Effect of Zearalenone on Apoptosis of In Vitro-Cultured Chicken Splenic Lymphocytes (Post-print)

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Abstract

This study aimed to investigate the effects of zearalenone (ZEA) on apoptosis in in vitro cultured chicken spleen lymphocytes. The ZEA exposure concentrations were 0 (control), 0.10, 0.40, 1.60, 6.25, and 25.00 μg/mL. After 48 h of exposure, the apoptosis rate, necrosis rate, mitochondrial membrane potential, intracellular reactive oxygen species (ROS), mRNA expression levels of B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), Bak-1, p53, and cysteine-aspartic protease-3 (caspase-3), as well as the corresponding apoptotic gene protein contents in the cell culture supernatant were measured in in vitro cultured chicken spleen lymphocytes. The results showed that, compared with the control group, ZEA exposure caused significant or extremely significant increases ($P<0.01$ or $P<0.05$) in the apoptosis rate, necrosis rate, ROS content, levels of p53, Bax, Bak-1, and caspase-3 in the cell culture supernatant, and mRNA expression levels of p53, Bax, Bak-1, and caspase-3 in cells, with these increases rising as the toxin concentration increased; whereas the mitochondrial membrane potential and Bcl-2 content in the supernatant of the ZEA groups were extremely significantly lower than those of the control group ($P<0.01$). Therefore, ZEA exposure can promote apoptosis in in vitro cultured chicken spleen lymphocytes in a dose-dependent manner.

Full Text

Effects of Zearalenone on Apoptosis of Chicken Splenic Lymphocytes in Vitro

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Abstract: This study investigated the effects of zearalenone (ZEA) on apoptosis of chicken splenic lymphocytes in vitro. Chicken splenic lymphocytes were treated with ZEA at concentrations of 0 (control), 0.10, 0.40, 1.60, 6.25, and 25.00 μg/mL for 48 h. The apoptosis rate, necrotic rate, mitochondrial membrane potential, intracellular reactive oxygen species (ROS) levels, mRNA expression levels of B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), Bak-1, p53, and cysteine-aspartic protease-3 (caspase-3), as well as the corresponding apoptotic protein concentrations in cell culture supernatants were measured. The results showed that compared with the control group, ZEA exposure significantly or extremely significantly increased the apoptosis rate, necrotic rate, ROS content, supernatant levels of p53, Bax, Bak-1, and caspase-3, and intracellular mRNA expression of p53, Bax, Bak-1, and caspase-3 ($P<0.05$ or $P<0.01$) in a dose-dependent manner. Conversely, mitochondrial membrane potential and Bcl-2 content in the supernatant were extremely significantly lower in ZEA-treated groups than in the control group ($P<0.01$). These findings indicate that ZEA promotes apoptosis in cultured chicken splenic lymphocytes in a dose-dependent fashion.

Keywords: zearalenone; chicken splenic lymphocytes; apoptosis; mitochondria; reactive oxygen species

Introduction

Zearalenone (ZEA), also known as F-2 toxin, is an estrogenic mycotoxin produced by Fusarium fungi (primarily *Fusarium graminearum*, but also *F. tricinctum*, *F. equiseti*, *F. nivale*, and *F. roseum*). It is widely found in moldy cereal crops and animal-derived food products. ZEA has been shown to promote cell apoptosis. Kim et al. [1] reported that ZEA caused severe damage to mouse spermatogenic cells, with varying degrees of apoptotic bodies appearing, and the severity increased with exposure time and concentration, being more pronounced in spermatogonia and spermatocytes. Yu et al. [2] found that in MCF-7 breast cancer cells, ZEA inhibited cell proliferation activity by regulating the expression of B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax) genes, thereby suppressing apoptosis, and ZEA could significantly increase the activity and mRNA expression of cytochrome P450 family 1 subfamily A polypeptide 1 (CYP1A1) enzyme. Deng et al. [3] also demonstrated that ZEA promoted Bcl-2 mRNA and protein expression while inhibiting Bax expression, enhanced the proliferation viability and mitotic index of human breast cancer cell line MCF-7, and inhibited estrogen depletion-induced apoptosis in MCF-7 cells through regulation of Bcl-2 and Bax expression. Fu [4] fed rats different doses of ZEA and found that ZEA affected the conformation of p53 gene

exon 8 in rats, causing base pair mutations in two bands of the exon, primarily pyrimidine-purine transversion mutations. The p53 gene is closely related to cell cycle regulation, cell transformation, DNA replication, and induction of programmed cell death, and it regulates apoptosis through the Bcl-2 family [5]. In Yu et al.' s [6] study on the mechanism of ZEA-induced death in mouse RAW264.7 macrophages, ZEA treatment caused loss of mitochondrial membrane potential and changes in Bcl-2 and Bax proteins in mitochondria, release of cytochrome C and apoptosis-inducing factor from the cytoplasm, and catalase inhibition reduced ZEA-induced effects in RAW264.7 cells.

