclei (Fig. 4C) and the proportions of apoptotic nuclei (Fig. 4D) in blastocysts were observed between the different groups. The results suggest that embryos are more sensitive to BEA after the four cell stage.

3.6. Gene expression in oocytes and cumulus cells

To study the mechanism of toxicity, COCs were cultured for 22 or 44 h in the presence of 0–10 μM BEA, and after separation of cumulus cells and oocytes, gene expression was examined by qPCR. As targeted markers for the ionospheric nature of BEA, expression of genes associated with ABC transporter activity (*ABCB1, ABCG2*), mitochondrial function (*MT-CO1*) apoptosis (*BAX, BCL2, CASP3*) and

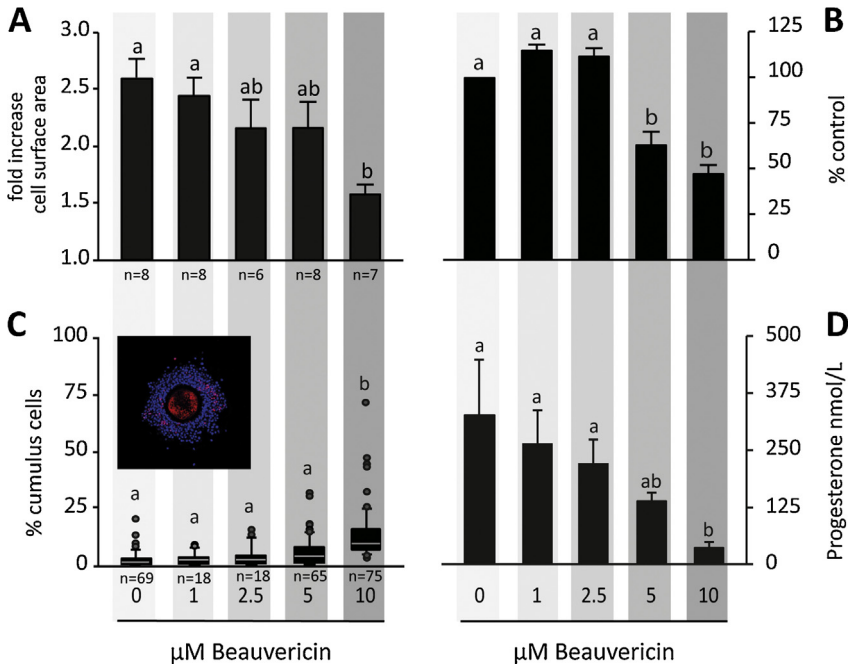

Fig. 3. Expansion (A), mitochondrial activity (B), cell membrane integrity (C) and progesterone synthesis (D) in cumulus cells of porcine COCs exposed to beauvericin during oocyte maturation. Cumulus expansion is expressed as the fold increase of the COC surface area after 22 h of culture with the COC surface area at the start of culture. Mitochondrial activity was recorded with the Alamar blue assay and expressed as percentage of mitochondrial activity in control cumulus cells. Cumulus cell membrane integrity in COCs is expressed as number (EtHD-1 stained nuclei + fragmented nuclei)/number of DAPI stained nuclei * 100% (EtHD-1: red, DAPI: blue; see insert in C). Concentration of progesterone (D) was determined in culture media. Bars or boxplots with different letters (ab) are significantly different ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

