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Individual and combined effects of *Fusarium* toxins on apoptosis in PK15 cells and the protective role of *N*-acetylcysteine

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ABSTRACT

Deoxynivalenol (DON), zearalenone (ZEN) and fumonisin B_1 (FB_1) are among the most toxicologically important *Fusarium* toxins commonly found in nature that lead to nephrotoxicity in animals. The present study investigated that the individual and combined effects of subcytotoxic DON (0.25 μM), ZEN (20 μM) and FB_1 (10 μM) on oxidative stress and apoptosis in porcine kidney cells (PK15). In addition, the protective effect of *N*-acetylcysteine (NAC) against the toxicity of *Fusarium* toxins was also evaluated. Our results showed that the activities of glutathione reductase (GR) and total superoxide dismutase (SOD) were affected by DON, ZEN and FB_1, and this change in activity induced reactive oxygen species (ROS) and malondialdehyde (MDA) production, increased apoptosis and regulated the mRNA expression of Bax, Bcl-2, caspase-3, caspase-9, cytochrome c (cyto c) and P53. This study demonstrated the complexity of combined mycotoxin infection since the combination of toxins exhibited more profound defects in the oxidative stress responses and apoptosis. Moreover, NAC reduced the oxidative damage and inhibited the apoptosis induced by *Fusarium* toxins. It was concluded that oxidative damage and apoptosis through the mitochondria-dependent channel were the mechanisms of *Fusarium* toxin mediated toxicity, and NAC reversed these damages to some extent.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by a range of fungi that can lead to numerous adverse health effects in animals and humans (Wentzel et al., 2016). The Food and Agriculture Organization of the United Nations (FAO) estimated that up to 25% of the world's food crop is contaminated with mycotoxins. Aflatoxins, ochratoxins, trichothecenes, zearalenone, fumonisins, tremorgenic toxins, and ergot alkaloids are the mycotoxins of greatest agro-economic importance (Hussein and Brasel, 2001). Among these mycotoxins, DON, ZEN and FB_1 are the most toxicologically important toxins that occur frequently in food and animal feeds (Martins et al., 2006; Monbaliu et al., 2010; Garrido et al., 2012).

DON is one of the most common *Fusarium* trichothecene mycotoxin. This large family of toxins is generally associated with feed refusal, vomiting, and lesions of the gastrointestinal tract in animals (Diaz, 2005). Pathophysiologic effects associated with DON include altered neuroendocrine signaling, proinflammatory gene induction, disruption of the growth hormone axis, and altered gut integrity (Pestka, 2010). Moreover, DON significantly affects fetal development and the innate immune system (Kinser et al., 2005; Collins et al., 2006; Pestka and Amuzie, 2008).

ZEN is a *Fusarium* fungal metabolite with oestrogenic properties (Diaz, 2005). Currently, studies not only focused on ZEN-induced lesions of the reproductive organs but also demonstrated the histopathological damage, oxidative stress and inflammatory cytokines production in pregnant animals and their offspring (Jia et al., 2015; Yin et al., 2015; Zhang et al., 2015). In addition, intake of ZEN could induce different degrees of hematotoxicity and negatively affects immune function (Yang et al., 2016). *In vitro* studies showed that ZEN induced obvious apoptosis in endometrial stromal cells (ESCs) via the Bcl-2 family and caspases-dependent signaling pathway (Hu et al., 2016).

Fumonisins (FUM) can inhibit lipid synthesis in biological membranes and cause a lethal condition, equine leucoencephalomalacia, that is characterized by massive atrophy of the brain and sudden death (Diaz, 2005). Swine are less sensitive to FUM, which is characterized by

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pulmonary oedema. FB_1 is a potent toxin which causes disorders in lipid metabolism, renal filtration perturb, rhabdomyolysis and blood lymphocyte cell death (Kouadio et al., 2013). Moreover, FB_1 causes brain hyperexcitability in vivo and mitochondrial dysfunction may represent a potential underlying mechanism (Poersch et al., 2015).

NAC, a well-known thiol antioxidant that acts as a glutathione precursor and reacts with OH, NO_2, CO_3 (−), and thiyl radicals, is widely used in clinical practice (Samuni et al., 2013). It has anti-inflammatory effects, impacts apoptosis and neurogenesis, and reverses models of mitochondrial toxicity (Asevedo et al., 2014). Supplementation with NAC (50 μM) resulted in significant cytoprotection, a reduction in ROS generation, higher antioxidant levels that were similar to that of control cells and the inhibition of DNA strand breaks induced by hypoxia (Jayalakshmi et al., 2005). Moreover, NAC has protective effects against DNA damage and carcinogenesis (Flora et al., 2001).