The primary target organ of ZEA in the immune system is the spleen, where it can induce apoptosis of splenic lymphocytes, thereby reducing immune function. Ma et al. [7] reported that ZEA had a significant pro-apoptotic effect on lipopolysaccharide-activated mouse splenic lymphocytes in vitro, with the effect showing dose dependence. However, few studies have investigated the effects of ZEA on apoptosis in chicken splenic cells. Therefore, this study used primary cultured chicken splenic lymphocytes as a model to investigate the effects of Fusarium toxin ZEA on apoptosis and its regulatory genes in chicken splenic lymphocytes at the cellular level, aiming to elucidate the impact and molecular mechanisms of ZEA on chicken splenic lymphocytes.

Materials and Methods

1.1 Experimental Materials

Experimental animals: Healthy Isa Brown roosters aged 40-60 days were provided by the Animal Center of the College of Veterinary Medicine, Northeast Agricultural University. Fetal bovine serum (FBS) was purchased from Gibco. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ZEA, and phenol red-free RPMI-1640 medium were purchased from Sigma. The apoptosis detection kit [Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining] was purchased from ACTGene (USA). 2 ,7 -dichlorodihydrofluorescein diacetate (DCFH-DA) and Rhodamine 123 were purchased from Sigma (USA). Trizol reagent kit, M-MLV reverse transcriptase, DNA extraction kit, and RNase were purchased from Invitrogen. rTaq enzyme and other PCR reagents were purchased from TaKaRa (Dalian) Biotech. Ethidium bromide (EB) was purchased from Sigma. Chicken Bax, Bak-1, Bcl-2, p53, and cysteine-aspartic protease-3 (caspase-3) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai Biological Company.

1.2.1 Preparation, Culture, and Treatment of Splenic Lymphocyte Suspension

Under aseptic conditions, chicken spleens were removed and placed in a petri dish containing phosphate-buffered saline (PBS). The spleens were gently washed with PBS to remove blood residues, and surrounding connective tissues were carefully stripped away. The spleens were then transferred to another

PBS-filled petri dish containing a 200-mesh screen. Using forceps, the spleen was placed on the 200-mesh copper net and gently ground with the plunger of a 20 mL disposable syringe. The filtrate was appropriately diluted to prepare a cell suspension, which was then transferred to a centrifuge tube pre-filled with chicken lymphocyte separation medium. The cell suspension was slowly layered over the chicken lymphocyte separation medium at a 1:1 volume ratio and centrifuged at 2000 r/min for 15 min at room temperature. Lymphocytes were collected with a Pasteur pipette, washed with cold PBS, and centrifuged at 1500 r/min for 5 min at 4 °C. After discarding the supernatant, the cells were washed once more with toxin-free RPMI-1640 complete medium (with FBS), resuspended, counted, and cell viability was assessed using trypan blue (viability >95%).

Preliminary experiments determined that the half-maximal inhibitory concentration (IC_{50}) of ZEA at 48 h was $(23.91\$\pm4.96)g/mL.BasedontheIC\_\{50\}\$$, ZEA treatment concentrations were selected as follows: Z1 group 0.10 μg/mL, Z2 group 0.40 μg/mL, Z3 group 1.60 μg/mL, Z4 group 6.25 μg/mL, and Z5 group 25.00 μg/mL [8].

1.2.2 Flow Cytometry Measurement of Apoptosis and Necrosis Rates

After 48 h of toxin exposure, cells were collected and centrifuged at 1500 r/min for 3 min, then washed three times with PBS. Following centrifugation, 500 L of Binding Buffer was added to prepare a cell suspension, followed by addition of 10 L Annexin V-FITC and 5 L PI. The mixture was incubated at room temperature in the dark for 5-15 min. Apoptosis and necrosis rates were measured by flow cytometry, with samples without Annexin V-FITC and PI serving as reagent controls. The excitation wavelength was 488 nm. Green fluorescence of Annexin V-FITC was detected through the FITC channel (FL-1, 530 nm), while red fluorescence of PI was detected through the PI channel (FL-2, 585 nm). Each sample was counted for 10,000 cells, and data were analyzed using standard computer programs of the flow cytometer.

