Effects of Deoxynivalenol and Zearalenone Co-exposure on Intracellular Homeostasis in In Vitro Cultured Chicken Splenic Lymphocytes (Postprint)

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Date: 2017-11-07T00:00:00+00:00

Abstract

The present study was designed to investigate the effects of combined exposure to deoxynivalenol (DON) and zearalenone (ZEA) on intracellular homeostasis in in vitro cultured chicken spleen lymphocytes. The lymphocytes were exposed to combined treatments of 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 for 48 hours, after which the activities of cell membrane ATPases (Ca2+-ATPase, Na+/K+-ATPase), intracellular pH, Ca2+ levels, and mRNA expression levels of calmodulin (CaM) were determined. A blank control group without toxin supplementation was simultaneously established. The results demonstrated that in the toxin-treated experimental groups, intracellular Ca2+ levels and CaM mRNA expression levels increased in a concentration-dependent manner, 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 induced a series of intracellular homeostasis imbalances in in vitro cultured chicken spleen lymphocytes, including intracellular acidification and ionic balance disruption, 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 intracellular homeostasis in chicken splenic lymphocytes cultured *in vitro*. Primary chicken splenic lymphocytes were exposed to combined treatments of DON and ZEA at the following concentrations: 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, the activities of cellular membrane ATPases (Ca^2 -ATPase and Na /K -ATPase), intracellular pH, Ca^2 levels, and calmodulin (CaM) mRNA expression levels were measured. A blank control group without toxin addition was also included. The results demonstrated that intracellular Ca^2 levels and CaM mRNA expression increased with rising toxin concentrations in all treatment 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 cellular membrane Ca^2 -ATPase and Na /K -ATPase decreased with increasing toxin concentrations, with all treatment groups exhibiting significant or highly significant reductions relative to the control ($P < 0.05$ or $P < 0.01$). These findings indicate that combined exposure to DON and ZEA induces intracellular acidification, ionic imbalance, and other disruptions to intracellular homeostasis in chicken splenic lymphocytes cultured *in vitro*, with effects showing clear dose-dependency.

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

1.1 Experimental Materials

The following reagents were used: fetal bovine serum (FBS) (Gibco, USA); DON, ZEA, and phenol red-free RPMI 1640 medium (Sigma, USA); Cell Counting Kit-8 (CCK-8) (Dojindo, Japan); intracellular pH fluorescent probe BCECF-AM (Dojindo, Japan); intracellular calcium ion 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 (Ca^2 -ATPase and Na /K -ATPase) activity assay kits (Nanjing Jiancheng Bioengineering Institute, China).

1.2 Experimental Methods

Preparation of splenic lymphocyte suspension: Under aseptic conditions, spleens were harvested from healthy Isa Brown roosters aged 40–60 days provided by 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 residual blood, and carefully stripped of surrounding connective tissue. Each spleen was then transferred to another PBS-filled dish containing a 200-mesh screen and gently ground using the plunger of a 20 mL disposable syringe. The filtered suspension was appropriately diluted and layered at a 1:1 volume ratio over chicken lymphocyte separation medium in a centrifuge tube. 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 supernatant was discarded, and the pellet was resuspended in toxin-free RPMI 1640 complete medium (supplemented with fetal bovine serum), washed once more, and finally resuspended to prepare a cell suspension at 5×10 cells/mL. Cell viability exceeding 95% as determined by trypan blue exclusion indicated successful preparation.

Determination of combined exposure concentrations: Using the CCK-8 assay, we evaluated the effects of DON and ZEA individually on the viability of chicken splenic lymphocytes cultured *in vitro*. After 48 hours of exposure, the half-maximal inhibitory concentration (IC) was determined to be (30.82 ± 10.48) μg/mL for DON and (23.91 ± 4.96) μg/mL for ZEA. Based on these IC values and considering that preliminary experiments showed severe lymphocyte damage at high concentrations of either toxin alone, we selected lower concentrations for combined exposure in the formal experiment. The final combined exposure doses were established as: 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 toxin addition was also included.

1.3 Determination of Intracellular Ca^2 Levels

After 48 hours of exposure culture, 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, and Fluo-3/AM fluorescent probe was added to a final concentration of 1 μmol/L. After mixing, the suspension was incubated at 37 °C for 30 minutes in the dark, washed three times with PBS, and analyzed by

flow cytometry to determine mean fluorescence intensity (excitation wavelength 488 nm, emission wavelength 530 nm).

