Toxicology and Applied Pharmacology 237 (2009) 41–48

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

# The food contaminant deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression

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ARTICLE INFO

*Article history:*
Received 1 February 2009
Revised 3 March 2009
Accepted 4 March 2009
Available online 14 March 2009

*Keywords:*
Mycotoxin
Deoxynivalenol
Barrier function
Intestinal permeability
Tight junction
Proteins

ABSTRACT

'The gastrointestinal tract represents the first barrier against food contaminants as well as the first target for these toxicants. Deoxynivalenol (DON) is a mycotoxin that commonly contaminates cereals and causes various toxicological effects. Through consumption of contaminated cereals and cereal products, human and pigs are exposed to this mycotoxin. Using *in vitro*, *ex vivo* and *in vivo* approaches, we investigated the effects of DON on the intestinal epithelium. We demonstrated that, in intestinal epithelial cell lines from porcine (IPEC-1) or human (Caco-2) origin, DON decreases trans-epithelial electrical resistance (TEER) and increases in a time and dose-dependent manner the paracellular permeability to 4 kDa dextran and to pathogenic *Escherichia coli* across intestinal cell monolayers. In pig explants treated with DON, we also observed an increased permeability of intestinal tissue. These alterations of barrier function were associated with a specific reduction in the expression of claudins, which was also seen *in vivo* in the jejunum of piglets exposed to DON-contaminated feed. In conclusion, DON alters claudin expression and decreases the barrier function of the intestinal epithelium. Considering that high levels of DON may be present in food or feed, consumption of DON-contaminated food/feed may induce intestinal damage and has consequences for human and animal health.
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## Introduction

Food safety is a major issue in Europe. In this respect, much attention needs to be paid to the possible contamination of food by fungi and the risk of toxin production. Mycotoxins are secondary metabolites produced by fungi that may contaminate all stages of the food chain. Their global occurrence is regarded as an important risk factor for human and animal health as up to 25% of the world crop production may be contaminated with mycotoxins (Oswald et al., 2005). In human, the toxicological syndromes caused by ingestion of mycotoxins range from death, induction of cancer and growth impairment (Bryden, 2007). Consumption of fungal toxins may also decrease resistance to infectious diseases (Oswald et al., 2005).

Deoxynivalenol (DON) is a mycotoxin of the trichothecenes family that is mainly produced by *Fusarium graminearum* and *F. culmorum*. DON is commonly detected in cereals and grains, particularly in wheat, barley, maize and their by-products. It is the most prevalent contaminating trichothecene in crop production in Europe and North America (CAST, 2003; SCOOP, 2003; Pestka and Smolinski, 2005). Recent surveys including 11,022 cereals samples from 12 European countries indicated that 57% of the samples were positive for DON contamination (SCOOP, 2003). Similarly, a survey including 630 samples collected in 25 states by the Federal Grain Inspection Service in United States, revealed that about 40% of the wheat samples and 57% of the barley samples contained levels greater than 2 mg/kg (Trucksess et al., 1995). Furthermore, this toxin is resistant to milling, processing and heating and, therefore, readily enters the food chain (Sugita-Konishi et al., 2006). Widespread human exposure to DON has also been demonstrated using a glucuronide metabolite as a urinary biomarker (Turner et al., 2008).

DON exhibits toxic effects in humans as well as in all animal species investigated so far (Pestka and Smolinski, 2005). Acute high dose toxicity of DON is characterized by effects such as diarrhea, vomiting, leukocytosis, hemorrhage, circulatory shock and ultimately death. Chronic low dose toxicity is characterized by anorexia, reduced weight gain, nutrients malabsorption, neuroendocrine changes and immunologic alterations (Pestka and Smolinski, 2005; Pinton et al., 2008). At the cellular level DON interacts with the peptidyltransferase at the 60S ribosomal subunit level, triggering a translational arrest (Pestka et al., 2004). As a result, protein synthesis is impaired, but also

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doi:10.1016/j.taap.2009.03.003

a so-called “ribotoxic stress” is induced (Pestka et al., 2004), resulting in the activation of mitogen-activated protein kinases.

