Effects of four *Fusarium* toxins (fumonisin B$_1$, $\alpha$-zearalenol, nivalenol and deoxynivalenol) on porcine whole-blood cellular proliferation

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**Abstract**

The in vitro effects of four *Fusarium* toxins, fumonisin B$_1$ (FB$_1$), $\alpha$-zearalenol ($\alpha$-ZEA), nivalenol (NIV) and deoxynivalenol (DON), on mitogen-induced cell proliferation were determined in swine whole-blood cultures. Considering the lack of sufficient toxicological data both on single and in combination effects, in vitro studies may contribute to risk assessment of these toxins. Incubation with increasing concentrations of FB$_1$ did not produce any consequence on proliferation; in contrast $\alpha$-ZEA, NIV and DON showed an inhibitory effect. Dose–response curves for each mycotoxin were generated. NIV was found to be the most potent toxin followed by DON and $\alpha$-ZEA. The effects of both FB$_1$ + $\alpha$-ZEA and NIV + DON mixtures were also analysed to investigate possible interactions. The results indicated that combination of FB$_1$ + $\alpha$-ZEA produces a synergistic inhibition of porcine cell proliferation; whereas there is no interaction between DON and NIV on porcine whole-blood proliferation, at tested concentrations.

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1. Introduction

The most important *Fusarium* toxins that may potentially affect human and animals’ health are fumonisin B$_1$ (FB$_1$), zearalenone (ZEA) and trichothecenes such as nivalenol (NIV) and deoxynivalenol (DON).

*Fusarium* fungi are commonly found on cereals grown in the temperate areas of America, Europe and Asia (Creppy, 2002). A recent data collection on the occurrence of *Fusarium* toxins in food in the European Union showed a 57% incidence of positive samples for DON and 16% for NIV out of several thousands of analysed samples (Schothorst and van Egmond, 2004). *Fusarium* toxins elicit a wide spectrum of toxic effects, including the capacity to modify normal immune functions both in humans and animals. Notably the capacity of these mycotoxins to alter immunity is exerted at levels below those causing overt toxicity (Oswald et al., 2005).

Fumonisin B$_1$ is the etiological factor of several mycotoxicosis in both domestic and laboratory animals (Colvin and Harrison, 1992; Gelderblom et al., 1991; Kuiper-Goodman, 1998) and it has been correlated to the incidence of human oesophageal cancer (Sun et al., 2007). The pathophysiological effects of FB$_1$ are attributable to disruption of the sphingolipids metabolism since this mycotoxin is inhibitor of ceramide synthase (Ramasamy et al., 1995; Yoo et al., 1996). The effects of fumonisin B$_1$ on immune system remain controversial: it causes immune-suppression in poultry (Li et al., 2000), swine (Harvey et al., 1995), bovine (Osweiler et al., 2003) and immune-stimulation in rodent species (Dombrink-Kurtzman et al., 2000; Dresden-Osborne et al., 2002; Sharma et al., 2004). Recently, an in vivo study showed that FB$_1$ alters immune functions in broilers by decreasing bursa weight and changing gene expression of cytokines (Cheng et al., 2006).

Zearalenone (ZEA) is a macrocyclic lactone with high binding affinity toward estrogenic receptors and,
consequently, it causes estrogenic effects in pigs (Dieckman and Green, 1992). It has been suggested as a causative agent of infertility and reduced milk production in cattle (D’Mello and MacDonalds, 1997). In particular, α-zearalenol (α-ZEA), a metabolite of ZEA, was found more active than ZEA (Minervini et al., 2001). The immune system is a potential target for estrogenic endocrine disruptors considering that its cells express estrogen receptors (Igarashi et al., 2001). In spite of that, only few studies have been carried out on the immune effects of zearalenone and its metabolites. In particular immune alterations were found at high concentrations of this Fusarium toxin in vitro such as reduction of both mitotic index and cell survival of bovine lymphocytes (Lioi et al., 2004; Yu et al., 2005).