1.2.3 Measurement of Intracellular Reactive Oxygen Species (ROS) Content

After 48 h of toxin exposure, cells were collected and centrifuged at 1500 r/min for 3 min, then washed three times with PBS. The fluorescent dye DCFH-DA, which specifically binds to intracellular hydrogen peroxide (H_2O_2), was used to detect intracellular ROS. Cells were suspended in PBS, and DCFH-DA stain was added to a final concentration of 100 mol/L. The mixture was incubated at 37 °C in the dark for 30 min, washed three times with PBS, and the mean fluorescence intensity was measured by flow cytometry (excitation wavelength 488 nm, emission wavelength 530 nm).

1.2.4 Measurement of Mitochondrial Membrane Potential

After 48 h of toxin exposure, cells were collected and centrifuged at 1500 r/min for 3 min, then washed 2-3 times with PBS. The fluorescent dye Rhodamine 123, which specifically binds to mitochondria, was used to detect mitochondrial membrane potential. Cells were suspended in PBS, and Rhodamine 123 stain was added to a final concentration of 5 g/mL. The mixture was incubated at 37 °C in the dark for 30 min, washed three times with PBS, and the mean fluorescence intensity was measured by flow cytometry (excitation wavelength 488 nm, emission wavelength 530 nm).

1.2.5 Determination of Bcl-2, p53, Bax, Bak-1, and Caspase-3 Contents in Supernatant (ELISA Method)

After 48 h of toxin exposure, cell culture supernatants were collected and centrifuged at 3000 r/min for 20 min. The supernatants were collected and stored at -20 °C for later use. The contents of Bcl-2, p53, Bax, Bak-1, and caspase-3 in the supernatants were measured according to the ELISA kit instructions.

1.2.6 Determination of mRNA Expression of Apoptosis Regulatory Genes Bcl-2, p53, Bax, Bak-1, and Caspase-3

Total tissue RNA was obtained following the RNA extraction kit instructions. An appropriate amount of total RNA was reverse transcribed according to the PrimeScript RT-PCR Kit instructions to convert extracted RNA into cDNA, which was stored at -80 °C for later use or used immediately for PCR. Based on the full gene sequences of chicken β-actin (L08165), Bcl-2 (Z11961.1), p53 (X13057.1), Bax (XM_{422067}.2), Bak-1 (NM_{001030920}.1), and caspase-3 (NM_{204725}.1) published in GenBank, specific upstream and downstream primers were designed using Prime 5.0 software, subjected to homology search via GenBank Blast, and synthesized by Invitrogen (Shanghai). Primer sequences and parameters are shown in Table 1 .

The PCR reaction system for related gene cDNA included cDNA, upstream and downstream primers, high-fidelity enzyme, Premix, and ddH_2O. Reaction conditions were: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 34 s, with termination at 4 °C. PCR amplification products were electrophoresed on 1.5% agarose gel for 2 h, and gel images were recorded using a gel imaging system.

1.2.7 Data Analysis

The REST software (Pfaffl) was used to analyze the mRNA expression abundance of target genes in toxin-treated samples. SPSS 13.0 software was used for significance F-tests and correlation analysis of data. All measurements were repeated with three different batches of cells, with each sample repeated three times per batch. Data are expressed as mean ± standard deviation, with $P<0.05$ considered statistically significant.

Results

2.1 Effects of ZEA on Apoptosis and Necrosis Rates of Chicken Splenic Lymphocytes

The effects of ZEA on apoptosis and necrosis rates of chicken splenic lymphocytes are shown in Table 2 . Significant differences in apoptosis rates were observed among all groups ($P<0.05$). For necrosis rates, except for no significant difference between Z2 and Z3 groups ($P>0.05$), extremely significant differences were found among all other groups ($P<0.01$). When ZEA concentration reached 6.25 μg/mL or higher, both apoptosis and necrosis rates slowed down. Except for the Z5 group showing decreased apoptosis and necrosis rates compared to the Z4 group, both rates increased with increasing ZEA concentration in all other treatment groups, with all ZEA groups showing extremely significantly higher apoptosis and necrosis rates than the control group ($P<0.01$). At the same concentration, the apoptosis rate of splenic lymphocytes was higher than the necrosis rate, indicating that ZEA exposure primarily induced apoptosis in splenic lymphocytes.

