Effects of Combined Exposure to Deoxynivalenol and Zearalenone on Homeostasis in In Vitro Cultured Chicken Splenic Lymphocytes Postprint

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Abstract

This study aimed to investigate the effects of combined exposure to deoxynivalenol (DON) and zearalenone (ZEA) on the intracellular homeostasis of in vitro cultured chicken spleen lymphocytes. The lymphocytes were subjected to combined exposure with 0.012 50 μg/mL DON + 0.006 25 μg/mL ZEA, 0.050 μg/mL DON + 0.025 μg/mL ZEA, 0.2 μg/mL DON + 0.1 μg/mL ZEA, and 0.8 μg/mL DON + 0.4 μg/mL ZEA, respectively. After 48 h, the activities of cell membrane ATPases (Ca2+-ATPase, Na+/K+-ATPase), intracellular pH, Ca2+ levels, and mRNA expression levels of calmodulin (CaM) were measured. A blank control group without toxin addition was also established. The results showed that in the toxin-treated experimental groups, intracellular Ca2+ levels and CaM mRNA expression levels increased with increasing toxin concentrations, and all toxin-treated groups were significantly or extremely significantly higher than the blank control group ($P<0.05$ or $P<0.01$). Intracellular pH and the activities of cell membrane Ca2+-ATPase and Na+/K+-ATPase decreased with increasing toxin concentrations, and all toxin-treated groups were significantly or extremely significantly lower than the blank control group ($P<0.05$ or $P<0.01$). It was concluded that combined exposure to DON and ZEA caused intracellular acidification, ion balance disruption, and a series of other intracellular homeostasis imbalances in in vitro cultured chicken spleen lymphocytes in a dose-dependent manner.

Full Text

Effects of Combined Exposure to Deoxynivalenol and Zearalenone on Homeostasis of Chicken Splenic Lymphocytes Cultured *in Vitro*

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Abstract: This study investigated the effects of combined exposure to deoxynivalenol (DON) and zearalenone (ZEA) on the intracellular homeostasis of chicken splenic lymphocytes cultured *in vitro*. Lymphocytes were exposed to four combined toxin doses: 0.01250 μg/mL DON + 0.00625 μg/mL ZEA, 0.050 μg/mL DON + 0.025 μg/mL ZEA, 0.2 μg/mL DON + 0.1 μg/mL ZEA, and 0.8 μg/mL DON + 0.4 μg/mL ZEA. After 48 hours of culture, we measured the activities of cellular membrane ATPases (Ca^{2+}-ATPase and Na^+/K^+-ATPase) and determined intracellular pH, Ca^{2+} levels, and calmodulin (CaM) mRNA expression. A blank control group without toxins was also included. The results demonstrated that intracellular Ca^{2+} levels and CaM mRNA expression increased significantly with rising toxin concentrations, with all treated groups showing significant or highly significant differences compared to the blank control ($P<0.05$ or $P<0.01$). Conversely, intracellular pH and the activities of both Ca^{2+}-ATPase and Na^+/K^+-ATPase decreased significantly with increasing toxin doses, with all treated groups significantly or highly significantly lower than the control ($P<0.05$ or $P<0.01$). These findings indicate that combined exposure to DON and ZEA disrupts intracellular homeostasis in cultured chicken splenic lymphocytes, causing intracellular acidification, ionic imbalance, and other disturbances in a dose-dependent manner.

Keywords: deoxynivalenol; zearalenone; combined exposure; splenic lymphocyte; homeostasis

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Deoxynivalenol (DON) and zearalenone (ZEA) are the two most prevalent mycotoxins contaminating animal feed. Mycotoxins not only impair animal growth and reproductive performance but also cause immunosuppression and increased disease susceptibility. As the fundamental unit of living organisms, cells require stable intracellular environments to maintain normal function [1]. When intracellular homeostasis is disrupted, metabolic pathways for carbohydrates, lipids, and proteins become disordered, leading to abnormal gene expression, impaired protein synthesis, and structural and functional cellular abnormalities [1]. The spleen serves as a crucial immune organ, and *in vitro* culture of chicken splenic lymphocytes has become an important research model [4]. Our preliminary experiments revealed that combined exposure to DON and ZEA induces apoptosis in cultured chicken lymphocytes (data not shown). During apoptosis, disruption of intracellular homeostasis commonly occurs, with alterations in redox status, pH, and ion concentration representing both characteristic features of apoptosis and factors that can further promote the apoptotic process [4]. Previous studies from our group demonstrated that individual exposure to either DON [4] or ZEA [4] disrupts intracellular homeostasis and induces apoptosis in chicken splenic lymphocytes. However, few reports have examined the effects of combined DON and ZEA exposure on intracellular homeostasis in these cells. Using primary

