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Comparative *in vitro* cytotoxicity of modified deoxynivalenol on porcine intestinal epithelial cells

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ABSTRACT

The gastrointestinal tract is the first target after ingestion of the mycotoxin deoxynivalenol (DON) via feed and food. Deoxynivalenol is known to affect the proliferation and viability of animal and human intestinal epithelial cells. In addition to DON, feed and food is often co-contaminated with modified forms of DON, such as 3-acetyldeoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) and deoxynivalenol-3-β-D-glucoside (DON3G). The goal of this study was to determine the *in vitro* intrinsic cytotoxicity of these modified forms towards differentiated and proliferative porcine intestinal epithelial cells by means of flow cytometry. Cell death was assessed by dual staining with Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI), which allows the discrimination of viable (FITC−/PI−), apoptotic (FITC+/PI−) and necrotic cells (FITC+/PI+). Based on the data from the presented pilot *in vitro* study, it is concluded that cytotoxicity for proliferative cells can be ranked as follows: DON3G ≪ 3ADON < DON ≈ 15ADON.

1. Introduction

Cereals, such as wheat, maize and oats are often infected by *Fusarium* species. These fungi may produce trichothecenes, a class of mycotoxins. Deoxynivalenol (DON) is the most prevalent trichothecene in cereals and feed in Europe and North America (Gareis et al., 2003). In addition to its presence in raw cereals, DON is particularly stable towards most food-processing techniques and thus can persist in processed food and feed (Hazel and Patel, 2004). After consumption of food/feed contaminated with DON, and of mycotoxins in general, the gastrointestinal tract is the first target of these compounds.

DON alters the viability and proliferation of animal and human IECs. At low doses (inhibitory concentration for 50% of cells or IC_{50} = 0.3–1.5 mg/L) inhibition of cell proliferation is observed, at higher concentrations (IC_{50} = 3–15 mg/L) cytotoxic effects and apoptosis can be seen in human, pig and rat IECs (Bianco et al., 2012; Diesing et al., 2011a, 2011b; Vandenbroucke et al., 2011). Factors influencing cytotoxicity such as status of the cells (differentiated versus proliferative) and exposure site (apical versus basolateral) have been investigated for pig IECs. It was observed that proliferative cells were 10-times more sensitive and that basolateral exposure resulted in a 4-times higher cytotoxicity compared to apical exposure (Diesing et al., 2011a, 2011b, 2012). Furthermore, DON inhibits the absorption of certain nutrients (e.g. glucose and amino acids) in human (Maresca et al., 2002) and animal (Awad et al., 2007, 2014; Hunder et al., 1991; Marin et al., 2011) intestinal epithelial cells (IECs). DON is also known to affect the tight-junctions of the IECs, thereby compromising the intestinal barrier function (Antonissen et al., 2015a; Diesing et al., 2011a, 2011b; Goossens et al., 2012; Maresca et al., 2002; Pinton et al., 2010, 2012). Additionally, DON may cause intestinal inflammation (Maresca and Fantini, 2010; Maresca et al., 2008), an increase in bacterial translocation (Maresca and Fantini, 2010; Vandenbroucke

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et al., 2009, 2011), a decrease in the number of goblet cells and a diminished mucus production (Antonissen et al., 2015b; Obremski et al., 2008; Pinton et al., 2015). These processes may facilitate the crossing of microbial antigens, thus indirectly affecting the IECs innate immunity (Maresca and Fantini, 2010; Maresca et al., 2008). The latter may also be directly affected by DON via the activation of signalling pathways (Cano et al., 2013; Van De Walle et al., 2008; Maresca, 2013; Van De Walle et al., 2010; Vandenbroucke et al., 2011).

Food and feed containing DON is often co-contaminated with its modified forms. These modified mycotoxins predominantly consist of 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON) and deoxynivalenol-3-β-D-glucoside (DON3G). Both 3ADON and 15ADON are fungal precursors in the biosynthesis of DON. DON3G is produced *in planta* as phase II metabolite in an attempt to detoxify and eliminate DON. Although the incidences and mean concentrations of 3ADON (22%, 15 μg/kg), 15ADON (31%, 37 μg/kg) and DON3G (55%, 85 μg/kg) in unprocessed cereals are generally lower than those of DON (84%, 458 μg/kg) (Broekaert et al., 2015a), concentrations exceeding 1000 μg/kg have been reported for all these modified forms (Gareis et al., 2003), amounts not to be neglected as the tolerable daily intake (TDI) for DON and its acetylated forms in humans is 1 μg/kg bodyweight (bw). Moreover, DON3G/DON ratios larger than 1 have been observed in some hard red spring wheat samples (Sasanya et al., 2008).

The *in vivo* fate, potential hydrolysis and disposition of 3ADON, 15ADON and DON3G have recently been investigated in pigs, based on toxicokinetic modeling (Broekaert et al., 2015b, 2016; Nagl et al., 2014). In contrast, the intrinsic cytotoxicity of these modified mycotoxins has not been as extensively investigated as DON. Therefore, the goal of the current study was to comparatively determine the *in vitro* cytotoxicity of 3ADON, 15ADON and DON3G towards both differentiated and proliferative IECs. As pigs are among the most sensitive species to DON toxicity, a porcine intestinal epithelial cell line derived from the jejunum (IPEC-J2) was chosen for this purpose.

