Reproductive Toxicity and Mechanism of Action of Zearalenone and Deoxynivalenol in Female Animals (Postprint)

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Abstract

Fusarium mycotoxins zearalenone (ZEA) and deoxynivalenol (DON) are widely present in contaminated grains and feeds. In vivo and in vitro studies have demonstrated that both ZEA and DON can affect the reproductive performance of female animals, causing alterations in reproductive organs, fetal morphology, germ cell maturation rate, and sex hormone secretion. This review summarizes the effects of ZEA and DON on the reproductive performance of female animals and their potential mechanisms of action.

Full Text

Reproductive Toxicities and Functional Mechanisms of Female Animals Induced by Deoxynivalenol and Zearalenone

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Abstract: Fusarium mycotoxins, including zearalenone (ZEA) and deoxynivalenol (DON), are widespread contaminants in cereals and feedstuffs. Both in vivo and in vitro studies have demonstrated that ZEA and DON can impair the reproductive performance of female animals, causing alterations in reproductive organs, fetal morphology, germ cell maturation rates, and sex hormone secretion. This review summarizes the effects of ZEA and DON on the reproductive performance of female animals and their potential mechanisms of action.

Keywords: zearalenone; deoxynivalenol; female animal; reproductive toxicity; functional mechanism

Zearalenone (also known as F-2 toxin, ZEA) and deoxynivalenol (also known as vomitoxin, DON) are mycotoxins produced by *Fusarium*, a common contaminant of grains and feedstuffs. The toxic effects of ZEA and DON have attracted significant attention in many countries and regions worldwide. The European Union has established DON as an important reference value for feed contamination by mycotoxins and as a marker for maximum toxin content limits, stipulating a maximum DON level of 8 mg/kg in cereal grains and their products, and no more than 2 mg/kg in feed for calves under 4 months of age [1]. The United States has set a maximum DON limit of 5 mg/kg for feed grains and by-products (except corn). In China, the maximum DON limit is 1 mg/kg in certain compound feeds (for pigs, calves, and lactating animals) and 5 mg/kg in feed for cattle and poultry, while the maximum ZEA limit in corn is 0.5 mg/kg. Among the various known mycotoxins, Fusarium toxins represent one of the most significant threats to animal health, reducing feed nutritional value and causing substantial economic losses [2]. In recent years, cases of decreased reproductive capacity, reduced production performance, immunosuppression, and secondary diseases in animals due to consumption of mycotoxin-contaminated feed have become increasingly severe, seriously hindering the development of animal husbandry [3]. This review summarizes the effects of Fusarium toxins ZEA and DON on the reproductive performance of female animals and their toxic mechanisms of action.

1 Contamination Status of ZEA and DON

Reports of Fusarium mycotoxin poisoning in animals have emerged from Europe, Asia, Africa, and the Americas [4]. Njobeh et al. [5] investigated mycotoxin contamination in 92 compound feed samples from South Africa, finding high contamination rates of ZEA and DON at concentrations of 30–610 g/kg and 124–2,352 g/kg, respectively, with mixed contamination being common. Steit et al. [6] analyzed 83 feed and feed ingredient samples, identifying 139 different secondary metabolites, with Fusarium toxins being the most prevalent; the positive detection rates for ZEA and DON were 49% and 75%, respectively. In tests conducted by Biomin in the first half of 2013 on 79 corn samples, 70 wheat samples, and 21 bran samples, DON was identified as the principal mycotoxin contaminant, with contamination rates exceeding 87%; notably, DON contamination in bran was far more severe than in corn, reaching 100% [7].

Investigations into mycotoxin contamination in feed ingredients from Sichuan, a region with hot and humid climate conditions, revealed that DON was detected in wheat and wheat by-products at rates of 72.7% and 71.4%, respectively, with a maximum concentration of 1,562 g/kg; ZEA detection rates were 87.0% and 100%, respectively, with a maximum concentration of 3,711.6 g/kg [8]. Huang et al. [9] found that although ZEA and DON contamination levels decreased in 2013 compared to the previous year, contamination of wheat and wheat bran

remained severe. These findings demonstrate that contamination by Fusarium toxins ZEA and DON is widespread and serious, with DON posing a greater hazard.

2 Effects on Female Reproductive Function

2.1 Effects of ZEA on Female Reproductive Function

ZEA possesses a structure similar to estrogen and can bind to estrogen receptors, exerting estrogenic effects with a potency approximately one-tenth that of estrogen. ZEA can cause ovarian lesions, interfere with ovulation, prolong the estrous interval, reduce litter size or cause infertility, and induce nymphomania, pseudopregnancy, and endometrial lesions in sows [10].