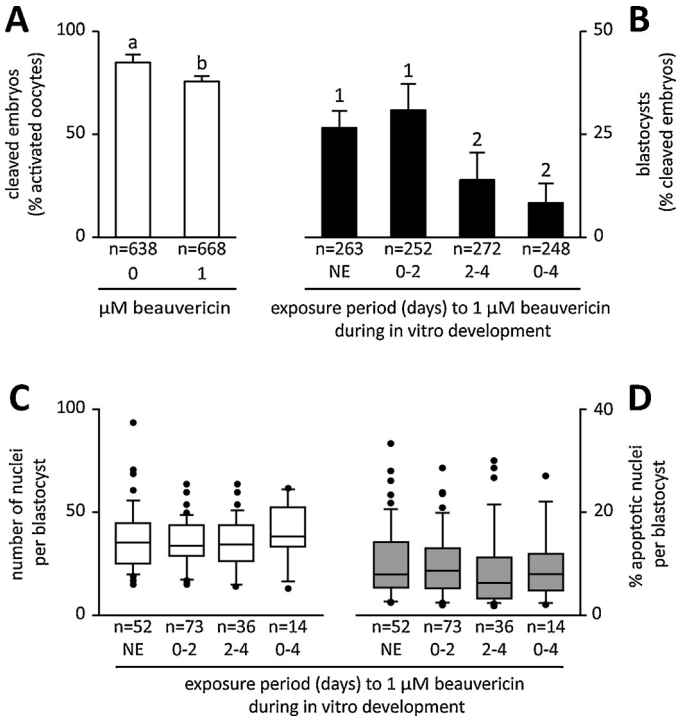

Fig. 4. Development of parthenogenetic embryos. Activated oocytes were exposed to 0 or 1 µM beauvericin (BEA) for two days and analyzed for cleavage (A). Control and BEA exposed cleaved embryos were activated and cultured without BEA (groups NE and 0–2) or with 1 µM BEA (groups 2–4 and 0–4) for two days. After four days culture, all groups were cultured for another two days without BEA and the percentage of blastocysts was determined (B). In cultured blastocysts total (C) and apoptotic (D) nuclei per blastocyst are depicted in boxplots (25–75 percentiles) Bars represent means and standard error of the means and were calculated from 5 replicates. Identical bars with different letters (a,b) or numbers (1,2) are significantly different ($P < 0.05$).

autophagy (*BECN*) were analyzed. Since ionophores can evoke quick responses in cells [39], expression of *ABCB1*, *ABCG2* and *MT-CO1* was also investigated after 4 h BEA exposure. In cumulus cells and oocytes, after a 4 h exposure period, BEA did not significantly influence the expression of *ABCB1, ABCG2* or *MT-CO1* (Fig. 5A-F). After 44 h exposure to BEA, in cumulus cells expression of *ABCB1* increased significantly, while that of *ABCG2* tended to increase and *MT-CO1* expression remained constant (Fig. 5ACE). In oocytes, expression of both *ABCB1* and *ABCG2* genes remained constant, and that of *MT-CO1* increased significantly after 22 h exposure to BEA (Fig. 5BDF). Expression of *BAX1, BCl2, CASP3* and *BECN1* remained similar with increasing BEA concentrations during 22 h exposure in oocytes, whereas in cumulus cells only expression of *BCL2* was significantly increased (Fig. S3).

Since progesterone synthesis in cumulus cells was negatively correlated with the concentration of BEA, expression of genes coding for key enzymes in steroidogenesis was investigated as well. Expression of *CYP11A1*, a gene located on mitochondrial DNA, was significantly increased at the highest concentration of BEA (Fig. 5D), whereas expression of *HSD3B1* and *CYP19A* tended to decrease with higher BEA concentrations (Fig. 5EF). It can be concluded that cumulus cells control intracellular BEA by MDR1 activity, and this was apparently not the case with oocytes, where mitochondrial function was altered. Both in oocytes and in cumulus cells, the pathways leading to apoptosis or autophagy were not activated. Further, there is evidence that BEA will interfere with progesterone synthesis in cumulus cells due to overexpression of *CYP11A1* in mitochondria.