cultured chicken splenic lymphocytes as a model, this study focused on changes in cellular membrane ATPase activities (Ca^{2+}-ATPase and Na^+/K^+-ATPase), intracellular Ca^{2+} levels, pH, and calmodulin (CaM) mRNA expression following combined DON and ZEA exposure, providing theoretical insights into the mechanisms underlying toxin-induced apoptosis.

1.1 Experimental Materials

Fetal bovine serum (FBS) (Gibco, USA); DON, ZEA, and phenol red-free RPMI1640 medium (Sigma, USA); Cell Counting Kit-8 (CCK-8) (Dojindo, Japan); intracellular pH fluorescent probe BCECF-AM (Dojindo, Japan); intracellular calcium fluorescent probe Fluo-3/AM (Molecular Probes, USA); activated Taq polymerase and other PCR reagents (TaKaRa, Japan); Trizol reagent kit and M-MLV reverse transcriptase (Invitrogen, USA); ethidium bromide (EB) (Sigma, USA); Triton X-100 (Sigma, USA); Tris-HCl buffer (Sigma, USA); intracellular protein assay kit (Lowry method) and cellular membrane ATPase activity assay kits (Ca^{2+}-ATPase and Na^+/K^+-ATPase) (Nanjing Jiancheng Bioengineering Institute, China).

1.2 Experimental Methods

Preparation of splenic lymphocyte suspension: Under aseptic conditions, spleens were harvested from healthy 40-60-day-old Isa roosters obtained from the Animal Center of Northeast Agricultural University College of Veterinary Medicine. Spleens were placed in Petri dishes containing phosphate-buffered saline (PBS), gently washed to remove blood residue, and stripped of connective tissue. Each spleen was then transferred to another PBS-filled dish containing a 200-mesh screen and gently ground with the plunger of a 20 mL syringe. The filtered cell suspension was appropriately diluted and layered over chicken lymphocyte separation medium at a 1:1 volume ratio. After centrifugation at 2,000 r/min for 15 minutes at room temperature, lymphocytes were collected with a Pasteur pipette, washed with cold PBS, and centrifuged again at 1,500 r/min for 5 minutes at 4 °C. The pellet was resuspended in toxin-free RPMI1640 complete medium (supplemented with fetal bovine serum), washed once more, and finally resuspended to prepare a cell suspension at 5$×10^{6}$ cells/mL. Trypan blue staining confirmed cell viability exceeding 95%, indicating successful preparation.

Determination of combined DON and ZEA exposure concentrations: Using the CCK-8 assay, we evaluated the effects of individual DON and ZEA exposure on the viability of cultured chicken splenic lymphocytes. After 48 hours of exposure, the half-maximal inhibitory concentration (IC_{50}) was $(30.82\pm10.48)g/mLforDONand(23.91\pm4.96)g/mLforZEA.BasedontheseIC_{50}$ values and considering that high concentrations of individual toxins caused severe lymphocyte damage in preliminary experiments, we selected low concentrations for combined exposure in the formal experiment. The final combined exposure doses were: 0.01250 μg/mL DON + 0.00625 μg/mL ZEA (DZ1 group),

0.050 μg/mL DON + 0.025 μg/mL ZEA (DZ2 group), 0.2 μg/mL DON + 0.1 μg/mL ZEA (DZ3 group), and 0.8 μg/mL DON + 0.4 μg/mL ZEA (DZ4 group). A blank control group without toxins was also established.