The intestinal tract represents the first barrier to ingested chemicals or food contaminants and is also the first line of defense against intestinal infection. The gut barrier is formed to a large extent by tight junctions that seal the luminal end of the intercellular space and limit transport by this paracellular route to relatively small hydrophilic molecules. These are multiple transmembrane, scaffolding and signaling proteins including Zonula Occludens-1 (ZO-1), occludin, and one or more claudin isoforms (Harhaj and Antonetti, 2004). Following ingestion of mycotoxin-contaminated food, intestinal epithelial cells could be exposed to high concentrations of toxin (Maresca et al., 2002; Bouhet and Oswald 2005; Sergent et al., 2006). However the effects of DON on the gastrointestinal tract have been poorly studied. DON was found to interfere with the TEER of human intestinal Caco-2 cells (Kasuga et al., 1998; Sergent et al., 2006) and to affect nutrients absorption (Maresca et al., 2002).

Using *in vitro*, *ex vivo* and *in vivo* approaches, the aims of the present study was to evaluate whether doses of DON commonly seen in contaminated food, could affect the barrier function of the intestine. We evaluated the effects of DON on (i) trans-epithelial electrical resistance, (ii) paracellular permeability and (iii) expression of tight junction proteins.

## Materials and methods

*Cell culture and reagents.* The Caco-2 and IPEC-1 cell lines were derived from a human colon adenocarcinoma (ATCC HTB-37, Rockville, USA) and the small intestine of a newborn unsuckled piglet (Gonzalez-Vallina et al., 1996; Bouhet et al., 2004) respectively. Caco-2 were grown in DMEM medium (Sigma, St Quentin Fallavier, France) supplemented with penicillin (100 UI/mL), streptomycin (100 µg/mL), 15% fetal bovine serum (Eurobio, Les Ulis, France), 2 mM L-glutamine (Eurobio), MEM non-essential amino acids (Sigma). The same medium was used during the differentiation process. IPEC-1 were grown and differentiated as previously described (Bouhet et al., 2004). Both cell lines were maintained by serial passages.

Purified DON (Sigma) was dissolved in DMSO and stored at −20 °C before dilution in cell culture media. Control samples were treated with DMSO.

*Animals.* Animal experimentations were carried out in accordance with European Guidelines for the Care and Use of Animals for Research Purposes. Crossbred piglets, fed *ad libitum* with free access to water were killed by electrocution and exsanguination. Six animals (15–20 kg) fed with mycotoxin-free diet were used for the Ussing chamber experiments. Evaluation of claudin-4 expression was performed on 10 growing pigs fed for 5 weeks with either mycotoxin-free diet or DON-contaminated diet (2.85 mg DON/kg feed).

*Measurement of trans-epithelial electrical resistance (TEER).* Caco-2 or IPEC-1 cells were seeded at $10^5$ cells in 0.3 $cm^2$ polyethylene terephtalate membrane inserts with 0.4 µm pore size (Becton Dickinson, Pont de Claix, France) in culture media and reached confluence within 2 days. Differentiation media was then used and changed every other day until complete differentiation. When differentiated, cells were treated with 0, 10, 20, 50 and 100 µM (Caco-2 cells) and 0, 10, 20, and 50 µM (IPEC-1 cells) DON and the TEER was measured during 14 days with a Millicell-ERS Voltohmmeter (Millipore, Saint-Quentin-en-Yvelines, France). TEER values were expressed as $k\Omega \times cm^2$.

*Cytotoxicity assay.* The cytotoxic effect of DON on Caco-2 or IPEC-1 cells was evaluated by measuring the activity of lactate dehydrogenase (LDH) released in the culture media using the CytoTox-96® Assay Kit (Promega, Charbonnières, France). Indeed, release of LDH correlates with the number of lysed cells and is widely used in cytotoxicity studies (Bouhet et al., 2004). Briefly, Caco-2 and IPEC-1 cells were seeded in 0.4 µm pore inserts and differentiated as described above before adding 0–100 µM DON for 48 h. LDH activity was then measured according to the manufacturer's instructions.