3.7. Beauvericin reduces Rh123 efflux in zygotes

To determine whether BEA can interfere with MDR1 activity, the uptake and efflux of Rh123 was measured in activated oocytes. BEA increased Rh123 accumulation in a concentration dependent manner and reduced the efflux of Rh123 (Fig. 6A-C). Since MDR1

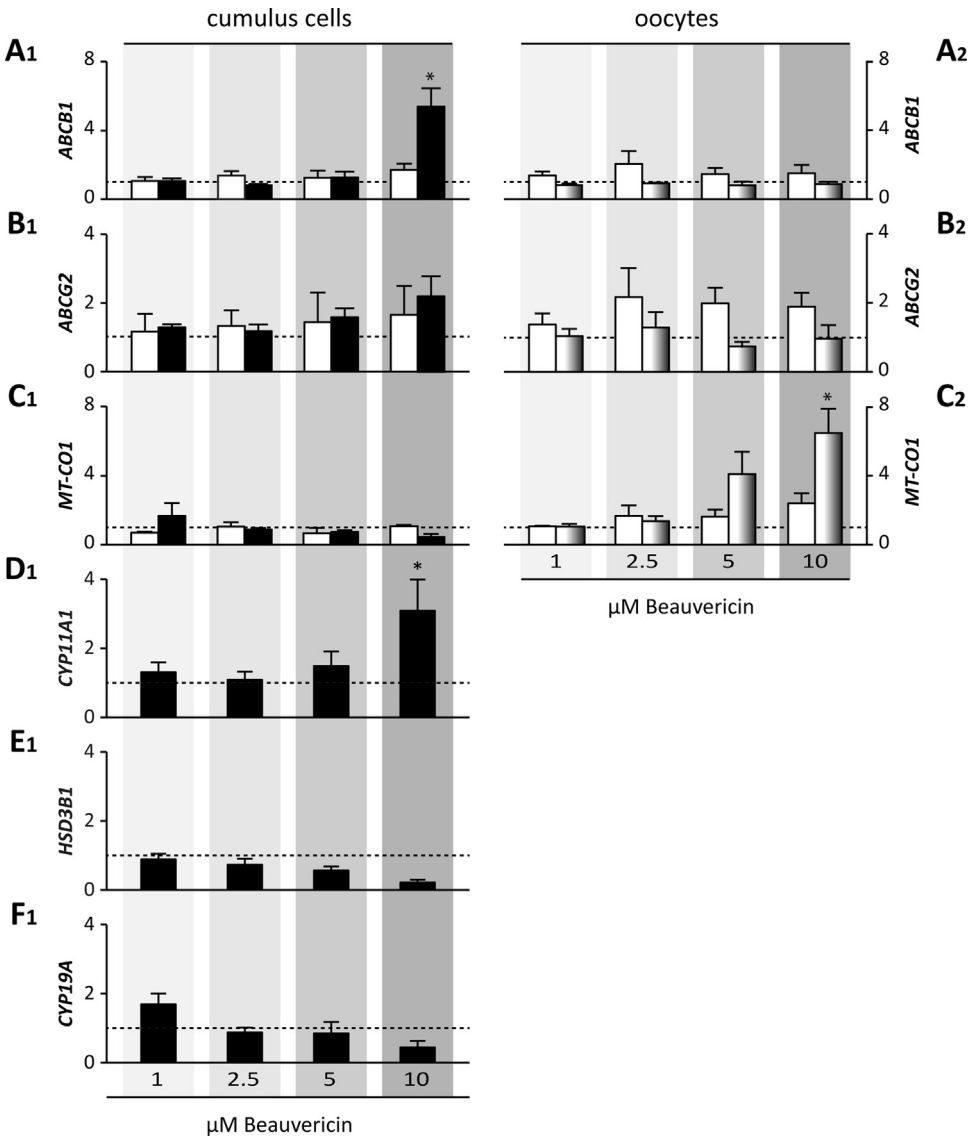

Fig. 5. Relative expression of *ABCB1*, *ABCG2*, *MT-CO1*, *CYP11A1*, *HSD3B1* and *CYP19A* in cumulus cells (A_1-F_1) and maturing oocytes (A_2-C_2). Cumulus cells and oocytes were exposed to beauvericin for 4 h (white bars) and for 22 h (white-black gradient bars) or 44 h (black bars). Experiments consisted of 4–6 replicates. Within identical bars, bars indicated with * are significantly different from control oocytes ($P < 0.05$).

activity is ATP dependent, ATP concentrations in activated oocytes were determined, and BEA tended to reduce ATP levels in zygotes (Fig. 6D). These results indicate that MDR1 activity is reduced by BEA as a result of lower ATP levels and the developing embryo would become more sensitive to external stimuli.

