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# Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2

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ABSTRACT

The Fusarium derived mycotoxin deoxynivalenol (DON) is frequently found in cereals used for human and animal nutrition. We studied effects of DON in non-transformed, non-carcinoma, polarized epithelial cells of porcine small intestinal origin (IPEC-1 and IPEC-J2) in a low (200 ng/mL) and a high (2000 ng/mL) concentration. Application of high DON concentrations showed significant toxic effects as indicated by a reduction in cell number, in cellular reduction capacity measured by MTT assay, reduced uptake of neutral red (NR) and a decrease in cell proliferation. High dose toxicity was accompanied by disintegration of tight junction protein ZO-1 and increase of cell cycle phase G2/M. Activation of caspase 3 was found as an early event in the high DON concentration with an initial maximum after 6–8 h. In contrast, application of 200 ng/mL DON exhibited a response pattern distinct from the high dose DON toxicity. The cell cycle, ZO-1 expression and distribution as well as caspase 3 activation were not changed. BrdU incorporation was significantly increased after 72 h incubation with 200 ng/mL DON and NR uptake was only transiently reduced after 24 h. Low dose effects of DON on intestinal epithelial cells were triggered by mechanisms different from those responsible for the high dose toxicity.



## 1. Introduction

Deoxynivalenol (DON) is a trichothecene primarily produced by the plant pathogen *Fusarium graminearum* and *Fusarium culmorum* and most prevalent in crops like wheat, oat or barley. DON contaminated products represent a serious problem in animal nutrition. It has been found that pigs are the most susceptible species and DON ingestion leads to reduced growth and thus to economical loss (Pestka et al., 2008). Typically, feed contamination of 20 ppm DON triggers acute toxic effects in pigs characterized by vomiting (Young et al., 1983). On the other hand, concentrations as low as 1 ppm DON lead to reduced feed intake and weight gain (Rotter et al., 1994). Whereas the DON contamination in feed is a measurable quantity, an approximation of the effective DON concentration on the apical enterocyte border is difficult. In human intestine DON concentrations were approximated between 160 ng/mL and 2000 ng/mL (Sergent et al., 2006).

The intestinal mucosa represents a pivotal border between the organism and its environment. The large contact surface allows efficient nutrient absorption, acts as an important barrier for pathogens and toxins and participates in the innate immune response (Mariani et al., 2009; Pitman and Blumberg, 2000). In the gastrointestinal tract DON comes into contact with the epithelial surface and is rapidly absorbed (Prelusky et al., 1988). The effects of high DON concentrations on the enterocyte border comprise cell death and loss of the epithelial barrier integrity. In jejunal explant cultures of pigs morphological changes were observed with DON concentrations of 1500 ng/mL including lysis of enterocytes (Kolf-Clauw et al., 2009). DON concentrations within the same range decreased the transepithelial electrical resistance (TEER) of Caco-2 and IPEC-1 cells cultured on permeable supports. Additionally, an altered claudin-3 and claudin-4 expression was observed after application of approximately 9000 ng/mL DON for 48 h (Pinton et al., 2009). However, lower concentrations of the mycotoxin were not tested in this investigation.

*Abbreviations:* Abs, absorbance; ANOVA, analysis of variance; BrdU, 5-bromo-2-deoxyuridine; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle medium; DON, deoxynivalenol; $EC_{50}$, half maximal effective concentration; EGF, epidermal growth factor; FCS, fetal calf serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IPEC-1, intestinal porcine epithelial cell line 1; IPEC-J2, intestinal porcine epithelial cell line J2; ITS, insulin-transferrin-selenium; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NR, neutral red; PBS, phosphate buffered saline; PI, propidium iodide; SD, standard deviation; SEM, standard error of the mean; ZO-1, zonula occludens-1 protein.

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The effects of DON on cellular physiology are complex and depend on the tested DON concentration and cell type. In early investigations DON binding to ribosomes and inhibition of translation has been reported (Ehrlich and Daigle, 1987). This ribotoxic effect of DON is not an inherent ribosome-degrading property of the toxin because ribosomal RNA is not cleaved in a cell free system (Li and Pestka, 2008).