Nivalenol and deoxynivalenol belong to the trichothecenes group, very stable compounds both during storage/milling and processing/cooking of food (Grove, 1988). Different effects have been associated to trichothecenes, including impaired delayed-type hypersensitivity, phagocyte activity (Pestka et al., 1994; Rotter et al., 1996) and modulation of host response to enteric infections (Li et al., 2005). In vitro analyses showed that trichothecenes can both suppress and stimulate immune functions (Bondy and Pestka, 2000). In particular, NIV inhibited proliferation of human lymphocytes (Forssell and Pestka, 1985; Thuవancer et al., 1999).

Despite the major sensitivity of swine to Fusarium toxins (Pestka and Smolarski, 2005), a very limited number of studies have been carried out to assess the immunotoxicity of Fusarium toxins in this species. In vitro investigations on pig peripheral blood mononuclear cells indicated that FB1 decreased IL-4 and increased IFN-gamma synthesis at both protein and mRNA levels (Taranu et al., 2005) and induced apoptosis of primary swine alveolar macrophages (Liu et al., 2002) and porcine renal epithelial cells (Gopee and Sharma, 2004). Moreover, FB1 altered the proliferation and the barrier function of porcine intestinal epithelial cells (Bouhet et al., 2004, 2006). Tornoy et al. (2003) showed that FB1 had no significant effect on the humoral and cellular specific and non-specific immune responses in pigs fed with contaminated feed at different levels of mycotoxin; on the other hand Marin et al. (2006) reported that FB1 was immunosuppressive and its effect was higher in males.

In vivo and in vitro studies in pig showed that DON induced a significant inhibition of lymphocytes proliferation and immunoglobulin secretion (Guyarts et al., 2006); whereas Accensi et al. (2006) did not observe any immune effects when pigs were fed with feed contaminated by low doses of DON.

Considering that food and feed commodities are often contaminated by more than one mycotoxin, (Speijers and Speijers, 2004), studying the interactions between different mycotoxins can be useful. It is known that Fusarium toxins can exert additive and synergistic effects (Tajima et al., 2002); but mycotoxins may also act as antagonists (Koshinsky and Khachatourians, 1992). Theumer et al. (2003) showed different in vitro effects of a mixture of aflatoxin B1 (AFB1) and fumonisin B1 in comparison to the individual toxins. Another study on Penicillium mycotoxins showed that the majority of examined mixtures produced in vitro less-than-additive effects (Bernhoft et al., 2004). In the present study we investigated the effects of fumonisins B1, α-zearalenol, nivalenol and deoxynivalenol on porcine immune response by proliferation assay performed using whole blood. Then, the effects of fumonisin B1, α-zearalenol, nivalenol and deoxynivalenol were assessed in binary combination.

2. Materials and methods

2.1. Reagents

RPMI-1640 medium, l-glutamine, streptomycin, penicillin, 2-mercaptoethanol and non-essential aminoacids were purchased from Cambrex Bioproducts Europe (Verviers, Belgium). Fumonisin B1 (F-1147), α-zearalenol (Z-0166), deoxynivalenol (D-0156), nivalenol (N-7769) and concanavalin A (C 0412) were purchased from Sigma (St. Louis, MO, USA).

2.2. Experimental animals

Twenty-six clinically healthy castrated male pigs of Norwegian Landrace breed, 8–10 months old, were used as blood donors. Blood samples for cell proliferation tests were collected from vena cava cranialis into heparinized sterile Vacutainer™ tubes (7 ml containing 150 IU Li–Heparin).

2.3. Whole-blood cell proliferation assay

Blood samples (20 ml) were diluted 1:20 (v/v) with RPMI-1640 containing penicillin (100 units/ml), streptomycin (100 μg/ml), 5 × 10^-5 M 2-mercaptoethanol, 1% (w/v) non-essential aminoacids and 2% pooled and heat-inactivated (56 °C for 30 min) swine serum.

Diluted blood was plated in 96-well flat-bottomed culture plates at 100 μl/well and single test mycotoxins added at a range of final concentrations (FB1, 0.5–80 μM; α-ZEA, 0.5–20 μM; DON and NIV, 0.0625–2 μM). In binary combinations mycotoxins were applied at same concentrations (1:1 ratio) at a range of final combinations (FB1 + α-ZEA mixture, 0.5–20 μM; NIV + DON mixture, 0.0625–1 μM).