3.8. Beauvericin alters embryo development before the four-cell stage

It has been suggested that the distribution of mitochondria and actin in blastomeres indicates the developmental capacity of embryos [40,41]. Since BEA induced mitochondrial dysfunction, the distribution of mitochondria, actin and chromatin was examined in two-day old embryos. After culture for 48 h most control embryos exhibited normal distribution of actin and mitochondria (Fig. 7A,B). When cultured for 48 h in the presence of 2.5 µM BEA, however, all embryos were degenerated. Embryo viability, as determined by Resazurin reduction, was significantly decreased after 48 h culture in presence of 1.0 and 2.5 µM BEA (Fig. 7C). From these results it can be concluded that adverse embryo development cannot exclusively be addressed to a disturbed mitochondrial distribution, but BEA already impaired embryo development before the embryo reached the four-cell stage.

4. Discussion

Beauvericin is considered as an emerging mycotoxin in several feed and food commodities. With the present study we demonstrated an impaired development of *in vitro* cultured oocytes and embryos after exposure to BEA at concentrations similar to those that have been measured in blood plasma after oral consumption of BEA-contaminated feed [42]. The impairment of developmental capacity was most prominent when embryos were exposed to BEA 48 h after the onset of activation. Within COCs, oocytes were more sensitive for BEA than cumulus cells, and toxicity in cumulus cells was comparable to what has been described for other somatic cell lines [8,12,15,17]. This is not only the first detailed study with BEA, but also the first study in which the toxicity of an ionophoric compound on maturing oocytes and developing embryos has been investigated

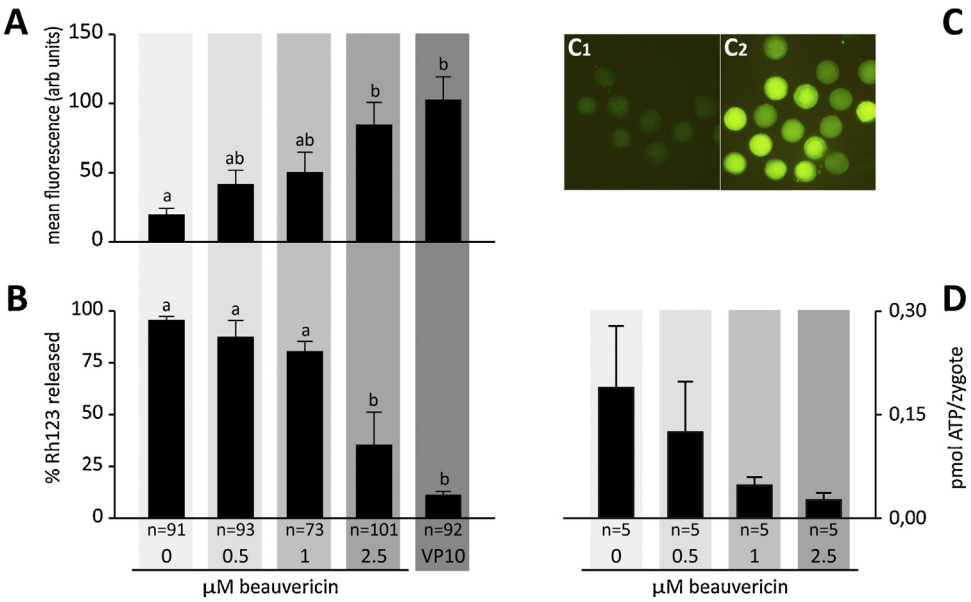

Fig. 6. Accumulation (A) and efflux (B) of rhodamine 123 in parthenogenetic embryos exposed to beauvericin (BEA) and the prototypic MDR-1 inhibitor verapamil (VP10; 10 μM) for 4 h after electrical activation. Fluorescence from rhodamine 123 was determined from digitized images (C), that were made 30 min after exposure (C1, control; C2, BEA 2.5 μM). ATP was also determined in BEA exposed parthenogenetic embryos (D). Bars represent means and standard errors of the means and are calculated from four replicates. Bars indicated with different letters (a,b) are significantly different ($P < 0.05$).