The ribotoxicity of DON is paralleled by reduced protein biosynthesis as shown with 3000 ng/mL DON in renal proximal tubular epithelial cells (RPTEC) (Königs et al., 2007). In comparison to epithelial cells, cellular components of the immune system seem to be more sensitive to DON mediated effects. In the murine macrophage cell line RAW264.7 low concentrations of DON (250 ng/mL) triggered phosphorylation of AKT and ERK, finally leading to an activation of caspase 3 and internucleosomal DNA fragmentation (Zhou et al., 2005).

So far, only few effects of low DON concentrations on epithelial cells or the epithelium of pigs were described. A reduced villus length was observed in pig jejunal explant cultures in response to 300 ng/mL DON (Kolf-Clauw et al., 2009). DON induced arrest in G2/M cell cycle in human HCT-116 and Intestinal-407 epithelial cells in the concentration range of 250 ng/mL and 1000 ng/mL (Yang et al., 2008).

Because swine is the most susceptible species, and acute toxicity as well as chronic effects triggered by long-term exposure of DON have been reported, we opted for the use of an *in vitro* cell culture that resembles the *in vivo* situation as closely as possible. As recently shown, intestinal porcine epithelial cells IPEC-1 and IPEC-J2 are promising cell culture models which retained most of their original epithelial nature (Mariani et al., 2009). Both cell lines, isolated from the small intestine of neonatal piglets are not carcinoma derived or genetically modified. IPEC-J2 originate from jejunum solely whereas IPEC-1 cells were isolated from both jejunum and ileum, representing a more distal epithelial cell fraction of the gut (Gonzalez-Vallina et al., 1996; Schierack et al., 2006).

We hypothesize that high and low DON concentrations trigger a different pattern of cellular responses in intestinal epithelium. In this context we have analyzed the effect of DON on IPEC-1 and IPEC-J2 including the estimation of cellular viability, growth capacity, induction of apoptosis, organization of tight junction and cell cycle analysis. The applied DON concentrations (typically between 200 and 2000 ng/mL) cover the expected concentrations on the intestinal border *in vivo* (Sergent et al., 2006).

## 2. Materials and methods

### 2.1. Cell culture conditions

IPEC-1 and IPEC-J2 cell lines were used in this study (Gonzalez-Vallina et al., 1996; Rhoads et al., 1994). They represent non-transformed intestinal porcine epithelial cell lines continuously maintained in cell culture. Cells were cultured in Dulbecco's modified eagle medium (DMEM/Ham's F-12 [1:1]) supplemented with 5% fetal calf serum (FCS), 1% insulin-transferrin-selenium (ITS), 16 mmol/L HEPES (all PAN-Biotech, Germany) and 5 ng/mL epidermal growth factor (EGF; BD Biosciences, Germany), incubated at 39 °C and 5% $CO_2$ (Schierack et al., 2006). Cell cultures were regularly tested and found to be free of mycoplasma contamination (Venor® GeM Mycoplasma Detection Kit; Minerva Biolabs, Germany). The cells were routinely seeded at a density of $0.6 \times 10^5$ mL$^{-1}$ (IPEC-1) and $0.5 \times 10^5$ mL$^{-1}$ (IPEC-J2) with 7.5 mL medium in plastic tissue culture flasks (75 cm$^2$ Nunc, Denmark) and passaged every 3–4 d for a maximum of 20 times (IPEC-1 passages 103–123; IPEC-J2 passages 78–98). Cells formed a confluent monolayer within 4 d and were then used in experiments.

### 2.2. Preparation of DON

The obtained DON (D0156; Sigma–Aldrich, Germany) was diluted in absolute ethanol (99.6%; Roth, Germany) to a 0.2 mg/mL stock solution and working dilutions were prepared in cell culture medium. A final concentration of 1% ethanol corresponding to the ethanol concentration of 2000 ng/mL DON solution was tested in all assays and results were not significantly different from control.

### 2.3. Assays in 96 well plate format

Analysis of cellular viability (cell count, lactate dehydrogenase (LDH) assay, neutral red (NR) uptake, MTT assay), proliferation (BrdU assay) and apoptosis (luminescence caspase 3/7 assay) were performed in 96 well plate format. IPEC-1 and IPEC-J2 cells were seeded in 96 well plastic tissue culture plates (Nunc, Denmark) and grown for 4 d until confluence. Medium was removed and after washing once with PBS, fresh medium was added containing increasing final concentrations of DON (100–4000 ng/mL). Cells were incubated for 24 h, 48 h or 72 h. For long term experiments (14 d) treatment was performed in the same manner, but with lower DON concentrations (50–500 ng/mL) and a regular exchange of medium + DON after every 3–4 d. All assays were performed in triplicates and in at least three independent experiments using a multiplate reader (Sunrise™ or Infinite M200, TECAN, Germany).