FB1 was dissolved in phosphate buffered saline (PBS), NIV and α-ZEA in methanol, whereas DON in ethanol. Each mycotoxin was then further diluted in complete culture medium. In preliminary studies, it was verified that final concentration of solvents in the culture medium did not affect cell proliferation.

Proliferation was induced by Concanavalin A (Con A) at an optimised concentration of 10 μg/ml (data not shown). The drug concentration in each well was calculated based on the final volume of 200 μl/well. Cell culture was incubated at 37 °C in a 5% CO₂-humidified air incubator for 72 h. All assays were performed in triplicate.

Eighteen hours prior to harvesting, cells were pulsed with 1 μCi/well [3H]-thymidine. Cultures were harvested on filters using a semiautomatic cell harvester (Filtermate, Packard, Danvers, MA). [3H]-thymidine incorporation was assessed by a microplate liquid scintillator (Top Count NXT™, Packard, Danvers, MA). Results were expressed as counts per minute (cpm).
2.4. Statistical analysis

Results were expressed as mean ± standard deviation (SD). Differences among the various treatment groups were determined by one-way analysis of variance (ANOVA). Multiple comparison of treatment means was made using the Tukey test. The criterion for significance was $P < 0.05$ for all studies.

2.5. Isobole method analysis

The type of interaction between FB1 and α-ZEA mycotoxins was determined as previously described by us (Luongo et al., 2006). In particular this type of calculation can be applied when one of the two micotoxins does not have any effect when examined alone, according to Berenbaum (Berenbaum, 1989). The interaction between NIV and DON mycotoxins was analysed according to Bernhoft (Bernhoft et al., 2004). In brief, effects of mycotoxins were determined at a range of six different concentrations: 100%, 80%, 60%, 40%, 20% and 10%, where 100% concentrations were chosen on the basis of preliminary runs. Toxins in combinations were used at fixed ratios (100 + 100%, 80 + 80%, 60 + 60%, 40 + 40%, 20 + 20%). Results giving IC$_{50}$ were extrapolated and transferred to isobole diagrams to illustrate the interactive effects. By using this particular combination of toxin concentrations an additive effect is seen when the mixture 50 + 50% of the two toxins causes the same response as 50% concentration of each toxin separately; that means that the additive effects follow the diagonal line between the concentration of each single mycotoxin. An independent effect is seen when the mixture 100 + 100% causes the same response as 100% concentration of each single mycotoxin; that means that the independent effect is present as vertical and horizontal lines from the effective concentration of each single toxin. If the combined effect of toxins in the diagram constitutes a curve with an upward bow, the effect can be either less-than additive, independent or antagonistic on the basis of statistical evaluation. A curve with a downward bow represents a synergistic effect. Student’s t-test was used for statistical analysis of the estimated combined effects. The criterion for significance was $P < 0.05$.

3. Results and discussion

3.1. Effects of administration of single mycotoxins on porcine whole-blood proliferation

Fusarium toxins are important contaminants, both alone and in mixture, in food and feed. Among farm animals, swine is the most susceptible species to Fusarium toxins as fumonisin B1, α-zearalenol and some trichothecenes (Pestka and Smolinski, 2005). However a very limited number of studies have been carried out to assess their toxicity on pig immune system (Oswald et al., 2005). Consequently, there is need to develop easy and accurate cell tests to assess the hazard of exposure of swine.

In the present study we addressed this issue by using a whole-blood proliferation test. The use of whole-blood lymphocyte proliferation was reported by Bloemena et al. (1989) and optimised subsequently by Fasanmade and Jusko (1995) in rat. This approach offers a series of advantages over the analysis of single cell populations, such as the reduction in the time of assay as well as the generation of data that resembles in vivo responses (Yancy et al., 2001; Thies et al., 1999; Edfors-Lilja et al., 1998).

In preliminary time-dependent experiments 72 h was chosen as optimal incubation time for all tested mycotoxins. Furthermore, in preliminary tests, it was found that 10 µg Con A/ml were optimal to induce porcine whole-blood proliferation (data not shown).