In the female reproductive cycle, oocyte maturation is considered as a key process, since it will deliver a fertilizable oocyte loaded with mRNAs and proteins to support early embryo development. To evaluate oocyte developmental potential, oocytes were parthenogenetically activated by electric pulse instead of fertilization with sperm. It has been established that parthenogenetic and IVF embryos are virtually identical towards the blastocyst stage in several animal models [43–46]. Since oocyte activation by spermatozoa may cause variability in IVF outcome, oocytes were activated by a standardized electrical pulse [47–49]. During development of the zygote to the blastocyst stage, the embryonic genome is activated and mitochondrial activity increases which is crucial for the embryo and fetal development. However, cytotoxicity tests only evaluating mitochondrial activity or membrane integrity of individual cells will provide only limited information and therefore these tests should be combined with functional bioassays including cumulus cell expansion, oocyte meiosis and early embryo development [50]. The cumulus-oocyte complex, a unique somatic-germ cell combination, provides an opportunity to study, in a more physiological way, specific mechanisms in female fertility and reproductive toxicity. The use of pig ovaries is a further advantage as pigs are rather sensitive to mycotoxins and because the pig reproductive physiology is more similar to that of humans than that of rodents [5,51].

In our study, cumulus cells, oocytes and embryos showed different sensitivity to BEA, suggesting that different mechanisms may contribute to the overall toxic effects. No changes in gene expression involved with apoptosis were observed in cumulus cells, oocytes and blastocysts, suggesting that apoptosis through the mitochondrial pathway is less likely. However, the chromatin morphology of degenerated cumulus cells exposed to BEA had a fragmented appearance that was different from that of degenerated oocytes exposed to BEA. Possibly other apoptotic pathways were activated, such as the Fas signaling pathway that has been reported in bovine cumulus cells during IVM [52]. During oocyte maturation and early embryo development, mitochondria are redistributed and clustered around the nuclear region, thereby controlling local uptake and release of Ca^{2+} and ATP [53]. It can be hypothesized that the incorrect distribution and morphology of mitochondria, affects ATP- and Ca^{2+} –dependent pathways controlling meiosis resulting in degeneration of the oocyte or the embryo [41]. Developing embryos were most sensitive to BEA and their vulnerability for this toxin remained similar when exposure to BEA had already occurred during oocyte maturation. Indeed, at the four cell-stage, corresponding to the activation of the embryonic genome, the embryo was most sensitive, since MDR1 cannot be translated due to destroyed oocyte specific transcripts. However, BEA did not alter blastocyst quality since similar fractions of apoptotic nuclei and blastocyst diameters were observed in non- and BEA-exposed blastocysts. These results suggest that during BEA exposure the embryo becomes less sensitive for BEA when it has entered a further stage of development. After blastocyst formation, blastomeres exhibit architecture and gene expression patterns different from those in early stage of development [54].

Abnormal oocyte maturation, and hence early embryo development, can be the result of hampered cumulus cell function, partially with regards to the progesterone synthesis [55]. We observed few dead cumulus cells in BEA-exposed COCs, but a ten-fold reduction in progesterone synthesis and oocytes had a significantly reduced developmental capacity. Exposure to BEA led to reduced expression of *CYP11A* and *HSD3B1*, transcripts giving rise to key enzymes for the synthesis of progesterone. However, reduced progesterone levels in culture media may also be the result of hampered ABC transporter function in cumulus cells, since MDR1 facilitates the transport of steroids over the cell membrane [25,32]. These findings indicate that BEA caused misbalanced progesterone levels in cumulus cells leading to abnormal meiosis.

