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Combined effects of deoxynivalenol and zearalenone on oxidative injury and apoptosis in porcine splenic lymphocytes *in vitro*

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ABSTRACT

Deoxynivalenol (DON) and zearalenone (ZEA) are the two most common mycotoxins in animal feed. In this study, we examined oxidative injury and apoptosis of porcine splenic lymphocytes induced by DON or ZEA and their combination *in vitro*. Based on IC_{50} values, porcine splenic lymphocytes were treated with 0.06, 0.3, 1.5, and 7.5 μg/mL DON, 0.08, 0.4, 2, and 10 μg/mL ZEA, or both DON and ZEA at 0.06 and 0.08 μg/mL, 0.3 and 0.4 μg/mL, and 1.5 and 2 μg/mL, respectively. After 48 h of DON and/or ZEA exposure, the cells were analyzed for antioxidant functions, apoptosis, and mRNA and protein expression of apoptosis-related genes p53, Bcl-2, Bax, caspase-3, and caspase-8 to determine their apoptosis and oxidative damage effects and mechanisms. The results showed that, compared with the control group, SOD, CAT, GPx, GSH, and Bcl-2 mRNA and protein expression levels were significantly reduced in exposed groups ($P < 0.05$ or $P < 0.01$). Furthermore, MDA contents, apoptosis rates, and p53, Bax, caspase-3 and caspase-8 protein and mRNA expression levels were increased significantly ($P < 0.01$). The effects of DON and ZEA were dose dependent and synergistic in combination. These data suggest that DON and ZEA induce oxidative damage and apoptosis of porcine splenic lymphocytes.

1. Introduction

Fungal toxin is a toxic secondary metabolite produced in grain fields, during harvesting and storage, and in the process of feed processing and storage (Marzocco et al., 2009). Among *Fusarium* toxins, zearalenone (ZEA) and deoxynivalenol (DON) are two of the most serious kinds of toxins in food and raw materials. Single *Fusarium* toxin have reproductive toxicity and different degrees of genotoxicity, immunotoxicity, and carcinogenicity (Desjardins, 2006). Some studies have evaluated the combined effects of exposure to ZEA and DON stimultaneously *in vivo*. For example, diet contaminated with DON, NIV and ZEA are fed to pigs for 28 days. The indexes of the experimental animals showed a significant increase on histological changes on the intestine, liver and lymphoid organs. And a significant increase on lymphocyte apoptosis was observed in lymph nodes and spleen in the experimental animals (Gerez et al., 2015). In addition, in our previous studies, mice were administered an intraperitoneal injection for 4 consecutive days with different concentrations of ZEA alone, DON alone, or ZEA + DON. DON and/or ZEA induced apoptosis, dysfunction, and oxidative stress in mouse kidney, brain and spleen. DON and/or ZEA also affected the immunity of mice. Furthermore, the combination of DON + ZEA exhibited a synergistic effect (Liang et al., 2015; Ren et al., 2016a, 2016b, 2014, 2016c). However, reports of the combined effects of DON and ZEA *in vitro* are few.

Swine are more sensitive to DON and ZEA than other species. Thus, in this study, to explore immunotoxicity of DON and ZEA, and their induction of apoptosis and oxidation damage, antioxidant functions, apoptosis, and mRNA and protein expression of apoptosis-related genes p53, B cell lymphoma gene-2 (Bcl-2), Bax, caspase-3, and caspase-8 were examined in primary cultured porcine spleen cells exposed to

Abbreviations: Bcl, 2 B-cell lymphoma-2; Caspase, Cysteinyl aspartate specific proteinase; CAT, Catalase; CCK-8, Cell Counting Kit-8; DON, Deoxynivalenol; GSH, Glutathione; GSH-Px, Glutathione peroxidase; IC_{50}, 50% inhibitory concentration; MDA, Malondialdehyde; p53, tumor protein 53; PBS, phosphate-buffered saline; ROS, Reactive oxygen species; RPMI, Roswell Park Memorial Institute; RT-PCR, reverse transcription-polymerase chain reaction; SOD, Superoxide dismutase; ZEA, Zearalenone

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DON and ZEA alone or in combination. Our results may have implications in animal husbandry, human health, food safety, environmental protection, and the development of mold inhibitors.

2. Materials and methods

2.1. Chemicals

Purified DON and ZEA were purchased from Pribolab Pte. Ltd (Singapore). Roswell Park Memorial Institute (RPMI) 1640 medium, and 2′, 7′ -dichlorofluorescein diacetate (DCFH-DA), and rhodamine 123 were purchased from Sigma-Aldrich (St Louis, MO, USA). Histopaque 1077 and the Cell Counting Kit-8 (CCK-8) were obtained from Dojindo Laboratories (Tokyo, Japan). The annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit was purchased from BD Pharmingen (Lexington, KY, USA). Kits for detecting glutathione (GSH), malonaldehyde (SOD), catalase (CAT) were obtained from the Nanjing Jiancheng Bioengineering Institute (China). Porcine Bcl-2, p53, Bax, caspase-3, and caspase-8 ELISA kits were obtained from Qiyi Biological Technology Co., Ltd. (Shanghai, China). Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Trizol reagent and the DNA extraction kit were purchased from Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The SYBR PrimeScript RT-PCR Kit II was purchased from Takara (Shiga, Japan). All other reagents were analytical grade.

2.2. Cell isolation and culture

All study procedures were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University. Ten 30–40-day-old healthy weaned piglets (Duroc × Large White × Landrace) were purchased from New Hope Group (China).