We found that treatment with increasing concentrations of FB1 did not produce any significant effect on the overall cell proliferation of whole blood following Con A stimulation, although a wide range of concentrations were used (0.5–80 µM) (Fig. 1A). These data are in apparent contrast with our previous results on Jurkat cells, a model of human T lymphocytes, where we showed that this mycotoxin enhanced cellular proliferation (Luongo et al., 2006). Nevertheless the effects of this mycotoxin on the immune functions remain controversial, as some authors found that FB1 inhibited cellular proliferation (Osweiler et al., 2003; Li et al., 2000; Harvey et al., 1995), whereas others observed that FB1 stimulated cellular proliferation (Sharma et al., 2004; Dresden-Osborne and Noblet, 2002; Dombrink-Kurtzman et al., 2000) and such effects can be explained by the use of different cellular models. On the other hand, incubation of cells in the presence of increasing concentrations of α-ZEA (0.5–20 µM) caused a marked inhibitory effect on the mitogen-induced proliferation even at very low doses (Fig. 1B). These results are in agreement with previous findings in L929 cells (Tajima et al., 2002).

Similarly, increasing concentrations of the two examined trichoethecenes (NIV and DON) caused inhibition of cells’ proliferation, following Con A stimulation (Fig. 1C and D). In particular, NIV concentrations, ranging from 0.0625 to 2 µM, induced a strong inhibition that became significant and dose-dependent already at the lowest concentration (Fig. 1C). DON (0.0625–2 µM) instead induced a consistent inhibitory effect starting from 0.125 µM (Fig. 1D). NIV was found to be the most potent inhibitor of cell proliferation, followed by DON and α-ZEA, respectively. These data are in agreement with other studies (Rocha et al., 2005; Meky et al., 2001; Thuvander et al., 1999) and also with our in vitro results in Jurkat cells (Luongo et al., 2006; Severino et al., 2006).

3.2. Interactive effects of mycotoxin co-administration

Exposure to mycotoxins often occurs simultaneously. In spite of the importance of their natural co-occurrence, there are only a few studies about the interactions among Fusarium toxins, both in vivo and in vitro (Luongo et al., 2006; Severino et al., 2006; Speijers and Speijers, 2004).

In mixture, FB1 and α-ZEA were tested at constant ratio and the effects of the mixture were compared to the effects of the individual compounds.
Fig. 1. Influence of FB1 (A), α-ZEA (B), NIV (C) and DON (D) on in vitro porcine whole-blood cell proliferation. Porcine whole-blood cells stimulated with 10 µg/ml of Concanavalin A (Con A) were incubated in the presence of increasing concentrations of four mycotoxins for 72 h. DNA synthesis was measured by [3H]-thymidine incorporation and expressed as counts per minute (cpm). Control (ctr): cells incubated in the absence of mycotoxins. Each value represents the mean ± SD of three independent experiments. The criterion for significance was \( P < 0.05 \) for all studies.

Fig. 2. Influence of FB1 and α-ZEA (A) and NIV and DON (B) co-administration on in vitro porcine whole-blood cells proliferation. Porcine cells were cultured in the presence of the mixture of increasing concentrations of four mycotoxins for 72 h. Toxins were used at the same concentration as indicated on x-axis. The results are expressed as counts per minute (cpm) and represent the mean ± SD of three independent experiments. The criterion for significance was \( P < 0.05 \) for all studies.
For α-ZEA, results reported in Fig. 2A indicated that co-administration of FB1 was associated to an increased inhibition of proliferation of blood immune cells. In particular, even if FB1 alone did not produce any effect, in combination with α-ZEA decreased the concentration eliciting 50% inhibition (IC₅₀), suggesting interaction. The inhibition curves of α-ZEA alone and in combination with a fixed amount of FB1 (10 μM) were shown in Fig. 3A. The calculated Da/da ratio (Luongo et al., 2006) for IC₅₀ was less than 1, indicating a synergistic interaction (Table 1). These data are in agreement with our previous findings in Jurkat cells (Luongo et al., 2006).