The efflux transporter MDR1 protects oocytes and developing embryos against toxins and forms an ATP dependent primary defense that is located in the cell membrane [27–30]. A standard assay to measure MDR activity is the Rhodamin 123 efflux assay. Following exposure to increasing concentrations of BEA we found significant differences in the efflux of Rhodamine 123 in zygotes, accompanied by a decreasing cytoplasmic ATP content, suggesting that reduced MDR1 activity is a consequence of ATP depletion rather than a direct interaction between BEA and MDR1 protein. During embryo development, after cryopreservation or toxin exposure, *ABCB1* transcript abundance and MDR1 activity have shown

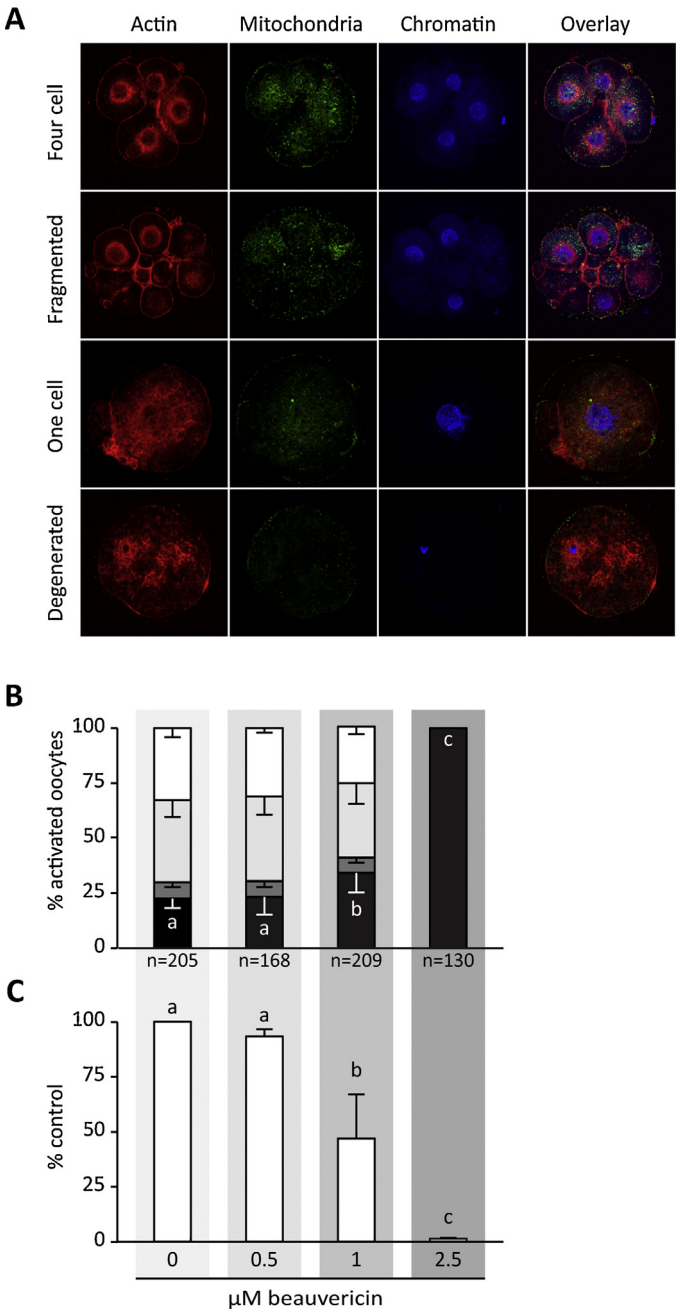

Fig. 7. Morphology and viability of porcine parthenogenetic embryos cultured in presence of beauvericin (BEA) for two days after electrical activation. Based on distribution of actin and mitochondria, and structure of chromatin embryos were classified in four groups: two-four cell embryos, fragmented embryos, non-cleaved zygotes (one cell) and degenerated embryos (A). In four replicate experiments embryos from different classes were counted and depicted in stacked bars where black parts represent degenerated embryos, dark gray parts non cleaved zygotes, light gray parts fragmented embryos with decondensed nuclei and white parts four cell embryos. (B). Resazurin reduction in embryos exposed to different concentrations of BEA (C). Identical bars or bar parts with different letters (a,b) are significantly different ($P < 0.05$).