DON, ZEN and FB_1 are likely to damage the host simultaneously because of their co-occurrence in contaminated cereal grains. As the main excretory organ, the kidney may be exposed to high concentrations of the toxin after the ingestion of mycotoxin-contaminated food. Although some researchers have discussed the individual effects of DON, ZEN or FB_1 in different *in vitro* models including intestinal porcine epithelial cell lines (IPEC) (Dänicke et al., 2010), the Chinese Hamster Ovary (CHO-K1) cell line (Ferrer et al., 2009) and both human and pig lymphocytes (Mwanza et al., 2009). However, the reports on the combined and interactive effects of DON, ZEN and FB_1 on kidney cell are limited. Therefore, this research aimed to investigate the individual and combined effects of DON, ZEN and FB_1 on the activity of antioxidant enzymes, the level of MDA and ROS and the mRNA expression of apoptosis-related genes in PK15 cells. In addition, the protective role of NAC was investigated.

2. Materials and methods

2.1. Chemicals

DON, ZEN, FB_1, NAC, [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich (St. Louis, MO, USA). DON, ZEN and FB_1 were dissolved in DMSO and NAC was dissolved in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4.5 g/L) obtained from GE Healthcare Life Sciences (Beijing, China). Fetal bovine serum (FBS) was obtained from Gibco-Life Technology (Eggenstein, Germany). The final concentration of the DMSO used as a solvent in the culture medium was 0.5%. All other chemicals were purchased from Beyotime Biotechnology (Nantong, China).

2.2. Cell culture

The porcine kidney cell line (PK15) was obtained from the Harbin veterinary research institute (Harbin, China). Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution at 37 °C in a humid atmosphere of 5% CO_2. Cells (2×10^5/ml) were seeded in 5 ml of medium in a 25 cm^2 flasks and cultured for 36 h. Thereafter, the cultured cells were either passaged or used in experiments. Cell monolayers were washed with phosphate-buffered saline (PBS) and trypsinized with 1 × trypsin/EDTA.

2.3. Preliminary concentration–response experiment: cell viability assay by MTT

The MTT assay was performed to assess cell viability (Mosmann, 1983). Cells were seeded at a density of 2000–4000 cells/well in 96-well plates and incubated for 24 h. Subsequently, the cells were rinsed with PBS and treated with *Fusarium* toxins (0.25–8 μM of DON, 5–160 μM ZEN and 5–160 μM FB_1) in serum-free media for 24 h. At the end of the incubation, the cells in each well were washed with PBS, and then, 100 μl (0.5 mg/ml) of MTT solution was added to each well. After a 4 h incubation at 37 °C, the MTT solution from each well was discarded and 100 μl of DMSO was added to each well and shaken for 5 min to solubilize the formazan formed in the viable cells. The absorbance was measured at 570 nm using a GENios microplate reader (Tecan Austria GmbH, Austria). The viability of the cells treated with *Fusarium* toxins was expressed as a percentage compared to the control (non-mycotoxin treated). From these data, subcytotoxic concentrations were used for subsequent study. In addition, to assess the protective role of NAC in *Fusarium* mycotoxins-induced cytotoxicity, NAC (0.5, 1.0, and 2.0 mM) was co-administered with DON, ZEN or FB_1 for 24 h.

Table 1
Design matrix from three *Fusarium* toxins and NAC.

Group	Treatment			
	DON 0.25 μM	ZEN 20 μM	FB_1 10 μM	NAC 0.5 mM
1	−	−	−	−
2	+	−	−	−
3	−	+	−	−
4	−	−	+	−
5	+	+	−	−
6	+	−	+	−
7	−	+	+	−
8	+	+	+	−
9	−	−	−	+
10	+	−	−	+
11	−	+	−	+
12	−	−	+	+
13	+	+	−	+
14	+	−	+	+
15	−	+	+	+
16	+	+	+	+

+: Mycotoxin/NAC treatment.
−: No mycotoxin/NAC treatment.

2.4. Inscribed central composite design for complete mixtures

An inscribed central composite design including a fractional factorial part was applied with four factors (DON, ZEN, FB_1 and NAC) to minimize the number of possible toxin combinations from 4^4 (all possible combinations of every concentration of each toxin/NAC) to 16. The design matrix is shown in Table 1. The cell viability was determined by MTT assay as described above.

2.5. Antioxidant enzymes activity assays

Cells were seeded at a density of 2.4×10^5 cells/well in 6-well plates and incubated for 24 h. Subsequently, cells were rinsed with PBS and treated as described in Table 1. After 24 h incubation, protein concentration and the activity of glutathione reductase (GR) and total superoxide dismutase (SOD) were assessed using kits (Beyotime Biotechnology) according to the manufacturer's instructions. The results were expressed as U per mg protein.

2.6. Lipid peroxidation assay

The level of lipid peroxidation was measured via the 2-thiobarbituric acid (TBA) color reaction for malondialdehyde (MDA) by using the kit (Beyotime Biotechnology) according to the manufacturer's instructions. The results were expressed as nmol per mg protein.

2.7. Evaluation of ROS level

Cells were seeded at a density of 2.4×10^5 cells/well in 6-well plates and incubated for 24 h. After treatment with or without *Fusarium*