1.3 Determination of Intracellular Ca^{2+} Levels

After 48 hours of toxin exposure, cells were collected by centrifugation at 1,500 r/min for 3 minutes and washed three times with PBS. The cell pellet was resuspended in PBS containing the intracellular calcium fluorescent probe Fluo-3/AM at a final concentration of 1 μmol/L, mixed thoroughly, and incubated at 37 °C in the dark for 30 minutes. Following three additional PBS washes, the mean fluorescence intensity was measured by flow cytometry (excitation wavelength 488 nm, emission wavelength 530 nm).

1.4 Determination of Intracellular pH

Following 48 hours of toxin exposure, cells were harvested by centrifugation at 1,500 r/min for 3 minutes and washed three times with PBS. The pellet was resuspended in serum-free RPMI1640 medium to prepare a cell suspension at 5$×10^{6}$ cells/mL, and the intracellular pH fluorescent probe BCECF/AM was added to a final concentration of 2 μmol/L. After incubation in a CO_2 incubator (dark, 37 °C) for 30 minutes, cells were collected and washed three times with serum-free RPMI1640 medium, then resuspended in PBS for flow cytometric analysis. Excitation was performed at 488 nm, and intracellular pH was displayed on a two-dimensional dot plot (X-axis 525 nm, Y-axis 610 nm) based on fluorescence intensity. According to the standard curve, intracellular pH was calculated as the ratio of green to red fluorescence intensity, with at least 10,000 cells analyzed per sample [7].

1.5 Determination of Cellular Membrane Ca^{2+}-ATPase and Na^{+}/K^{+}-ATPase Activities

After 48 hours of toxin exposure, cells were collected by centrifugation at 1,500 r/min for 3 minutes and washed three times with PBS. Each sample was treated with 500 μL of 0.1 mol/L Tris-HCl buffer (pH 7.4) containing 0.1% Triton X-100 and sonicated at 4 °C. The lysate was centrifuged at 1,000 g for 10 minutes, and the supernatant was collected for protein quantification. The protein concentration was adjusted to 3–5 mg/mL with phosphate-free saline. The supernatant was then used to determine the activities of Na^{+}/K^{+}-ATPase and Ca^{2+}-ATPase using the phosphorus determination method, following the kit instructions.

1.6 Determination of Intracellular CaM mRNA Expression

Total RNA was extracted from chicken splenic lymphocytes using the Trizol method and reverse-transcribed into cDNA using M-MLV reverse transcriptase. Specific primers for chicken β-actin (L08165) and CaM (NM205005) were designed using Prime 5.0 software based on GenBank sequences, verified by

BLAST homology search, and synthesized by Invitrogen (Shanghai). Primer sequences and parameters are listed in . The reverse transcription reaction system (30 µL) contained 10 µL total RNA, 1 µL M-MLV reverse transcriptase, 1 µL RNase inhibitor, 4 µL dNTP, 2 µL Oligo dT, 4 µL DTT, and 8 µL 5× Buffer. The reaction was performed at 42 °C for 30 minutes, followed by enzyme inactivation at 99 °C for 5 minutes and cooling at 5 °C for 5 minutes. The cDNA products were briefly centrifuged and stored at -20 °C until use. Real-time PCR was performed using a Bio-Rad CFX96 system with the following conditions: Taq polymerase activation at 95 °C for 30 seconds, followed by 40 cycles of denaturation at 95 °C for 10 seconds and annealing/extension at 60 °C for 30 seconds.

1.7 Statistical Analysis

Differences in target gene mRNA expression levels between toxin-treated and control samples were analyzed using REST software (Pfaffl, 2001) with the following formula:

Ratio = (E_{target}^ΔCT_{target}) / (E_{ref}^ΔCT_{ref})

where Ratio represents the expression ratio, E_{target} is the amplification efficiency of the target gene, E_{ref} is the amplification efficiency of the reference gene, ΔCT_{target} is the difference in cycle threshold values for the target gene, and ΔCT_{ref} is the difference for the reference gene. Data were further analyzed using SPSS 13.0 software for significance testing (F-test) and correlation analysis. All measurements were repeated across three independent cell batches, with three replicates per group within each batch. Results are expressed as mean ± standard deviation.