Piglets in good condition were anesthetized with an intramuscular injection of “Sumianxin” anesthetic mixture (haloperidol, dihydroetorphine, and 2, 4-dimethylaniline thiazole) using 0.8 mL/kg body weight as the dose. After laparotomy, spleen samples were removed aseptically. The spleen samples were then soaked in 75% alcohol for 5 min, and cleaned with phosphate-buffered saline (PBS) three times. After stripping the surrounding connective tissue and fat, splenic tissue was cut into suitable fragments and dispersed to a single cell suspension using a 200-mesh gauze sieve. The cell suspension was gradually added to a centrifuge tube with the same volume of lymphocyte separation medium. After centrifugation for 20 min at 400 × *g*, we collected the second layer of cells. To obtain relatively pure lymphocytes, the cell sample was washed twice with RPMI-1640 medium at 500 × *g* for 5 min at room temperature. More than 95% of cells were viable, based on trypan blue dye exclusion. Finally, the density of spleen lymphocytes was adjusted to 3×10^6 cells/mL, and the cells were then exposed in RPMI-1640 medium containing 10% FBS, HEPES (Wuhan Boster), 100 U/mL penicillin, and 100 μg/mL streptomycin, and incubated with 5% CO_2 at 37 °C.

2.3. CCK-8 bioassay

Cell viability was quantified using the CCK-8 bioassay as described elsewhere (Wang et al., 2016). Splenic lymphocytes were seeded into 96-well culture plates (3×10^6 cells/well) and treated with various concentrations of DON or ZEA (25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195, 0.0976, and 0.0488 μg/mL) for 48 h at 37 °C with 5% (v/v) CO_2. At the indicated time points, 10 μL CCK-8 solution was added to each well, and then the plates were incubated for 4 h. The optical density (OD) was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Germany). All samples were tested as five independent replicates. The index of cell viability in the presence of DON was calculated with the following formula: index of viability (%) = ([OD of cells treated with DON]/[OD of cells without DON treatment]) × 100.

2.4. Preparation of cell lysates

To monitor various parameters (excluding the CCK-8 bioassay), splenic lymphocytes were cultured in six-well culture plates (6×10^6 cells/mL) and treated with DON alone (0.06, 0.3, 1.5, and 7.5 μg/mL; IC_{50} = 1.48 ± 0.20 μg/mL), ZEA alone (0.08, 0.4, 2, and 10 μg/mL; IC_{50} = 2.03 ± 0.31 μg/mL), or DON and ZEA (0.06 and 0.08, 0.3 and 0.4, 1.5 and 2 μg/mL, individually) at 37 °C in a humidified atmosphere with 5% (v/v) CO_2 for 48 h (Ren et al., 2015). After 48 h of incubation, cells were collected and centrifuged (400 × *g* for 3 min, abandoning the supernatant), then washed with PBS three times. Lysis buffer was added (with protease and phosphatase inhibitor) for every 1 mL containing 10^7 cells, sonicated on ice, and centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was collected in 1.5 mL eppendorf tubes and used for GPx, GSH, SOD, MDA, p53, Bc1-2, Bax, caspase-3, and caspase-8 analyses. Cells were collected to analyze the rate of apoptosis and for DNA and RNA isolation. Both the supernatants and the cells were frozen at −80 °C until use. All samples were tested as five independent replicates. For each index, five different samples were used.

2.5. Determination of antioxidant indexes

Intracellular GPx assay: Test was carried out according to the kit brochures operation, and the spectrophotometric absorbance was assessed at 412 nm for GPx.

Intracellular GSH assay: the micro enzyme labeled method was used. The spectrophotometric absorbance was assessed at 405 nm for GSH according to manufacturer’s instructions.

Intracellular SOD assay: SOD in the cytosol was measured by the xanthine oxidation method at 450 nm according to the manufacturer’s instructions.

An intracellular CAT assay was performed using the ammonium molybdate colorimetry method. The spectrophotometric absorbance was assessed at 405 nm for CAT according to the manufacturer’s instructions.

Intracellular MDA assay: the thiobarbituric acid method. The spectrophotometric absorbance was assessed at 532 nm for MDA according to the manufacturer’s instructions.

2.6. Determination of apoptotic cells

Apoptosis induction by DON was analyzed by annexin V-binding and propidium iodide (PI) uptake. After treatment for 48 h, 400 μL binding buffer (BD Pharmingen) was added to the harvested cells. Resuspended cells were then incubated with 10 μL annexin V-FITC and 20 μL PI for 15 min in the dark at room temperature. Apoptosis was measured by flow cytometry on 10,000 cells per sample.

2.7. Apoptosis-related protein assay

The concentration of p53, Bc1-2, Bax, Caspase-3, and Caspase-8 in supernatants were measured using enzyme linked immune-absorbent assays (ELISA) development kits for chicken p53, Bc1-2, Bax, Caspase-3, and Caspase-8 according to the manufacture instructions.

2.8. Quantification of p53, Bc1-2, Bax, caspase-3, and caspase-8 mRNA

Total RNA was isolated from cells using Trizol reagent (Invitrogen Biotechnology Co.) according to the manufacturer’s instructions. RNA concentrations were determined using GeneQuant 1300 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The reverse transcription reaction (40 μL) consisted of 10 μg total RNA, 1 μL M-MLV reverse transcriptase, 1 μL RNase inhibitor, 4 μL dNTP, 2 μL Oligo dT, 4 μL dithiothreitol and