2.1.1 Effects on Female Germ Cells Early studies on porcine oocytes cultured in vitro demonstrated that the degeneration rate of porcine oocytes increased progressively with -ZEA and -ZEA concentrations (3.75–90 mol/L), with -ZEA exhibiting more pronounced effects than -ZEA [11]. Additionally, ZEA (10 g/L) reduced the number of secondary oocytes that underwent division to produce ova and second polar bodies, while significantly decreasing the ability of sperm to penetrate metaphase II oocytes, thereby inhibiting further oocyte development and reducing oocyte activation rates [12].

Recent in vitro studies have shown that ZEA inhibits the proliferation and viability of porcine and murine ovarian granulosa cells and oocytes, and reduces maturation rates by disrupting the cytoskeleton, inducing abnormal spindle morphology in oocytes, increasing abnormal chromosome numbers, and altering actin expression and distribution; these effects are dose-dependent in ovarian granulosa cells [13-14].

2.1.2 Effects on Sex Hormone Secretion ZEA is not only a ligand for estrogen receptors but also a competitive substrate for steroid synthesis and metabolic enzymes. Similar to steroid hormones, ZEA can participate in biotransformation as an important transcription factor. Both in vivo and in vitro studies have shown that ZEA affects progesterone and estrogen secretion, although whether these effects are inhibitory or stimulatory remains controversial.

Research has found that ZEA (75 g) and its derivatives -ZEA and -ZEA (at concentrations of 15 and 30 mol/L, respectively) can reduce progesterone content in porcine ovarian granulosa cells [15] and in the serum of female Beagle dogs, while increasing serum 17- estradiol levels in female Beagle dogs [16]. However, Cortinovis et al. [17] found that addition of 9.4 mol/L ZEA to cultured porcine ovarian granulosa cells promoted progesterone secretion without affecting estradiol content. These findings suggest that ZEA promotes progesterone secretion at low concentrations but inhibits it at high concentrations. Furthermore, the differing activities of ZEA and its metabolites may represent another

important factor contributing to variations in progesterone and estradiol levels.

2.1.3 Other Toxic Effects In vivo studies have demonstrated that ZEA causes morphological and functional abnormalities in the reproductive organs of female animals, primarily manifesting as disordered estrous cycles, endometrial and glandular hyperplasia, vascular dilation, and ovarian tissue damage [18-20]. Feeding weaned piglets (21 days old) a diet containing 1 mg/kg ZEA for 22 days resulted in vulvar enlargement and increased genital organ weight [21]. Sexually immature gilts that received long-term (48 days) oral administration of low-dose ZEA (20 and 40 g/kg) exhibited uterine wall tissue hyperplasia, congestion, redness, and swelling [22]. These symptoms in pre-pubertal gilts are closely related to the estrogenic effects of ZEA.

Pregnant sows fed diets contaminated with ZEA (10 and 50 mg/kg) showed a linear relationship between ZEA dose and anestrus duration; as ZEA dosage increased, the weaning-to-estrus interval prolonged, and germ cell reduction increased [23]. Furthermore, feeding replacement gilts diets containing ZEA (3 mg/kg) caused infertility in the first parity [24]. These observations indicate that sensitivity varies among sows at different stages, with replacement gilts being most sensitive to ZEA; however, the mechanisms underlying these differences in porcine sensitivity remain unclear and require further investigation.

Koraichi et al. [25] administered daily subcutaneous injections of 1 mg/kg body weight ZEA to pregnant rats on days 7–20 of gestation, finding that ZEA affected the content of ATP-binding cassette (ABC) transporter substrates and estrogen receptor 1 (ESR1) mRNA expression levels in maternal tissues and fetal liver, thereby interfering with fetal development. Zhao et al. [26] fed female mice diets containing 20 mg/kg ZEA and observed that F0, F1, and F2 generations exhibited early estrus; reduced embryo implantation rates, pregnancy rates, and litter sizes; increased inter-pregnancy intervals; prolonged gestation in F1 and F2 generations; and decreased fertility in the F2 generation. These results demonstrate that ZEA can affect fetal growth and development by crossing the placental barrier, with toxic intensity depending on the maternal stage and intake dose.

The reproductive toxicity of ZEA in female animals primarily manifests as inhibition of ovarian granulosa cell and oocyte proliferation and viability, reduced maturation rates, interference with progesterone and estrogen synthesis, and induction of morphological and functional abnormalities in reproductive organs.

