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The mycotoxins deoxynivalenol and nivalenol show *in vivo* synergism on jejunum enterocytes apoptosis

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ABSTRACT

The mycotoxins deoxynivalenol (DON) and nivalenol (NIV), worldwide cereal contaminants, raise concerns for human and animal gut health, following exposure through contaminated food and feed. The aim of this work was to analyze the effects of DON and NIV, alone or associated, on the intestinal pig mucosa. Jejunal loops were used for testing DON and NIV individually and in combination (1:1) after a single exposure, for 24 h. For repeated exposure, piglets received a natural contaminated feed, with DON or with DON + NIV for 28 days. Histological investigations, proliferation and apoptosis assessments were conducted. Both experiments were concordant for the total-cell proliferation decreased at the villus tips after DON or DON + NIV at 10 μM acutely, or repeatedly, by 30–35% and 20–25%, respectively. In loops model, apoptotic enterocytes at villus tips increased dose-dependently after DON, NIV alone or DON + NIV in combination. The combination in loops at 10 μM showed higher effects on proliferation and apoptosis than DON alone, and synergism was shown for villus apoptotic enterocyte. These results are to be considered for NIV consumer risk assessment. Our results demonstrate the *in vivo* disruption of the intestinal balance proliferation/apoptosis explaining, at least partly, the disruption of intestinal barrier by these mycotoxins.

1. Introduction

The worldwide contamination of agricultural grain commodities by mycotoxins raise a high concern for food and feed safety (Streit et al., 2013). Mycotoxins are low-molecular-weight secondary metabolites produced by toxigenic fungi (Bouhet and Oswald, 2005).

The *Fusarium* fungi are commonly found on cereals grown worldwide in the temperate regions. They produce mycotoxins, including deoxynivalenol (DON) and nivalenol (NIV), often associated. *F. graminearum* and *F. culmorum* on wheat are both co-producers of DON and NIV (Logrieco et al., 2002; Bottalico and Perrone, 2002). These toxins cause a variety of toxic effects in both animals and human (Creppy, 2002). DON may have adverse health effects after acute or chronic exposure. Acute administration of DON at high dose decreases feed consumption (up to anorexia), and induces emesis of neurogenic origin (Gaigé et al., 2013). Repeated ingestion of low dose DON in pig induced intestinal changes and might predispose animals to infections by enteric pathogens (Pinton et al., 2009; Bracarense et al., 2012). DON is capable to disrupt proliferation, to induce programmed cell death and to alter genes expression (Pestka, 2010). Pig is the most

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sensitive species to DON and to NIV toxicity (EC, 2000; Pestka and Smolinski, 2005). Because of a digestive physiology very similar to that of human (Kararli, 1995), pig can be regarded as the most relevant animal model for extrapolating to human (Rotter et al., 1996).

NIV is considered to be one of the mycotoxins needing regulation (SCOOP EU, 2003; EFSA, 2013). However, the occurrence of NIV contamination is limited to some parts of Europe and Asia. Consequently, NIV has been poorly studied, and the health risks have not been evaluated (EFSA, 2013). *In vitro*, NIV inhibited proliferation of human lymphocytes (Thuvander et al., 1999). In young male pigs fed with 2.5 or 5 mg purified NIV/kg feed for 3 weeks, there was no sign of altered feed intake or body weight and no vomiting or clinical sign (Hedman et al., 1997).

Globally, mycotoxin co-occurrence is common (Schatzmayr and Streit, 2013), particularly in finished feed: for example 58% of 4548 samples contained two or more mycotoxins (Streit et al., 2013). DON and NIV usually co-occur in grains and grain products, and the DON concentration is generally higher than that of NIV (Schothorst and van Egmond, 2004; Yazar and Omurtag, 2008). DON and NIV were detected in 57% of 11,022 samples, and in 16% of 4166 food samples, respectively, from European Union (Schothorst and van Egmond, 2004). The combination of NIV with DON resulted in an additive effect *in vitro* on human lymphocytes (Thuvander et al., 1999). Interaction between DON and NIV were synergistic *in vitro* on Caco-2 cells (Alassane-Kpembi et al., 2013). Less additive effect was observed in *ex vivo* explants (Kolf-Clauw, data not published).

Research has been recently focused on gastrointestinal tract toxicity because it is the first organ exposed to food/feed contaminants (Haschek et al., 2010) playing multi-function roles in regulation, storage, propulsion, digestion, absorption, secretion, barrier activity and elimination (Haschek et al., 2010; Gelberg, 2012; Pinton and Oswald, 2014). In the approach of "3 Rs"-Replacement, Reduction and Refinement (Russel and Burch, 1959), alternatives to animals experiments are needed for this research. Many biological models have been used in toxicity study such as *in vitro* (cell lines: Pinton et al., 2009) and *ex vivo* (explants: Kolf-Clauw et al., 2009), in parallel to conventional animal experiments (Hedman et al., 1997). The intestinal loops, an *in vivo* model, have been developed previously in parasitology or in bacteriology studies (Pernthaner et al., 1996; Gerdts et al., 2001; Vandenbroucke et al., 2011). Jejunal loops were previously shown to allow to analyze the *in situ* effects of toxins on intestinal mucosa (Cheat et al., 2015).