1.4 Determination of Intracellular pH

Following 48 hours of exposure culture, cells were collected by centrifugation at 1,500 r/min for 3 minutes and washed three times with PBS. The pellet was resuspended in serum-free RPMI 1640 medium to prepare a cell suspension at 5 × 10 cells/mL, and BCECF/AM fluorescent probe was added to a final concentration of 2 μmol/L. The suspension was incubated in a CO incubator (dark, 37 °C) for 30 minutes, then collected and washed three times with serum-free RPMI 1640 medium. After final resuspension in PBS, samples were analyzed by flow cytometry with 488 nm excitation, and intracellular pH was determined from the ratio of green to red fluorescence intensity displayed on a two-dimensional dot plot (X-axis 525 nm, Y-axis 610 nm) based on a standard curve. At least 10,000 cells were analyzed per sample [7].

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

After 48 hours of exposure culture, cells were collected by centrifugation at 1,500 r/min for 3 minutes and washed three times with PBS. Each sample was resuspended in 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 physiological saline, and the supernatant was used to determine Na /K -ATPase and Ca^2 -ATPase activities using the phosphorus determination method according to 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 -actin (L08165) and CaM (NM205005) were designed using Primer 5.0 software based on sequences from GenBank, subjected to homology searches using GenBank Blast, 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 activation at 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 30 seconds.

1.7 Statistical Analysis

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

Ratio = (E_target^ΔC_t_target) / (E_ref^ΔC_t_ref)

where E_target is the amplification efficiency of the target gene, E_ref is the amplification efficiency of the reference gene, ΔC_t_target is the difference in threshold cycles for the target gene, and ΔC_t_ref is the difference in threshold cycles for the reference gene. Data were subjected to significance testing using F-tests and correlation analysis with SPSS 13.0 software. All measurements were repeated using three different batches of cells, with three replicates per group in each batch. Data are expressed as mean ± standard deviation.

2.1 Effects of Combined DON and ZEA Exposure on Intracellular Ca^2 Levels in Chicken Splenic Lymphocytes Cultured *In Vitro*

As shown in , after 48 hours of combined DON and ZEA exposure, intracellular Ca^2 levels in chicken splenic lymphocytes increased with rising toxin concentrations. All treatment groups showed highly significant differences compared to 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 pairwise comparisons between treatment groups were significant or highly significant ($P < 0.05$ or $P < 0.01$). These results indicate that combined exposure to DON and ZEA causes a dose-dependent increase in intracellular Ca^2 levels in chicken splenic lymphocytes cultured *in vitro*.

2.2 Effects of Combined DON and ZEA Exposure on Intracellular pH in Chicken Splenic Lymphocytes Cultured *In Vitro*

As shown in , intracellular pH in chicken splenic lymphocytes decreased progressively with increasing toxin concentrations following 48 hours of combined DON and ZEA exposure. All treatment groups were significantly or highly significantly lower than the blank control group ($P < 0.05$ or $P < 0.01$), with significant or highly significant differences observed between all treatment groups ($P < 0.05$ or $P < 0.01$). These findings demonstrate 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 in Chicken Splenic Lymphocytes Cultured *In Vitro*

As shown in , after 48 hours of combined exposure, CaM mRNA expression levels were slightly decreased in the DZ-1 group compared to the blank control (P

> 0.05), but were significantly or highly significantly increased in all other treatment groups ($P < 0.05$ or $P < 0.01$). Among treatment groups, CaM mRNA expression levels rose with increasing toxin concentrations, with significant or highly significant differences between groups ($P < 0.05$ or $P < 0.01$), except for the non-significant difference between DZ-1 and DZ-2 groups ($P > 0.05$). These results indicate that combined DON and ZEA exposure causes a dose-dependent increase in CaM mRNA expression levels (except for the DZ-1 group which was lower than control), suggesting a significant dose-dependent relationship.

2.4 Effects of Combined DON and ZEA Exposure on Cellular Membrane ATPase Activities in Chicken Splenic Lymphocytes Cultured *In Vitro*

As shown in , both Na /K -ATPase and Ca² -ATPase activities in chicken splenic lymphocytes decreased with increasing toxin concentrations after 48 hours of combined exposure. All treatment groups were highly 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 differences were highly significant ($P < 0.01$). These findings demonstrate that combined DON and ZEA exposure causes a dose-dependent reduction in cellular membrane ATPase activities. The decline in Ca² -ATPase activity was more pronounced than that of Na /K -ATPase activity with increasing toxin concentrations, indicating that Ca² -ATPase is more sensitive to DON and ZEA exposure.