2.2 Effects of DON on Female Reproductive Function

2.2.1 Effects on Female Germ Cells In vitro studies have shown that DON at concentrations of 1.88–3.40 mol/L significantly inhibits the proliferation of porcine and bovine oocytes and ovarian granulosa cells, reduces maturation rates, and decreases the proportion of metaphase II oocytes [11,17,27]. Schoevers et al. [28] added DON at concentrations of 0.02, 0.20, and 2.00 mol/L to

culture media for porcine cumulus-oocyte complexes, finding that 2.00 mol/L DON inhibited cumulus expansion and induced cumulus cell death. After 42 hours, this treatment reduced the formation of metaphase II porcine oocytes and caused meiotic spindle abnormalities, with significantly higher rates of aneuploid blastomeres compared to the control group, thereby inhibiting porcine oocyte development.

These findings indicate that the toxic effects of DON on oocytes and ovarian granulosa cells are primarily indirect, mediated by inhibiting cumulus cell proliferation and inducing cell death, thereby interfering with oocyte meiotic progression and suppressing the proliferation and differentiation of oocytes and ovarian granulosa cells. Cumulus cells play a crucial role in oocyte development, maturation, and ovulation by providing energy for oocyte meiosis [29].

2.2.2 Effects on Sex Hormone Secretion Cortinovis et al. [17] and Ranzenigo et al. [30] found that 1 g/mL DON inhibited progesterone and estradiol secretion in porcine ovarian granulosa cells induced by follicle-stimulating hormone (FSH). However, Medvedova et al. [31] observed that 1 g/mL DON stimulated progesterone secretion in cultured porcine ovarian granulosa cells. This discrepancy may be attributable to differences in culture conditions, as Medvedova et al. [31] used serum-containing culture medium (in which progesterone synthesis depends on exogenous cholesterol [32]), whereas Cortinovis et al. [17] and Ranzenigo et al. [30] used serum-free medium.

2.2.3 Other Toxic Effects DON may act as a potential endocrine disruptor, causing reduced feed intake, decreased production performance, impaired reproductive capacity, and can cross the placental barrier to affect embryos, causing malformed, weak, and dead fetuses. Díaz-Llano et al. [33] fed pregnant sows (91 ± 3 days) diets containing 5.5 mg/kg DON until 21 days postpartum, observing significant reductions in body weight of pregnant sows, significantly increased piglet mortality, and significantly decreased feed intake, body weight, and prolonged estrus intervals in lactating sows, though milk nutrient composition was unaffected.

Studies on fetal organ formation in pregnant female mice found that intraperitoneal injection of DON (1.6, 2.5, 3.3, 4.2, 5, and 10 mg/kg body weight) altered uterine tissue morphology, caused fetal developmental abnormalities, and increased fetal mortality and resorption rates [34]. Other researchers found that SD pregnant rats administered DON by gavage (2.5 and 5.0 mg/kg body weight) exhibited reduced fetal weight, shortened body length, increased rates of weak fetuses, skeletal dysplasia, malformations, and significantly increased early and late mortality. Additionally, as toxin dosage increased, saliva secretion in pregnant rats gradually increased, likely due to stimulation of the emetic reflex [35].

In summary, the reproductive toxicity of DON in female animals is similar to that of ZEA, primarily manifesting as inhibition of ovarian granulosa cell and

oocyte proliferation and viability, reduced maturation rates, interference with progesterone and estrogen synthesis, induction of morphological and functional abnormalities in reproductive organs, and embryotoxicity.

3 Mechanisms of Action

3.1 Hormone-Mediated Effects

ZEA and its metabolites exert estrogen-like effects by binding to estrogen receptors (ER) and stimulating ER-mediated signal transduction pathways. The classical estrogen receptors include two subtypes, ER and ER , which are distributed in both the cytoplasm and nucleus. Cytoplasmic ER acts as a carrier to transport estrogen into the nucleus, where it binds to estrogen response elements (ERE) to stimulate target gene transcription and exert corresponding biological effects.

ER possesses two transcription activation domains: a ligand-independent transcription activation domain (AF1) and a ligand-dependent transcription activation domain 2 (AF2), the latter of which can be activated by hormone induction; these domains work synergistically to regulate estrogen-responsive genes [36]. The tissue distribution and expression levels of ER and ER are related to animal species, sex, and age; ER is primarily expressed in the uterus, whereas ER is mainly expressed in ovarian cortical stromal cells and granulosa cells [37-38].

