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The Effect of Vomitoxin on IPEC-J2 Cell Apoptosis Postprint

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Abstract

This study utilized IPEC-J2 cells as an experimental model to investigate the effects of deoxynivalenol (DON) at different concentrations and treatment durations on apoptosis in porcine jejunal epithelial cells. The CCK-8 assay kit was employed to assess cytotoxicity and determine appropriate DON exposure times (6, 12, and 24 h). IPEC-J2 cells were treated with low (200 ng/mL) and high (2,000 ng/mL) concentrations of DON for 6, 12, and 24 h to examine the effects of DON on the mitotic cell cycle and early apoptosis. The results demonstrated: 1) DON significantly inhibited IPEC-J2 cell growth; at DON concentrations of 200 and 2,000 ng/mL, cell viability at 48 and 72 h was significantly lower than at 24 h ($P<0.05$); at a DON concentration of 2,000 ng/mL, cell viability at 72 h was significantly lower than at 48 h ($P<0.05$). 2) Different DON concentrations exerted distinct effects on the cell cycle; low-concentration DON primarily acted on the S phase of mitosis, interfering with DNA replication, whereas high-concentration DON mainly targeted the G2/M phase, disrupting the synthesis of relevant enzymes and spindle proteins. 3) Following 6 h of DON treatment, both low- and high-concentration DON groups exhibited significantly higher early and total apoptosis rates compared to the control group ($P<0.05$); after 12 h of DON treatment, the high-concentration DON group showed significantly higher early and total apoptosis rates than both the control and low-concentration DON groups ($P<0.05$). These findings indicate that DON suppresses IPEC-J2 cell proliferation, with low-concentration DON arresting mitosis in the S phase and high-concentration DON arresting mitosis in the G2/M phase. DON promotes early apoptosis and increases the total apoptosis rate, with this pro-apoptotic effect intensifying as DON concentration increases.

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Full Text

Effects of Deoxynivalenol on Apoptosis of IPEC-J2 Cells

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Abstract

This study investigated the effects of deoxynivalenol (DON) on apoptosis in porcine jejunal epithelial cells (IPEC-J2) under various concentrations and exposure durations. Using the Cell Counting Kit-8 (CCK-8) assay to assess cytotoxic effects on cell proliferation, appropriate DON exposure times (6, 12, and 24 h) were selected. IPEC-J2 cells were then treated with low (200 ng/mL) and high (2,000 ng/mL) concentrations of DON for 6, 12, and 24 h to examine effects on the cell mitotic cycle and early apoptosis. The results showed that: (1) DON significantly inhibited IPEC-J2 cell growth. At DON concentrations of 200 and 2,000 ng/mL, cell viability at 48 and 72 h was significantly lower than at 24 h ($P<0.05$). At 2,000 ng/mL, viability at 72 h was significantly lower than at 48 h ($P<0.05$). (2) Different DON concentrations affected the cell cycle differently. Low-concentration DON primarily acted on the S phase of mitosis, interfering with DNA replication, whereas high-concentration DON mainly affected the G2/M phase, disrupting synthesis of enzymes and spindle proteins. (3) After 6 h of DON exposure, both low- and high-concentration groups showed significantly higher early and total apoptosis rates compared to the control ($P<0.05$). After 12 h, the high-concentration group exhibited significantly higher early and total apoptosis rates than both the control and low-concentration groups ($P<0.05$). In conclusion, DON inhibits IPEC-J2 cell proliferation, with low concentrations arresting cells in S phase and high concentrations arresting them in G2/M phase. DON promotes early apoptosis and increases total apoptosis rates in a concentration-dependent manner.

Keywords: deoxynivalenol; IPEC-J2 cells; cell cycle; apoptosis; proliferation

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Deoxynivalenol (DON), belonging to the trichothecene family, is primarily produced by *Fusarium* species such as *F. graminearum*, *F. oxysporum*, and *F. nivale*, with additional production by certain *Cephalosporium* and *Trichoderma* species. Due to its emetic effects in swine, it is commonly known as vomitoxin [1-2]. The toxin was first identified in 1970 in scab-infected barley in Kagawa Prefecture, Japan, and its chemical structure was elucidated and named in 1972 by Japanese researchers [3]. Human consumption of DON-contaminated food causes acute poisoning symptoms including anorexia, vomiting, fever, diarrhea, unsteady gait, and slowed reflexes, with severe cases affecting the hematopoietic

system and potentially causing death [4]. The risk is particularly pronounced in China due to traditional dietary patterns with higher proportions of grains compared to Western diets. In 1998, the International Agency for Research on Cancer classified DON as a Group 3 carcinogen. The European Union mandates DON concentrations below 0.75 mg/kg in feed, while China' s limit is 1 mg/kg [5-6].