Results

2.1 Effects of Combined DON and ZEA Exposure on Intracellular Ca^{2+} Levels

As shown in , intracellular Ca^{2+} levels in chicken splenic lymphocytes increased with rising toxin concentrations after 48 hours of combined DON and ZEA exposure. All treated groups were extremely significantly higher than the blank control group ($P<0.01$). Except for the non-significant difference between DZ-2 and DZ-3 groups ($P>0.05$), all other inter-group comparisons showed significant or extremely significant differences ($P<0.05$ or $P<0.01$). These results demonstrate that combined DON and ZEA exposure causes a dose-dependent increase in intracellular Ca^{2+} levels in cultured chicken splenic lymphocytes.

2.2 Effects of Combined DON and ZEA Exposure on Intracellular pH

reveals that intracellular pH in chicken splenic lymphocytes progressively decreased with increasing toxin concentrations following 48 hours of combined exposure. All treated groups were significantly or extremely significantly lower

than the blank control group ($P<0.05$ or $P<0.01$), with significant or extremely significant differences observed between all treatment groups ($P<0.05$ or $P<0.01$). This indicates that combined DON and ZEA exposure induces a dose-dependent reduction in intracellular pH in chicken splenic lymphocytes.

2.3 Effects of Combined DON and ZEA Exposure on CaM mRNA Expression Levels

According to , CaM mRNA expression levels in chicken splenic lymphocytes were significantly or extremely significantly higher than the blank control group in all treated groups except DZ-1, which showed a slight non-significant decrease ($P>0.05$). Among the treated groups, CaM mRNA expression increased with toxin concentration, with significant or extremely significant differences between groups ($P<0.05$ or $P<0.01$), except for the non-significant difference between DZ-1 and DZ-2 ($P>0.05$). These findings suggest that combined DON and ZEA exposure leads to a dose-dependent increase in CaM mRNA expression (with the exception of the low-dose DZ-1 group) in cultured chicken splenic lymphocytes.

2.4 Effects of Combined DON and ZEA Exposure on Cellular Membrane ATPase Activities

demonstrates that both Na^+/K^+-ATPase and Ca^{2+}-ATPase activities in chicken splenic lymphocytes decreased progressively with increasing toxin concentrations after 48 hours of combined exposure. All treated groups were extremely significantly lower than the blank control group ($P<0.01$). Except for the significant difference between DZ-3 and DZ-4 groups ($P<0.05$), all other inter-group comparisons showed extremely significant differences ($P<0.01$). This indicates that combined DON and ZEA exposure causes a dose-dependent reduction in cellular membrane ATPase activities. Notably, the decline in Ca^{2+}-ATPase activity was more pronounced than that of Na^+/K^+-ATPase activity, suggesting greater susceptibility of Ca^{2+}-ATPase to DON and ZEA toxicity.

Discussion

Tonshin et al. [8] reported that DON affects oxidative phosphorylation in mouse liver mitochondria, altering mitochondrial membrane potential and levels of H^+, K^+, and other ions, causing mitochondrial swelling, increased K^+ permeability, and Ca^{2+} efflux. These effects impair mitochondrial membrane function and disrupt calcium homeostasis. Peng et al. [9] demonstrated that DON significantly blocks L-type, B-type, and T-type Ca^{2+} channels in cultured human cardiomyocytes, reducing channel opening probability and duration while prolonging closed states. These studies collectively show that DON disrupts calcium homeostasis and interferes with Ca^{2+}-related signal transduction in both mouse hepatocytes and human cardiomyocytes, leading to cellular dysfunction. Intracellular Ca^{2+} overload plays a critical role in apoptosis, primarily by activating Ca^{2+}/Mg^{2+}-dependent endonucleases and Ca^{2+}/CaM-dependent enzymes [10].

