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Original Research Article

Effects of purified zearalenone on selected immunological and histopathologic measurements of spleen in post-weanling gilts

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ABSTRACT

The present study was aimed at investigating the adverse effects of dietary zearalenone (ZEA) on the lymphocyte proliferation rate (LPR), interleukin-2 (IL-2), mRNA expressions of pro-inflammatory cytokines, and histopathologic changes of spleen in post-weanling gilts. A total of 20 crossbred piglets (Yorkshire × Landrace × Duroc) with an initial BW of 10.36 ± 1.21 kg (21 d of age) were used in the study. Piglets were fed a basal diet with an addition of 0, 1.1, 2.0, or 3.2 mg/kg purified ZEA for 18 d *ad libitum*. The results showed that LPR and IL-2 production of spleen decreased linearly ($P < 0.05$) as dietary ZEA increased. Splenic mRNA expressions of interleukin-1β (*IL-1β*) and interleukin-6 (*IL-6*) were linearly up-regulated ($P < 0.05$) as dietary ZEA increased. On the contrary, linear down-regulation ($P < 0.05$) of mRNA expression of interferon-γ (*IFN-γ*) was observed as dietary ZEA increased. Swelling splenocyte in 1.1 mg/kg ZEA treatments, atrophy of white pulp and swelling of red pulp in 2.0 and 3.2 mg/kg ZEA treatments were observed. The cytoplasmic edema in 1.1 mg/kg ZEA treatments, significant chromatin deformation in 2.0 mg/kg ZEA treatment and phagocytosis in 3.2 mg/kg ZEA treatment were observed. Results suggested that dietary ZEA at 1.1 to 3.2 mg/kg can induce splenic damages and negatively affect immune function of spleen in post-weanling gilts.

1. Introduction

Zearalenone (ZEA) is one of mycotoxins produced mainly by *Fusarium* fungi growing on grains and its derived products worldwide (Warth et al., 2012; Wei et al., 2012; Zinedine et al., 2007). Among farm animals, pigs, especially female pigs are susceptible to ZEA (EC, 2006), resulted in maximum limits of 0.1 mg ZEA/kg diet for piglets and gilts (EFSA, 2004).

The major toxicity of ZEA and its metabolites, such as α-zearalonol (α-ZOL), is attributed to their estrogenic effects on the genital organs and reproduction in gilts (Etienne and Jemmali, 1982; Jiang et al., 2010). Besides, ZEA has been shown to be toxic to multiple tissues in animals, such as hepatotoxicity in rabbits (Conkova et al., 2001) and piglets (Jiang et al., 2012), haematotoxicity in rats (Maaroufi et al., 1996), oxidative stress in mice (Ben Salah-Abbès et al., 2009) and gilts (Jiang et al., 2011), and cytotoxic effects on cultured Vero cells (Ouanes et al., 2008). Notwithstanding, the effects of ZEA on immune function have been well established in mice (Abbès et al., 2006a; Ben Salah-Abbès et al., 2008) and humans (Vlata et al., 2006), and *in vitro* (Berek et al., 2001). However, studies of ZEA on immune response of pigs mostly have been conducted with respect to feeding grains naturally contaminated with ZEA and other *Fusarium* mycotoxins (Swamy et al., 2004). Meanwhile, several changes of immunological parameters were induced by high ZEA concentrations (Abbès et al., 2006a; Ben Salah-Abbès et al., 2008), but such high doses are usually not found in cereals used for animals. Moreover, comprehensive studies regarding ZEA (1.1 to 3.2 mg/kg) effect on splenic damages in post-weanling gilts has not been previously reported.

Therefore, an experiment is conducted to examine whether or not the feeding of a purified ZEA-contaminated (1.1 to 3.2 mg/kg) diet to post-weanling gilts will influence lymphocyte proliferation rate (LPR), interleukin-2 (IL-2) production, mRNA expressions of pro-inflammatory cytokines and histopathologic changes of spleen.

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2. Materials and methods

2.1. Preparation of zearalenone contaminated diet

Purified ZEA (Fermentek, Jerusalem, Israel) was dissolved in acetic ether, poured onto talcum powder, which was left overnight to allow acetic ether evaporation. A ZEA premix was prepared by blending ZEA-contaminated talcum powder with ZEA-free corn, which was subsequently mixed at appropriate levels with a corn–soybean meal diet to create the experimental diets. All diets were prepared in 1 batch, stored in covered containers prior to feeding. A composite sample of each experimental diet was prepared for analysis of ZEA and other mycotoxins by the Asia Mycotoxin Analysis Center (Chaoyang University of Technology, Taichung, China) before and at the end of the feeding experiment. Deoxynivalenol (DON) was analyzed using high performance liquid chromatography (HPLC). Enzyme linked immunosorbent assay (ELISA) and fluorometry techniques were used to measure ZEA, fumonisins (FUM), and aflatoxin (AFL) levels. The detection limits of these mycotoxins were 1 μg/kg for AFL, 0.1 mg/kg for ZEA, 0.1 mg/kg for DON, including 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol, and nivalenol, and 0.25 mg/kg for FUM.

2.2. Experimental design, animals and management

The animals used in all experiments were cared for in accordance with the guidelines for the care and use of laboratory animals described by the Animal Nutrition Research Institute of Shandong Agricultural University and the Ministry of Agriculture of China. A total of 20 post-weanling piglets (Landrace × Yorkshire × Duroc) with an average body weight of 10.36 ± 1.21 kg (21 d of age) were used in the study. Gilts were randomly allocated into 4 treatments according to body weight after 7 days of adaptation. Pigs were fed a basal diet (Table 1) supplemented with an addition of 0, 1, 2, or 3 mg/kg purified ZEA for 18 days. Analyzed ZEA contents were 0, 1.1 ± 0.02, 2.0 ± 0.01 and 3.2 ± 0.02 (means ± SD) mg/kg in the control and the 3 experimental diets, respectively. Aflatoxin, DON, and FUM were not detected in the test diets.