NIV and DON interaction was analysed according to Bernhoft et al. (2004) and reported in Fig. 3B. The concentration of the two mycotoxins for IC₅₀ alone or in combination were extrapolated by the curves and reported in Fig. 3C and Table 1, clearly showing no interaction between NIV and DON. On the contrary, some previous in vitro studies showed a synergistic effect of combination NIV and DON on yeast growth inhibition (Madhyastha et al., 1994), and an additive toxicity in human lymphocytes cultures (Thuvander et al., 1999) caused by an inhibition of proliferative responses significantly higher than that caused by exposure to one toxin. Moreover, Tajima et al. (2002) reported that mixtures of different concentrations of these two trichothecenes can exert an interactive effect in L929 cells. A plausible explanation for such discrepancies could be that, as individual toxins show large differences depending on species, sex, in vivo or in vitro exposure systems, also the differences in combination toxicity can be influenced by the same factors. Furthermore, on the basis of data reported in Table 1 we inferred that NIV was the most potent inhibitor of cell proliferation followed by DON and α-ZEA.

In conclusion, results obtained with the pig whole-blood model demonstrated the immunomodulatory capacities exerted by Fusarium toxins, alone and in combination. We attributed immunosuppressive properties to NIV, DON and α-ZEA, also in agreement with our previous studies on the Jurkat cell line (Luongo et al., 2006; Severino et al., 2006). FB1 had no significant effects on porcine blood cell proliferation. Moreover, the data of the present work demonstrated that the interaction between FB1 and α-ZEA is synergistic as previously observed in Jurkat (Luongo et al., 2006) and according to literature data (Tajima et al.,

Fig. 3. Inhibition curves of α-ZEA alone and in combination with a fixed (10 μM) concentration of FB1 (A). Inhibition curves of NIV and DON singly and in combination (B). NIV and DON interaction was analysed according to Bernhoft et al. (2004). Isobole diagram illustrating combined effects of NIV and DON at concentration eliciting 50% inhibition (IC₅₀) (C). The points are mean concentration with standard deviation of the mean, based on dose–response curves shown in panel B. *, significantly different from the mixture.
Table 1

<table>
<thead>
<tr>
<th>Mycotoxin combination</th>
<th>Concentration (μM; mean ± SD)</th>
<th>T-Test (P &lt; 0.05)</th>
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<tr>
<td>α-ZEA</td>
<td>6.0 ± 1.5 [Da]</td>
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<tr>
<td>FB1 + α-ZEA</td>
<td>5.6 ± 2.9 [da]</td>
<td>0.04</td>
</tr>
<tr>
<td>NIV alone</td>
<td>0.25 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>DON alone</td>
<td>0.26 ± 0.60</td>
<td></td>
</tr>
<tr>
<td>NIV in (NIV + DON)</td>
<td>0.85 ± 0.11</td>
<td>0.01</td>
</tr>
<tr>
<td>DON in (NIV + DON)</td>
<td>0.43 ± 0.05</td>
<td>0.000001</td>
</tr>
</tbody>
</table>

Da = concentration of the mycotoxin α-ZEA giving 50% inhibition when examined alone; da = concentration of the mycotoxin α-ZEA in the mixture giving 50% inhibition. According to Berenbaum the effect is: additive when da/Da = 1; synergic when da/Da < 1; antagonist when da/Da > 1.

2002). On the contrary, there is no interaction between DON and NIV on porcine whole-blood proliferation, at tested concentrations.

Moreover, these data provide further information about the validity and utility of whole-blood cellular proliferation assays for the study of immunotoxicity in vitro (Thies et al., 1999; Edfors-Lilja et al., 1998).

In terms of risk assessment in foods and feeds, mycotoxins which show interactive effects are of more concern, in particular studying the molecular mechanisms of their toxicity to understand the real way by which Fusarium toxins can interact each other and exert immunomodulatory effects both in humans and animals. Finally, these studies can help to define or optimise the legal maximum levels of mycotoxins in food and feedstuffs.

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Conflict of interest

These authors have no conflicts of interest.

References