#### 2.3.1. Cell count

Cells were treated as described, trypsinized, pelleted and after resuspending in PBS counted in a Neubauer chamber.

#### 2.3.2. Alkaline phosphatase (AP) activity

The cellular enzymatic activity of alkaline phosphatase was detected by formation of blue-colored diformazan precipitate within 22 d of cultivation.

A working solution was prepared from 240 μL stock solution A (25 mg/mL nitro blue tetrazolium chloride [NBT] in 70% dimethylformamide [DMF]) and 60 μL B (50 mg/mL 5-bromo-4-chloro-3-indolylphosphate toluidine salt [BCIP] in 100% DMF) in 16 mL 0.1 M Tris–HCl. Wells were incubated with 200 μL working solution overnight in the dark, subsequently the solution was removed and the diformazan was dissolved for 3 h in 200 μL DMF. Absorbency was measured at 560 nm and a calibration curve was used for the diformazan calculation.

#### 2.3.3. LDH activity

Cellular membrane integrity (necrosis) was assessed in normal and FCS-free medium by measurement of LDH activity in the supernatant at an optical density of 492 nm using the colorimetric Cytotoxicity Detection Kit$^{PLUS}$ (Roche, Germany).

#### 2.3.4. NR uptake

Cellular viability was measured by NR uptake at an optical density of 546 nm. NR (Sigma, Germany) was added in a final concentration of 1:40 in cell media with or without FCS, incubated for 3 h, washed three times with PBS and destained with a solution of 1% acetic acid and 50% ethanol.

#### 2.3.5. Metabolic activity

Metabolic activity of cell culture was measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; Sigma–Aldrich, Germany) assay. After the accordant time periods 10 μL of MTT (in 5 mg/mL PBS) were added and the cells were incubated for 3 h additionally. After dissolving the crystalline formazan product (100 μL of 0.01 mol/L HCl/SDS for 3 h at room temperature in the dark) the optical density was measured at 570 nm. Antimycin (50 μmol/L) was tested as a positive control and MTT reduction capacity was found below 35% of the untreated control.

#### 2.3.6. Cell proliferation

DNA-synthesis during proliferation was quantified by BrdU (5′-brome-2′-deoxyuridine) incorporation during the last 6 h of incubation using the colorimetric BrdU ELISA (Roche, Germany) according to manufacturer's protocol. The optical density was measured at 450 nm. Mitomycin (10 μmol/L) was tested as a positive control and BrdU incorporation was found below 17% of the untreated control.

#### 2.3.7. Apoptosis

Cellular caspase 3 activity was measured by the luminescence Caspase-Glo® 3/7 Assay (Promega, Germany) as described in manufacturer's protocol.

### 2.4. Analysis of ZO-1 structure by immunofluorescence

IPEC-1 and IPEC-J2 cells were seeded in a Lab-Tek® Chamber Slide® System (Nunc, Germany) and experiments were performed with confluent cell layers (4 d). DON exposure was performed as described above and cells were fixed for 30 min with absolute ethanol at 4 °C followed by an incubation with acetone for 3 min. Cells were washed with PBS and blocked with 1% normal goat serum (NGS; Axxora, Germany). Primary rabbit anti-ZO-1 antibody (diluted 1:100; Invitrogen, Germany) and secondary Alexa fluor 488 labeled goat anti-rabbit antibody (1:200; Invitrogen, Germany) were used. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Partec, Germany). Fluorescence microscopy was performed using an Axiovert 200 M (Zeiss, Germany) with corresponding Axiovision software.

### 2.5. Protein isolation and immunoblot analysis of caspase 3 and ZO-1

IPEC-1 and IPEC-J2 cells were seeded in plastic tissue culture 6 well plates (Nunc, Denmark) and experiments were performed with confluent cell layers (4 d). Cell