Following ingestion, DON exerts neurotoxic effects in pigs, with even low concentrations increasing 5-hydroxyindoleacetic acid levels in the brain [7]. DON exhibits hemolytic effects on rat erythrocytes in a concentration-dependent manner, with a threshold below which hemolysis does not occur [8]. The toxin may exert cytotoxic effects on prokaryotic cells through three mechanisms: (1) penetration of the phospholipid bilayer to act at the subcellular level, (2) interaction with cell membranes, and (3) free radical-mediated lipid peroxidation, potentially acting through one or more simultaneous pathways [7]. Research demonstrates that DON exerts cytotoxic effects on bone marrow hematopoietic cells, with immunotoxicity dependent on concentration, lymphocyte subtype, tissue origin, and glucocorticoids [9]. Additionally, DON inhibits protein synthesis in thymocytes and exhibits embryotoxic and teratogenic effects. Animal studies show that long-term low-dose exposure can induce tumors in various organs [10]. Oral administration of 5 and 25 mg/kg DON to mice increased mRNA expression of pro-inflammatory cytokines IL-6, IL-1 , and TNF- . High DON concentrations inhibited proliferation and immunoglobulin production in cultured human peripheral lymphocytes in a dose-dependent manner, whereas low concentrations increased immunoglobulin levels [11-12].

Porcine intestinal epithelial cells play crucial roles in the immune system, participating in innate immune responses through barrier function, antimicrobial peptide synthesis, mucus secretion, and cytokine signaling. However, most knowledge of intestinal cell function derives from human cancer cell lines or classical animal models. Since species exhibit different cytokine responses to specific environmental stimuli, caution is required when extrapolating immune function models from mice and humans to pigs [13]. Therefore, fundamental research at the molecular level is essential.

This study employed the porcine jejunal epithelial cell line IPEC-J2 to evaluate DON cytotoxicity using CCK-8 assay for screening appropriate concentrations and exposure times, subsequently examining DON effects on cell cycle and apoptosis to characterize its impact on IPEC-J2 cell death.

1.1 Experimental Materials

DON (Sigma-Aldrich, USA); penicillin and streptomycin (Beyotime Institute of Biotechnology); fetal bovine serum (FBS), high-glucose Dulbecco' s Modified Eagle Medium (DMEM), 25% trypsin-EDTA, phenol red, and trypsin (Gibco, USA); phosphate-buffered saline (PBS) (HyClone, USA); Cell Counting Kit-8 (Dojindo Laboratories, Japan); Annexin V-FITC/PI apoptosis detection kit

(KeyGen Biotech, China); propidium iodide (PI) (Sigma, USA).

1.2 Experimental Instruments

Memmert CO incubator, Leica inverted fluorescence microscope, Eppendorf centrifuge (Germany); SW-CJ-IF laminar flow hood (Suzhou Purification Equipment Co., Ltd.); multifunctional microplate reader (Switzerland).

1.3 Cell Source

IPEC-J2 cells were provided by the Monogastric Animal Research Group, Institute of Subtropical Agriculture, Chinese Academy of Sciences.

1.4.1 DON Preparation

Original DON powder was dissolved in dimethyl sulfoxide (DMSO) to prepare a 1 mg/mL stock solution, filtered through a 0.22 m membrane, and then diluted with complete medium containing 10% FBS and 1% penicillin-streptomycin to final concentrations of 200 and 2,000 ng/mL. All procedures were performed in a biosafety cabinet.

1.4.2 Cell Culture

IPEC-J2 cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic at a seeding density of 3.0×10 cells/mL in 10 cm dishes and incubated at 37°C in a humidified atmosphere with 5% CO .

1.4.3 CCK-8 Assay Seeding and DON Treatment

Logarithmic-phase IPEC-J2 cells were trypsinized with 2.5 g/L trypsin and seeded into three 96-well plates designated for 24, 48, and 72 h timepoints at 1×10 cells/well. Each plate contained four groups (A, B, C, D) with two columns and ten replicates per group. Upon reaching appropriate confluence, medium was replaced with treatment solutions as detailed in Table 1 . Column 1 served as the blank control containing 100 L medium only, while other columns received 100 L of treatment medium.

Table 1 Groups and DON Concentrations

Group	DON Concentration
A (Blank)	Cell-free medium
B (Control)	DON-free medium
C	200 ng/mL
D	2,000 ng/mL

After 24, 48, or 72 h of DON exposure, medium was replaced with 100 L fresh medium containing 10% CCK-8 reagent and incubated for 2 h. Absorbance

(OD) was measured at 450 nm using a microplate reader. Cell viability was calculated as: Viability (%) = [(OD - OD) / (OD - OD)] × 100.