Intracellular Ca^{2+} overload is closely linked to mitochondrial function—mitochondria act as intracellular calcium stores and play a vital role in maintaining Ca^{2+} homeostasis [11], while ATP depletion resulting from impaired mitochondrial respiration directly contributes to elevated intracellular Ca^{2+} levels [12]. Conversely, Ca^{2+} overload can promote mitochondrial oxidative phosphorylation uncoupling and opening of the mitochondrial permeability transition pore, leading to inhibited oxidative phosphorylation, reduced proton motive force, mitochondrial swelling, and release of mitochondrial Ca^{2+} into the cytoplasm, thereby facilitating cell death [13]. Our study found that combined DON and ZEA exposure caused Ca^{2+} overload in cultured chicken splenic lymphocytes and mitochondrial dysfunction, which may represent an important mechanism underlying toxin-induced apoptosis. This finding aligns with Busk et al. [14], who used quantitative proteomics to demonstrate that ZEA affects oxidative phosphorylation pathways and induces mitochondrial dysfunction in human adrenocortical H295R cells.

Intracellular Ca^{2+} levels are regulated by multiple factors. In addition to mitochondrial regulation, cellular membrane Na^+/K^+-ATPase and Ca^{2+}-ATPase play crucial roles in maintaining Ca^{2+} homeostasis by transporting free cytoplasmic Ca^{2+} across the plasma membrane into the extracellular fluid and participating in other ion transport processes and ATP synthesis. Reduced activity of these enzymes leads to cytoplasmic Ca^{2+} overload [15]. Calmodulin (CaM) serves as a primary Ca^{2+} receptor in eukaryotic cells, transducing Ca^{2+} signals to regulate various cellular functions. When intracellular Ca^{2+} reaches certain levels (>10 μmol/L), Ca^{2+} binds to CaM, activating it; the activated CaM then stimulates Ca^{2+}-ATPase to maintain low cytoplasmic Ca^{2+} concentrations, functioning as a second messenger [16]. Our results show that combined DON and ZEA exposure at various doses significantly or extremely significantly reduced both Na^+/K^+-ATPase and Ca^{2+}-ATPase activities in chicken splenic lymphocytes, which may contribute to intracellular Ca^{2+} overload. Both ATPases are ATP-dependent enzymes requiring sufficient intracellular ATP for proper function; inhibition of mitochondrial respiration and subsequent ATP depletion can suppress their activities [17]. The reduced ATPase activities observed in our study likely resulted from DON and ZEA-induced oxidative damage to cellular membranes and interference with intracellular energy metabolism [18]. Additionally, CaM mRNA expression levels were significantly or extremely significantly higher in all treated groups compared to the control, suggesting that DON and ZEA may affect Ca^{2+} release from intracellular stores, leading to elevated Ca^{2+} levels that bind to and activate CaM. However, the concurrent reduction in Ca^{2+}-ATPase activity prevents timely extrusion of Ca^{2+} from the cell, resulting in Ca^{2+} overload and potentially triggering apoptosis.

Intracellular homeostasis is essential for normal cellular function. The plasma membrane Ca^{2+}-ATPase hydrolyzes one ATP molecule to transport 1–2 Ca^{2+} ions across the membrane while importing H^+ at a 1:2 ratio, maintaining electroneutrality and preventing membrane potential from affecting Ca^{2+} transport. The Na^+/Ca^{2+} exchanger in the plasma membrane represents another impor-

tant component of calcium homeostasis regulation. Intracellular pH regulation occurs through ion transport mechanisms and robust cytoplasmic buffering capacity, including Na^+/H^+ exchange, ATP-driven H^+ pumps, and various bicarbonate exchangers, with Na^+/H^+ exchange playing a predominant role [18]. The degree of intracellular acidification correlates quantitatively with apoptosis incidence, and studies have shown that pH changes directly participate in mitochondria-mediated apoptosis, with intracellular acidification promoting cytochrome c-mediated caspase activation [20]. Our findings demonstrate that combined DON and ZEA exposure reduces intracellular pH in cultured chicken splenic lymphocytes, suggesting that toxin-induced mitochondrial membrane damage is the primary cause of intracellular acidification, which in turn further promotes apoptosis.

In conclusion, combined exposure to DON and ZEA disrupts intracellular homeostasis in cultured chicken splenic lymphocytes, manifesting as Ca^{2+} overload (evidenced by upregulated CaM mRNA expression and increased intracellular Ca^{2+} levels), intracellular acidification, and reduced cellular membrane ATPase activities (Na^+/K^+-ATPase and Ca^{2+}-ATPase).

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