to be related with cellular quality of blastocysts [56,57]. However, exposure to BEA during embryo development did not lead to differences in *ABCB1* or *ABCG2* expression in blastocysts indicating that BEA exerts its toxic effect at earlier stages of development (such as the zygotes), and embryos that pass this 1st sensitive stage will develop normally to the blastocyst stage.

Mitochondria are essential for oocyte maturation and preimplantation embryo development. In human and pigs, mitochondrial morphology, DNA copy number, activity and redistribution in oocytes and embryos influence fertilization and embryo quality [22,41,58]. It has been described that glucose metabolism, Krebs cycle activity and transcription from the mitochondrial genome increase between the zygote and blastocyst stage, indicating a more active role of mitochondria in metabolism in later developmental stages [59–61]. Here we demonstrate that in early embryos BEA reduced mitochondrial activity, but mitochondrial distribution in blastomeres was not changed after BEA exposure. Although increased numbers of mitochondria have been associated with increased developmental competence, increased ATP production is not necessarily associated with improved embryo development. In contrast, it has been hypothesized that a 'quiet' embryo is of higher quality since damage caused by reactive oxygen species formed during oxidative phosphorylation will be reduced to a minimum [62].

The clinical significance of these findings remains to be elucidated, as the *in vivo* toxicity of BEA needs to be investigated in more detail. The only available toxicological information is the acute toxicity in mice, with an LD50 of 100 mg/kg b.w. for BEA. This places the substance into the group of chemicals with low acute toxicity. It needs to be considered, however, that these data might not be representative for all animal species, as for example major differences in the sensitivity of animal species were observed in the group of ionophoric polyethers used as coccidostatic agents, such as Monensin, Lasolocid and Salinomycon. They share with the hexadepsipeptides the ionophoric nature, which may affect cellular calcium homeostasis, as also demonstrated for BEA [6,7].

Considering the high prevalence of BEA contamination of feed products it is assumed that under practical conditions pigs are exposed to BEA frequently [5]. BEA is well absorbed after oral ingestion, rapidly transformed in the liver and excreted [42]. No data are available on blood serum levels (or concentrations in other body fluid) from large animals, including pigs. Experiments with rats revealed maximum serum levels (13.9 ± 4.9 µg/ml), which are in the same order of magnitude as the concentrations used in our experiments [42]. In the absence of kinetic data, the possible concentration of BEA in the follicular fluid cannot be predicted, but for a number of other mycotoxins the distribution into the follicular fluid has been clearly demonstrated [63]. It should be noted that the experimental protocol in this study reflects only a single exposure, and it cannot be excluded that more pronounced effects will occur in practice where repetitive exposure via contaminated feed is common.

Of special interest is the interaction of BEA with efflux transporters, like MDR-1. This finding indicates a need to investigate the interactions of BEA not only with other mycotoxins, but also with various medicinal products, as many of the drugs used in veterinary medicine are substrates of ABC transporters [64]. Distinct polymorphisms in the expression of these transporters may result in a species-specific sensitivity for BEA.

With regard to reproductive and developmental toxicity, again only very limited data have been reported so far. Monensin, inhibited the formation of gap junctions between blastomers at the eight cell stage towards the morula stage [18]. The observed effect of monensin in embryos during a specific period of development resembles the observed vulnerability of porcine embryos for BEA during the same period of development.

In conclusion, while there is currently no epidemiological evidence that BEA exerts direct toxic effects in farm animals and/or impairs animal productivity and reproduction, the biological potency and the specific effects described in this study on early embryo development clearly indicate that this frequently occurring food and feed contaminant deserves further attention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.reprotox.2016.07.017.

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