1.5.1 Cell Culture and DON Treatment

IPEC-J2 cells were seeded in 10 cm dishes and cultured at 37°C with 5% CO . Upon reaching appropriate density, medium was replaced with fresh medium containing 0, 200, or 2,000 ng/mL DON for 6, 12, or 24 h.

1.5.2 Cell Collection and Detection

Seventy percent ethanol was prepared and pre-chilled at -20°C. Cells were trypsinized with 2.5 g/L trypsin, collected in 15 mL tubes, and centrifuged at 1,000 rpm for 5 min at 20°C. After removing supernatant, cells were washed with 3-5 mL PBS and centrifuged again. Approximately 200 L PBS was retained to resuspend the cell pellet. While vortexing gently, 800 L pre-chilled 70% ethanol was added dropwise to fix cells. Cell cycle samples could be stored at -20°C for 3-4 days.

Fixed samples were centrifuged at 800 rpm for 5 min, supernatant was discarded, and cells were resuspended in 1 mL cold PBS, centrifuged again, and washed 1-2 times to remove ethanol. Cells were then stained with 150 L PI working solution for 30 min at 4°C in the dark. Samples were transferred to flow cytometry tubes and analyzed using a 488 nm argon laser with 630 nm bandpass filter. Ten thousand cells were collected via FSC/SSC scatter plots, and gating was applied to exclude doublets and debris. Percentages of cells in each cycle phase were determined from PI fluorescence histograms.

1.6.1 Cell Culture and DON Treatment

As described in Section 1.5.1.

1.6.2 Cell Collection and Detection

After 6, 12, or 24 h of DON treatment, medium was removed and cells were washed once with PBS. Cells were trypsinized, collected in 15 mL tubes, and centrifuged at 1,000 rpm for 5 min at 20°C. After two PBS washes (2,000 rpm, 5 min, 20°C), 1×10 to 5×10 cells were resuspended in 500 L binding buffer. Five microliters of Annexin V-FITC was added, mixed gently, followed by 5 L PI. Samples were incubated at room temperature in the dark for 5-15 min and analyzed by flow cytometry within 1 h.

1.7 Statistical Analysis

All data were analyzed using SPSS 22.0 general linear model univariate analysis with viability as the dependent variable and concentration and time as

fixed factors. P<0.05 was considered statistically significant and P<0.01 highly significant.

2.1 Effects of DON Concentration and Exposure Time on IPEC-J2 Cell Viability

The effects of various DON concentrations and exposure durations on IPEC-J2 cell viability are shown in Figure 1 [Figure 1: see original paper]. Compared with controls at each timepoint, both 200 and 2,000 ng/mL DON groups showed highly significant reductions in viability (P<0.01), with the 2,000 ng/mL group showing significantly lower viability than the 200 ng/mL group (P<0.01). Across timepoints, cell viability exhibited a time-dependent decrease at each DON concentration. At 200 and 2,000 ng/mL, viability at 48 and 72 h was significantly lower than at 24 h (P<0.05). At 200 ng/mL, no significant difference was observed between 48 and 72 h (P>0.05).

Figure 1 Effects of different DON concentrations and exposure times on IPEC-J2 cell viability. Different lowercase letters at each timepoint indicate significant differences (P<0.05); same letters indicate no significant difference (P>0.05). *** indicates highly significant differences between DON concentrations (P<0.01).

2.2.1 Cell Cycle Profiles

Representative cell cycle histograms for IPEC-J2 cells under different DON concentrations and exposure times are shown in Figure 2 [Figure 2: see original paper], with quantitative effects presented in Figure 3 [Figure 3: see original paper]. Low-concentration DON primarily affected the S phase of mitosis, interfering with DNA replication, whereas high-concentration DON predominantly impacted the G2/M phase, disrupting synthesis of enzymes and spindle proteins.

Figure 2 IPEC-J2 cell cycle histograms under different DON concentrations and exposure times. G0/G1 indicates pre-mitotic phase, S indicates metaphase, and G2/M indicates post-mitotic phase. Same applies to Figure 3.

Figure 3 Effects of different DON concentrations and exposure times on IPEC-J2 cell cycle distribution. Value columns with same lowercase letters indicate no significant difference (P>0.05); different letters indicate significant difference (P<0.05).