The diets used in the study were isocaloric and isonitrogenous with the only difference being in ZEA level. All nutrient concentrations were formulated to meet or exceed minimal requirements according to the NRC (1998). Animals were housed in cages equipped with 1 nipple drinker and 1 brick-shaped feeder in a temperature controlled room at Jinzhuyuan Farm (Yinan, Shandong, China). During the experimental period, the temperature in the nursery room was maintained between 26 and 28 °C. The mean relative humidity was approximately 65%. Gilts were fed *ad libitum* and allowed access to water freely through the entire experiment period.

Table 1
Ingredients and compositions of the basal diet (air-dry basis).

Ingredients	Content, %	Nutrients	Analyzed values, %
Corn	53.00	Gross energy, MJ/kg	17.12
Wheat middling	5.00	Crude protein	19.40
Whey powder	6.50	Calcium	0.84
Soybean oil	2.50	Total phosphorus	0.73
Soybean meal	24.76	Lysine	1.36
Fish meal	5.50	Methionine	0.46
L-lysine HCl	0.30	Sulfur amino acid	0.79
DL-methionine	0.10	Threonine	0.90
L-threonine	0.04	Tryptophan	0.25
Calcium phosphate	0.80		
Limestone (pulverized)	0.30		
Sodium chloride	0.20		
Premix[1]	1.00		

[1] Supplied per kg of diet: vitamin A, 3300 IU; vitamin D_3, 330 IU; vitamin E, 24 IU; vitamin K_3, 0.75 mg; vitamin B_1, 1.50 mg; vitamin B_2, 5.25 mg; vitamin B_6, 2.25 mg; vitamin B_{12}, 0.02625 mg; pantothenic acid, 15.00 mg; niacin, 22.5 mg; biotin, 0.075 mg; folic acid, 0.45 mg; Mn (from $MnSO_4 \cdot H_2O$), 6.00 mg; Fe (from $FeSO_4 \cdot H_2O$), 150 mg; Zn (from $ZnSO_4 \cdot H_2O$), 150 mg; Cu (from $CuSO_4 \cdot 5H_2O$), 9.00 mg; I (from KI), 0.21 mg; Se (from Na_2SeO_3), 0.45 mg.

2.3. Sample collection

Pigs were fasted for 12 h at the end of the experimental period. After the collection of blood samples, piglets were immediately euthanized and spleens were isolated, weighed, and gross lesions examined. Four samples of splenic tissues from each pig were quickly collected, the first portion was put in D-Hank's for LPR and IL-2 measurement, the second was stored at −80 °C for mRNA expressions of cytokines analysis, the third was fixed in 10% buffered formalin for histopathology evaluation, and the fourth was cut into 0.5 mm^3 and fixed in 2.5% polyoxymethylene–glutaraldehyde for ultrastructure analysis.

2.4. Splenic lymphocyte proliferation assay

Splenic samples in a proper amount of sterile D-Hank's were gently mashed by pressing with the flat surface of a syringe plunger against a stainless-steel sieve (200 meshes). After the red blood cells were disrupted, the splenocytes were washed twice. The resulting pellet was resuspended and diluted to 2.5×10^6 cells/mL with RPMI-1640 and fetal bovine serum after the cell viability was assessed by trypan blue exclusion. The solutions were incubated into 96-well culture plates with 190 μL cell suspension and 10 μL ConA per well. The plates were respectively incubated in a humid atmosphere with 5% CO_2 (Thermo) at 37 °C for 72 h. Briefly, 100 μL of methyl thiazolyl tetrazolium (MTT, 5 mg/mL) was added into each well at 4 h before the end of incubation. Then the plates were centrifuged at 1000 × *g* for 10 min at room temperature. The supernatant was removed carefully and 100 μL of dimethyl sulfoxide (DMSO) was added into each well. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance of cells in each well was measured by enzyme-linked immunosorbent assay reader (Model RT-6100, Leidu Co., Ltd., Shenzhen, China) at a wave length of 570 nm (A value) as the index of lymphocytes proliferation (Huang et al., 2013). Meanwhile, the lymphocytes proliferation rate was calculated as follows: Proliferation rate (%) = $100 \times A_{(\text{test group})}/A_{(\text{control group})}$.

2.5. Measurement of IL-2 of splenic lymphocytes

Lymphocytes were collected as described above, and then 500 μL cell suspensions were incubated in a humid atmosphere with 5% CO_2 (Thermo) at 37 °C for 24 h. The supernatants were collected and the concentrations of IL-2 were assayed by ELISA kit (BD Biosciences, Heidelberg, Germany). Briefly, ELISA plates were precoated with 50 μL of 1:500 diluted capture antibody overnight at 4 °C and afterwards washed and blocked with 100 μL assay diluents (phosphate buffered saline [PBS] with 10% fetal calf serum [FCS]) at room temperature for 1 h. After the washing step, 50 μL standard and culture supernatant (1:4 diluted with PBS) were incubated at room temperature for 1 h, washed and 50 μL detection antibody and streptavidin-HRP reagent were added and incubated at room temperature for 1 h. After subsequent washing steps, 50 μL substrate solutions were added and incubated in the dark for 10 to 30 min. The reaction was stopped by the addition of 50 μL 2 N H_2SO_4. The measurement was performed using an enzyme-linked immunosorbent assay reader (Model RT-6100, Leidu Co., Ltd., Shenzhen, China) at a wave length of 450 nm (OD value).

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