In our previous study, the digestive effects of DON and NIV were investigated in explants and in loops after 4-h exposure, and we identified villus apoptosis and proliferation as sensitive endpoints (Cheat et al., 2015). In the present study, we investigated these endpoints to compare the digestive effects of DON and NIV alone or associated, after a single 24-h exposure in loops, or after 28-day repeated exposure of pigs.

2. Materials and methods

2.1. Purified toxins

DON was acquired from Sigma (St Quentin Fallavier, France) and NIV from Waco Pure Chemical Industries LTD (Osaka, Japan). Stock solutions of these mycotoxins were dissolved in dimethyl sulfoxide (DMSO) at 30 mM DON and NIV for the loops experiments. These stock solutions were stored at −20 °C. Working dilutions were prepared in physiological saline solution. The concentrations at 0 (Ctrl), 1, 3 and 10 μM were used for the dose–response of individual or combined mycotoxins.

2.2. Animals and toxins exposure

2.2.1. Loops

For the loops experiment, two 2-month-old Large White female pigs weighting 20 and 25 kg were used and housed in the animal facility at INRA Nouzilly. The experimental procedures were conducted in accordance with European Guidelines for the Care and Use of Animals for Research Purposes and were approved by the Val de Loire local ethical committees for animal experimentation (C2EA-06). The animals were fasted for 6 h before loops surgery. A 2-m long segment of jejunum was surgically prepared, to constitute the loops as previously described (Girard-Misguich et al., 2011; Meurens et al., 2009). This segment was then subdivided into consecutive segments, designated as "loops" (10 cm long, 12 loops), separated by "inter-loops" of 4–5 cm long. Treatments, control (Ctrl), DON and NIV at 1 μM, 3 μM and 10 μM and DON + NIV (1:1) at 1 μM, 3 μM and10 μM concentration were used for each of the 2 pigs (1 loops/pig for each condition) by injecting 3 mL of each test condition into each loop. Then the intestine was returned into the abdominal cavity, which was sutured. The pigs were euthanized 24 h after the surgery by barbiturate intravenous overdose (Dolethal, 1000 mg per animal, Vetoquinol SA, 70204 Lure Cedex) and the jejunum loop were collected. These loops were washed twice with physiological serum prior to fixation in 10% formalin. In addition, jejunum normal segments (nL) were sampled and processed in parallel.

2.2.2. Animal experiment

For the animal experiment, 24-crossbred castrated-male piglets (4-week old) were acclimatized for 1 week in the animal facility of the Toxalim laboratory, INRA (Toulouse, France) before the experimental protocols. The mean weight was of 11.2 (±1.2) kg at the beginning of toxins exposure. Pigs were randomly allocated to 3 experimental batch pens. The experimental procedures were conducted in accordance with European Guidelines for the Care and Use of Animals for Research Purposes and were approved by the INRA local ethical committees for animal experimentation (Directive 63/2010/EU).

DON and NIV were given *via* a natural contaminated feed source. The levels of the other mycotoxins were below the detection limits or considered as negligible. A diet without toxins (Ctrl) or with natural single contamination (DON) at 3.50 mg DON/kg feed or co-contamination (DON + NIV) at 2.89 mg DON/kg with 0.72 mg NIV/kg feed were used to expose orally the animals for 28 days. Feed and water were provided *ad libitum* throughout the experimental period.

The animals were weighted before starting the experiment (day 0). The body weights and feed intake were measured weekly and at the end of the experiment period (day 28). Blood samples were collected at days 0, 7 and at 28 for biochemistry analysis: total serum protein (TP), albumin, fibrinogen, gamma-glutamyl transferase (GGT). At the end of the experiment, the pigs were fasted overnight prior to be subjected to electrical stunning and euthanized by exsanguination. The jejunum segments were immediately collected and processed similarly to the "Swiss rolls" procedure on a 15-cm segment (Moolenbeek and Ruitenberg, 1981), then fixed in 10% buffered formalin solution for histological procedure.

2.3. Histological assessment

2.3.1. Histological processing and architectural changes

A routine histological processing sequence was used. Paraffin sections from the loops and from the animal experiment, 4-μm thick were stained with HE to assess architectural changes and immunohistochemically labeled (IHC) to assess proliferation and