2.2 Effects of ZEA on Intracellular ROS and Mitochondrial Membrane Potential in Chicken Splenic Lymphocytes

The effects of ZEA on intracellular ROS and mitochondrial membrane potential in chicken splenic lymphocytes are presented in Table 3 . After 48 h of ZEA exposure, ROS content in chicken splenic lymphocytes increased with toxin concentration, with all ZEA groups showing extremely significantly higher ROS content than the control group ($P<0.01$). Mitochondrial membrane potential decreased with increasing toxin concentration (except for the Z3 group), with all ZEA groups showing extremely significantly lower membrane potential than the control group ($P<0.01$). Significant differences in ROS content were observed among all groups except between Z3 and Z4 ($P>0.05$), while mitochondrial membrane potential differed significantly among ZEA groups ($P<0.05$).

2.3 Effects of ZEA on Bcl-2, p53, Bax, Bak-1, and Caspase-3 Contents in Chicken Splenic Lymphocyte Culture Supernatants

The effects of ZEA on Bcl-2, p53, Bax, Bak-1, and caspase-3 contents in culture supernatants are shown in Table 4 . For Bcl-2 content, except for no significant difference between Z1 and Z2 groups ($P>0.05$), extremely significant differences were observed among all other groups ($P<0.01$). For p53 content, significant differences were found among all groups ($P<0.05$). For Bax content, no significant differences were observed among Z1, Z2, Z3, and control groups ($P>0.05$), while Z4 and Z5 groups showed extremely significant differences from each other and from the aforementioned groups ($P<0.01$). For Bak-1 content, significant differences were observed among all groups ($P<0.05$). For caspase-3 content, except for no significant difference between Z3 and Z4 groups ($P>0.05$), significant differences were found among all other groups ($P<0.05$). These results

indicate that p53, Bax, Bak-1, and caspase-3 contents increased with toxin concentration, while Bcl-2 content decreased, showing a significant dose-dependent relationship.

2.4 Effects of ZEA on mRNA Expression of Bcl-2, p53, Bax, Bak-1, and Caspase-3 in Chicken Splenic Lymphocytes

The effects of ZEA on mRNA expression levels are presented in Table 5 . For Bcl-2 mRNA expression, no significant differences in Bax/Bcl-2 ratio were observed among Z1, Z2, and Z3 groups ($P>0.05$), but these were significantly higher than the control group ($P<0.05$), while Z4 and Z5 groups were extremely significantly higher than all other groups ($P<0.01$). For p53 mRNA expression, all ZEA groups were extremely significantly higher than the control group ($P<0.01$). For Bax mRNA expression, significant differences were observed among all groups ($P<0.05$). For Bak-1 mRNA expression, except for no significant difference between Z2 and Z3 groups ($P>0.05$), extremely significant differences were found among all other groups ($P<0.01$). For caspase-3 mRNA expression, no significant differences were observed among control, Z1, Z2, and Z3 groups ($P>0.05$), while Z4 and Z5 groups were extremely significantly higher than all other groups ($P<0.01$), with an extremely significant difference between these two groups ($P<0.01$). These results demonstrate that, except for slightly lower caspase-3 mRNA expression in Z1, Z2, and Z3 groups compared to the control, Bax/Bcl-2 ratio and mRNA expression levels of Bax, Bak-1, and caspase-3 increased with toxin concentration, showing a significant dose-dependent relationship.

Previous studies from our laboratory have shown that ZEA can induce apoptosis in mouse kidney, brain, and liver cells in vivo [8-11], and in porcine splenic lymphocytes in vitro [12]. The current study demonstrates that ZEA can also induce apoptosis in cultured chicken splenic lymphocytes, consistent with our previous findings.

Mitochondria produce large amounts of ROS during oxidative metabolism. When free radical production exceeds the capacity of the antioxidant defense system, the body cannot effectively clear ROS from mitochondria, leading to accumulation. Excessive ROS can oxidize corresponding redox-sensitive sites on mitochondrial permeability transition pores, causing decreased mitochondrial membrane potential, mitochondrial swelling, and further ROS production, ultimately resulting in mitochondrial oxidative damage [13]. Our results show that ZEA exposure caused massive ROS accumulation and extremely significant decreases in mitochondrial membrane potential in chicken splenic lymphocytes, consistent with these findings.