*Paracellular tracer flux assay.* Caco-2 or IPEC-1 were grown and differentiated in 0.4 µm pore inserts and treated with DON for 48 h as described above. 4 kDa Fluorescein isothiocyanate-dextran (FITC-dextran) (Sigma) was dissolved in cell culture medium and used at a final concentration of 2.2 mg/mL in the apical cell compartment. After 1 h of incubation the amount of fluorescence was measured in the basal compartment with a fluorimeter (Perkin Elmer LS50B, Courtaboeuf, France). The excitation and emission wavelengths were 490 and 520 nm, respectively.

*Bacterial translocation.* IPEC-1 cells, seeded in 3 µm pore inserts, were differentiated and treated for 48 h with DON in antibiotic-free medium. 24 h later, $2 \times 10^6$ colony forming unit of the pathogenic *Escherichia coli* strain 28C (CIP 107983 (Oswald et al., 2003)) was inoculated onto the apical surface of the cell layer. At various time points, 100 µL were collected in the basolateral compartment, diluted 1:1 in PBS and plated on Luria Broth Agar (Sigma).

*Measurement of paracellular passage of FITC-dextran across porcine intestinal explants.* The intestinal tract was removed and 10-cm segments from the mid-jejunum were immediately kept at 4 °C in a Krebs–Henseleit buffer (Sigma). After longitudinal incision, the underlying serosal and muscular layers were stripped off, and epithelial layer were mounted in Ussing chambers (exposed area, 0.5 $cm^2$).

Intestinal explants were equilibrated for 10 min in Krebs–Henseleit buffer at 38 °C continuously gassed with carbogen (95% $O_2$, 5% $CO_2$) before adding DON in the mucosal compartment (final concentration 0–50 µM). Afterwards 500 µL of FITC-dextran was added to the mucosal side. 800 µL samples were taken from the serosal side of the chambers 1 to 8 h after the addition of DON and replaced in the chambers after fluorescence measurements (as described above).

*Confocal immunofluorescence microscopy.* Caco-2 and IPEC-1 cells, seeded in inserts (0.3 $cm^2$, 0.4 µm pores), were differentiated and treated for 48 h with 30 µM DON. Cells were fixed with PBS 3.7% formaldehyde (30 min, room temperature) or for claudin-3 with methanol (5 min, −20 °C), permeabilized with PBS 0.5% Triton-X-100 for 3 min, and blocked with 10% goat serum (30 min, room temperature). Samples were incubated (1 h, room temperature) with anti-human primary antibodies diluted 1:100. These were rabbit polyclonal anti-claudin-3 (Z23.JM), occludin (Z-T22) and ZO-1 (Z-R1) or mouse monoclonal anti-claudin-4 (3E2C1) (Zymed Laboratories, South San Francisco, CA, USA). We first verified that these antibodies cross-reacted with porcine IPEC-1 cells. Cells were washed with PBS 0.1% Tween®20 (v/v) and incubated (30 min, room temperature) with FITC-conjugated goat anti-rabbit (Southern Biotech, Birmingham, AL, USA) or FITC-conjugated goat anti-mouse (Sigma) diluted 1:100. After washing, inserts were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were captured using a laser scanning confocal microscope (Olympus-IX70, Rungis, France). Optical sections (0.5 µm) were obtained using Olympus FV500 FluoView Application Software. To compare the expression of tight junction proteins in control and treated cells, images were acquired using constant acquisition parameters (laser power, confocal aperture, photomultipliers voltage and gain).

*Immunohistochemistry.* Jejunum fragments (1 cm) obtained from control and DON-treated animals were rinsed in PBS, fixed in 4% neutralized formalin for 24 h, embedded in paraffin-wax, cut into 5-µm-thick sections and deparaffinized. Immunohistochemical staining for

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