2.3 Effects of DON on Apoptosis

DON effects on apoptosis are presented in Figure 4 [Figure 4: see original paper] and Tables 2 and 3 . At 6 h, early and total apoptosis rates increased with DON concentration, with both 200 and 2,000 ng/mL groups significantly higher than control (P<0.05). At 12 h, the 2,000 ng/mL group showed significantly higher early and total apoptosis rates than both control and 200 ng/mL groups (P<0.05). At 24 h, no significant differences were observed among groups

(P>0.05). Within each concentration, the 200 ng/mL group exhibited significantly lower early and total apoptosis rates at 12 and 24 h compared to 6 h (P<0.05), while the 2,000 ng/mL group showed significantly lower rates at 24 h compared to 6 and 12 h (P<0.05).

Figure 4 IPEC-J2 cell apoptosis scatter plots under different DON concentrations and exposure times. UL quadrant: necrotic cells (upper left); LL quadrant: viable cells (lower left); UR quadrant: late apoptotic cells (upper right); LR quadrant: early apoptotic cells (lower right); G: early apoptosis rate; T%: total apoptosis rate.

Table 2 Effects of different DON concentrations and exposure times on early and total apoptosis rates of IPEC-J2 cells

Time (h)	DON (ng/mL)	Early Apoptosis Rate	Total Apoptosis Rate
6	0	2.56±0.13	3.14±0.37
6	200	3.39±0.49	2.52±0.10
6	2000	3.09±0.37	2.66±0.22
12	0	2.66±0.23	3.23±0.10
12	200	2.80±0.08	2.38±0.31
12	2000	2.56±0.22	2.60±0.21
24	0	3.18±0.12	0.26±0.13
24	200	2.29±0.28	2.70±0.33
24	2000	2.60±0.34	

Values in the same column with different lowercase superscripts differ significantly (P<0.05); same or no superscripts indicate no significant difference (P>0.05).

Table 3 Effects of different DON concentrations and exposure times on early and total apoptosis rates of IPEC-J2 cells

DON (ng/mL)	Time (h)	Early Apoptosis Rate	Total Apoptosis Rate
200	6	2.56±0.13	2.65±0.22
200	12	2.80±0.08	2.38±0.31
200	24	2.29±0.28	2.70±0.33
2000	6	3.14±0.37	3.39±0.49
2000	12	2.66±0.22	3.23±0.11
2000	24	2.38±0.31	2.69±0.32

Same as Table 2.

3 Discussion

DON exhibits significant toxicity to both prokaryotic and eukaryotic cells, particularly affecting rapidly dividing cells including gastrointestinal epithelial cells, lymphocytes, thymocytes, splenocytes, and bone marrow hematopoietic cells, while inhibiting protein synthesis. Alm et al. [14] and Tiemann et al. [15] demonstrated that DON affects cell cycle distribution, inhibiting S phase entry and arresting cells in G0/G1 phase, exerting significant anti-proliferative effects. Our results confirm that DON markedly inhibits cell growth, with pronounced effects within 24 h. At low concentration (200 ng/mL), the inhibitory effect plateaued after 48 h, whereas high concentration (2,000 ng/mL) caused sustained suppression. IPEC-J2 cell viability decreased significantly with increasing DON concentration. Low-concentration DON primarily affected the S phase of mitosis, interfering with DNA replication, while high-concentration DON targeted the G2/M phase, disrupting synthesis of enzymes and spindle proteins. These findings align with previous reports [16].

Apoptosis is a gene-controlled, programmed cell death process that maintains internal environment stability. It proceeds through three stages: initiation, apoptotic body formation, and phagocytic clearance. The mechanism involves apoptotic signal reception, regulatory molecular interactions, protease activation, and execution of the death program, with cellular remnants being recycled. Our apoptosis data show that after 6 h DON exposure, both 200 and 2,000 ng/mL groups exhibited significantly elevated early and total apoptosis rates. At 12 h, only the high-concentration group maintained significantly elevated rates, while by 24 h the effect was no longer significant. Within each concentration, low-dose DON showed significantly reduced apoptosis rates at 12 and 24 h compared to 6 h, whereas high-dose DON demonstrated significantly reduced rates only at 24 h compared to 6 and 12 h. Notably, the pro-apoptotic effect of high-concentration DON persisted through 12 h, likely reflecting activation of distinct apoptotic mechanisms [17].

4 Conclusion

1. DON significantly inhibits IPEC-J2 cell growth in a concentration-dependent manner, with maximal anti-proliferative effects observed within 24 h.
2. DON concentration differentially affects the cell cycle: low concentrations arrest cells in S phase, interfering with DNA replication, while high concentrations arrest cells in G2/M phase, disrupting enzyme and spindle protein synthesis.
3. DON promotes early apoptosis and increases total apoptosis rates in a concentration-dependent manner.

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