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 Ca² efflux, which damages mitochondrial membrane function and disrupts calcium homeostasis. Peng et al. [9] demonstrated that DON significantly blocks B, L, and T-type Ca² channels in cultured human myocardial cells, reducing channel open probability and duration while prolonging closed states. These studies indicate that DON disrupts calcium homeostasis and interferes with Ca² -related signal transduction in both mouse hepatocytes and human myocardial cells, leading to cellular dysfunction. Intracellular Ca² overload plays a crucial role in apoptosis, primarily by activating Ca² /Mg² -dependent endonucleases and Ca² /CaM-dependent enzymes [10]. Intracellular Ca² overload is closely related to mitochondrial function; mitochondria serve as intracellular calcium stores and play a vital role in maintaining Ca² homeostasis [11]. ATP depletion resulting from impaired mitochondrial function directly mediates increased intracellular Ca² levels [12], while Ca² overload can promote mitochondrial oxidative phosphorylation uncoupling and opening of the mitochondrial permeability transition pore, leading to inhibition of oxidative phosphorylation, reduced proton motive force, mitochondrial swelling, and release of mitochondrial Ca² into the cytoplasm, thereby promoting cell death [13]. Our study found that combined DON and ZEA exposure caused intracellular Ca² overload and mitochondrial dysfunction in chicken splenic lymphocytes, which may represent

an important mechanism underlying DON and ZEA-induced apoptosis. This is consistent with the findings of Busk et al. [14], who used quantitative proteomics to demonstrate that ZEA affects oxidative phosphorylation pathways and mitochondrial function 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 intracellular Ca^{2} homeostasis by transporting free cytoplasmic Ca^{2} across the membrane to 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]. CaM is a major Ca^{2} receptor in eukaryotic cells that transmits 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; 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 highly significantly reduced 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 Na /K -ATPase and Ca^{2} -ATPase activities [17]. The reduced ATPase activities observed in this study may result 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 highly significantly higher in all treatment groups compared to the blank control, suggesting that DON and ZEA may affect Ca^{2} release from intracellular stores, increase intracellular Ca^{2} levels, promote Ca^{2} -CaM binding and CaM activation, and subsequently activate Ca^{2} -ATPase. However, reduced Ca^{2} -ATPase activity prevents timely extrusion of Ca^{2} from the cell, resulting in intracellular Ca^{2} overload, which may represent an important mechanism of apoptosis in chicken splenic lymphocytes.

Intracellular homeostasis is essential for normal cellular function. Cellular membrane Ca^{2} -ATPase hydrolyzes one ATP molecule to transport 1–2 Ca^{2} ions across the membrane to the extracellular space while transporting H into the cell at a 1:2 ratio, maintaining electroneutrality and preventing membrane potential from affecting Ca^{2} transport. The Na /Ca^{2} exchanger in the cellular membrane, particularly the plasma membrane, is considered an important component of calcium homeostasis regulation, while acid-base balance regulation is a prerequisite for homeostasis. Intracellular pH regulation is achieved through ion transport mechanisms and strong cytoplasmic buffering capacity, including Na /H exchange, ATP-driven H pumps, and various bicarbonate exchangers, with Na /H exchange playing a major role in pH regulation [18]. The degree of intracellular acidification correlates with apoptosis incidence in a dose-dependent manner, and studies have shown that intracellular pH changes directly participate in mitochondria-mediated apoptosis, with intracellular acid-

ification promoting cytochrome c-mediated caspase activation [20]. Our study found that combined DON and ZEA exposure reduced intracellular pH in chicken splenic lymphocytes, suggesting that DON and ZEA-induced mitochondrial membrane damage is the primary cause of intracellular acidification, which in turn promotes apoptosis.

In conclusion, combined exposure to DON and ZEA disrupts intracellular homeostasis in chicken splenic lymphocytes cultured *in vitro*, primarily manifested as intracellular Ca^2 overload (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).

Acknowledgments

We thank Professor Xu Shiwen, Professor Li Yanfei, Professor Li Jinlong, Mr. Wang Wei, Mr. Zhang Zhigang, Dr. Zhang Ziwei, Mr. Su Jian, Mr. Zhang Bo, and Mr. Guan Bo from Northeast Agricultural University College of Veterinary Medicine for their assistance during the experiment.

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