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Toxicity of beauvericin on porcine oocyte maturation and preimplantation embryo development

Eric J. Schoevers [a,*], Regiane R. Santos [b], Johanna Fink-Gremmels [b], Bernard A.J. Roelen [a]

[a] Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM Utrecht, The Netherlands
[b] Institute for Risk Assessment Sciences, Division Veterinary Pharmacology, Pharmacotherapy and Toxicology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 104, 3584 CM Utrecht, The Netherlands

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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.

* Corresponding author.
E-mail address: E.J.Schoevers@uu.nl (E.J. Schoevers).

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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