Apoptosis signal transduction is achieved through activation of the cysteine-aspartic protease system by specific death signals [14]. Caspase-3 is a key enzyme leading to apoptosis after stimulation by various inducers, and its activation marks the beginning of the execution phase of apoptosis. Under normal

conditions, it exists as a proenzyme in the cytoplasm, and when cells receive apoptotic stimuli, it is activated through a series of reactions to induce apoptosis [15-17]. ZEA induces apoptosis in porcine ovarian granulosa cells via a caspase-dependent apoptotic pathway [18]. Our results show that ZEA increased caspase-3 mRNA expression and secreted caspase-3 protein in cultured chicken splenic lymphocytes in a dose-dependent manner, consistent with these findings.

The p53 protein plays a crucial role in apoptosis. Primarily concentrated in the nucleolus, p53 can specifically bind to DNA and exhibits clear cell transformation inhibition, which is important for protecting the integrity of cellular genomic DNA. If DNA suffers external damage, p53 acts as a transcription factor to induce expression of a series of downstream genes, mediating cell cycle arrest at the G phase to allow sufficient time for DNA repair. If DNA damage is severe and repair fails irreversibly, p53 initiates the apoptosis program to induce cell death [19]. Ayed-Boussema et al. [20] reported that ZEA activates p53 and induces apoptosis in human hepatocytes (HepG2) through the p53 pathway in a dose-dependent manner. Yu et al. [6] demonstrated that p53 activation played a key role in ZEA' s toxic effects on RAW264.7 macrophages. Bouaziz et al. [21] showed that ZEA triggered p53-dependent apoptotic pathways in human liver cancer cells. Our results also indicate that ZEA altered p53 expression at the genetic level in chicken splenic lymphocytes, interfering with p53 apoptotic pathway regulation and thereby inducing apoptosis.

Bcl-2 family proteins play a vital role in regulating mitochondria-mediated apoptosis. Bcl-2 protein is mainly distributed on mitochondrial membranes, nuclear membranes, and endoplasmic reticulum membranes, participating in maintaining mitochondrial membrane integrity, preventing cytochrome C release, blocking "intrinsic activation" of apoptosis, and serving as an anti-apoptotic member [22]. When Bax forms homodimers (Bax-Bax), it induces apoptosis. As Bcl-2 expression increases, more Bax dimers dissociate and form more stable Bax-Bcl-2 heterodimers with Bcl-2, thereby neutralizing the apoptosis-regulating effect of Bax-Bax dimers. Thus, the intracellular Bax/Bcl-2 ratio is critical for determining cell survival after receiving stimulus signals. Bcl-2 overexpression leads to cell survival, while Bax overexpression leads to apoptosis [23]. Yuan et al. [24] reported that ZEA increased Bax mRNA expression and decreased Bcl-2 mRNA expression in mouse male germ cells. Yu et al. [25] found that ZEA significantly inhibited apoptosis in human breast cancer MCF-7 cells in a dose-dependent manner, with Western blot and multiplex RT-PCR analyses showing upregulation of anti-apoptotic Bcl-2 protein and mRNA levels and downregulation of pro-apoptotic Bax, indicating that ZEA has estrogenic activity and can promote MCF-7 cell cycle progression from G0/G1 to S phase, inhibiting apoptosis through Bcl-2 expression regulation. Our results show that under ZEA treatment, Bak-1 expression increased, Bax/Bcl-2 ratio increased, and corresponding protein contents also increased or decreased accordingly in chicken splenic lymphocytes.

Conclusion

1. This study demonstrated that with increasing ZEA concentration, the contents of apoptosis regulatory proteins p53, Bax, Bak-1, and caspase-3 increased, while Bcl-2 content decreased. ZEA upregulated Bcl-2 family genes (Bax and Bak-1), p53 gene, and caspase-3 gene expression, while inhibiting Bcl-2 gene expression within the Bcl-2 family.

2. ZEA induced increased apoptosis by activating p53 and caspase pathways, and this apoptosis was caused by oxidative stress.

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Note: Figure translations are in progress. See original paper for figures.