2. Experimental

2.1. Chemicals

DON, 3ADON and 15ADON (>99% purity) were purchased from Fermentek (Jerusalem, Israel). DON3G was enzymatically synthesized, purified and verified using nuclear magnetic resonance (NMR) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Michlmayr et al., 2015). No remaining DON (<0.05%) was detected in the produced DON3G. DON, 3ADON, 15ADON and DON3G were dissolved in ethanol yielding a stock solution of 10 mg/mL and stored at ≤−15 °C.

2.2. Cell line and culture conditions

The IPEC-J2 cell line is a continuous intestinal cell line derived from the jejunal epithelium isolated from a neonatal piglet. The IPEC-J2 cells are unique as they are derived from the small intestine and are neither transformed nor tumorigenic in nature (Vergauwen, 2015). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 (1:1) medium (Invitrogen™ Life Technologies, CA, USA), supplemented with 5% fetal calf serum (FCS, HyClone, UK), 1% (v/v) insulin/transferrin/Na-selenite (Gibco, Thermo Fischer Scientific, MA, USA), 1% (v/v) penicillin/streptomycin (Gibco), 1% (v/v) kanamycin (Gibco) and 0.1% fungizone (Bristol-Myers Squibb, Braine-l'Alleud, Belgium), further referred to as culture medium. The cells were routinely seeded at a density of 3×10^6 cells in 50 mL of medium in plastic tissue culture flasks (150 cm^2, Nunc, Denmark), maintained in a humidified incubator at 37 °C under 5% CO_2, and passaged twice weekly.

2.3. Cytotoxicity assay

For the cytotoxicity experiment, IPEC-J2 cells were seeded at 5×10^5 cells/well on 24-well plates in 1 mL of culture medium and allowed to grow for 2 or 21 days for proliferative and differentiated cells, respectively. The IPEC-J2 cell line in culture with addition of 5% FCS undergoes a process of spontaneous differentiation that leads to the formation of a polarized monolayer with high trans-epithelial electrical resistance (TEER) within 1–2 weeks (Vergauwen, 2015). Each well was washed twice with 1 mL of sterile Hank's buffered salt solution (HBSS, Gibco) in order to remove dead cells caused by the trypsin treatment/seeding protocol. Monolayers were exposed in 5-fold to DON, 3ADON, 15ADON or DON3G, all dissolved in ethanol and diluted with culture medium, for 72 h at the following concentrations: 0, 1, 5, 10, 15 and 20 μg/mL. Cells were exposed to maximum 0.2% ethanol in the medium during the 72 h exposure. In order to exclude a position effect on the plate, the order of the toxins was different for each 24-well plate. After incubation, the IPEC-J2 cells were trypsinized and cells together with their culture medium (containing detached cells) were joined in a flow cytometric tube. In order to remove the mycotoxins and cellular debris, each tube was centrifuged (10 min, 524× *g*, 4 °C) after which the pellet was re-suspended in 500 μL of HBSS. This was done three consecutive times. Next, the cells were centrifuged (10 min, 524× *g*, 4 °C) followed by an incubation for 10 min in the dark at room temperature with 100 μL of a solution containing 20 μL of a commercial Annexin-V-fluorescein isothiocyanate reagent (Annexin-V-FITC, Sigma-Aldrich, Belgium) and 20 μL of a 50 μg/mL propidium iodide solution (PI, Sigma-Aldrich) dissolved in 960 μL of incubation buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 140 mM NaCl and 5 mM $CaCl_2$. Cells were analysed by a FACSCanto flow cytometer (Becton, Dickinson and Company, Belgium). A positive apoptosis control was included by staining the cells solely with Annexin-V-FITC after a 4 h treatment with 1.9 μM staurosporine (ImmunoChemistry Technologies, LLC, MN, USA). A positive necrosis control was obtained by single staining the cells with PI after a 1 min incubation with RIPA buffer (PBS with 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1% NP-40). Additional controls were used to quantify and compensate for spectral bleed-through. Furthermore, for each condition one of the five replicates was not stained (no Annexin-V-FITC and no PI) to determine autofluorescence of IPEC-J2 cells. To obtain viable cells as a negative control for Annexin-V-FITC staining and to determine the distinction between positive and negative stained cells, the culture medium was removed and the cells were washed twice with HBSS before trypsin treatment to exclude nonviable cells. Cell death was assessed using dual staining with Annexin-V-FITC and PI which allows the discrimination of viable ($FITC^-/PI^-$), apoptotic ($FITC^+/PI^-$) and late apoptotic or necrotic cells ($FITC^+/PI^+$), see Fig. 1. The mycotoxin concentration resulting in 50% reduction in viable cells (IC_{50}) was calculated using linear regression.

2.4. Statistical analysis

Statistical analysis of the mean fluorescence (Annexin-V-FITC) intensity (MFI) consisted of a Levene's test for homogeneity of variances (P value > 0.01) followed by one-way ANOVA with post-hoc Scheffé tests (P value < 0.05).