Research has shown that germ cell proliferation is regulated by ER , while ER regulates cell differentiation and reproductive organ development [39]. Wang et al. [40] added 0.5–2.0 mg/kg ZEA to diets and observed upregulated ER gene transcription levels and downregulated ER transcription levels in the uterus and vaginal tissues of replacement gilts. These findings indicate that ZEA exerts both activating and antagonistic effects on estrogen receptors, with regulation of estrogenic activity primarily mediated by ER .

Estrogen receptor-mediated cell cycle regulation may represent another mechanism of ZEA reproductive toxicity. Quirk et al. [41] found that estradiol can induce the transition of ovarian granulosa cells from G0/G1 phase to S phase, increasing the relative percentage of S-phase cells. Due to its structural similarity to estrogen, ZEA may directly affect germ cell division and interfere with the cell cycle. Moreover, ovarian granulosa cells are most sensitive to ZEA toxicity and prone to apoptosis during the G1-to-S phase transition [42]. Therefore, ZEA can exert toxic effects by mediating the cell cycle through estrogen receptors.

3.2 Cell Apoptosis

Recent studies have confirmed that cell apoptosis is one of the mechanisms underlying ZEA- and DON-induced reproductive toxicity in female animals. Mitochondria serve as crucial sites for cellular energy synthesis, storage, and

metabolism, and occupy a central position in regulating apoptosis. Mitochondria harbor various pro-apoptotic soluble mitochondrial intermembrane proteins (SIMPs), including cytochrome C, apoptosis-inducing factor (AIF), and endonuclease G (EndoG), which are released from mitochondria following changes in mitochondrial membrane permeability to participate in apoptotic responses [43]. Mitochondria can mediate both caspase-dependent and caspase-independent apoptosis. Cytochrome C is typically involved in caspase-dependent apoptosis, whereas AIF and EndoG can induce apoptosis in a caspase-independent manner [44]. Mitochondrial changes during apoptosis include release of apoptosis-inducing proteins, loss of electron transfer function with reduced energy production, and disappearance of mitochondrial transmembrane potential. Mitochondria play a fundamental role as executors of apoptosis.

Studies have demonstrated that the mitochondrial pathway and cysteinyl aspartate-specific proteinase (caspase)-mediated pathway are critical for ZEA-induced apoptosis [45]. Zhu et al. [14] investigated the mechanism of ZEA-induced apoptosis in porcine ovarian granulosa cells and found significantly reduced mitochondrial membrane potential and upregulated expression of Caspase-3 and Caspase-9. These results indicate that ZEA induces apoptosis in porcine ovarian granulosa cells through a caspase-dependent mitochondrial apoptotic pathway. Additionally, ZEA can indirectly induce p53 gene activation in ovarian tissue, leading to upregulation of GADD45 and cell cycle arrest at the G2/M phase to facilitate DNA damage repair. If DNA damage is severe and repair fails, p53 initiates the apoptotic program to induce ovarian granulosa cell apoptosis. The mechanism involves p53 activation upregulating Bax and downregulating Bcl-2, disrupting mitochondrial membrane integrity and causing cytochrome c release into the cytoplasm to form the apoptosome complex and activate Caspase-9. Activated Caspase-9 cleaves and activates Caspase-3, which then cleaves death substrates, resulting in ovarian granulosa cell apoptosis [19,46].

Guerrero-Netro et al. [47] found that DON increased the phosphorylation levels of mitogen-activated protein kinase (MAPK) 3/1, MAPK14 (P38), and MAPK8 [c-Jun N-terminal kinase (JNK)] in cultured bovine ovarian granulosa cells, induced increased mRNA abundance of the pro-apoptotic protein FASL, and promoted apoptosis. Medvedova et al. [31] found that DON at concentrations of 10, 100, and 1,000 ng/mL did not affect Caspase-3 expression in cultured porcine ovarian granulosa cells. These findings suggest that DON-mediated apoptosis in bovine ovarian granulosa cells is associated with MAPK family kinases and promotes MAPK phosphorylation, whereas DON-induced apoptosis in porcine ovarian granulosa cells may occur through a caspase-independent pathway.

3.3 Oxidative Stress

Studies have shown that oxidative stress is one of the mechanisms by which ZEA and DON cause germ cell damage. Under normal conditions, reactive oxygen species (ROS) and free radicals maintain a balance with the antioxidant system;

when cells are damaged, this balance is disrupted, leading to oxidative stress. ZEA and DON can accelerate lipid peroxidation by accelerating free radical production and damaging the antioxidant system.

Capcarova et al. [48] cultured porcine ovarian granulosa cells to 75% confluence and monolayer formation (5–7 days) before adding low, medium, and high doses of DON and ZEA (10/10, 100/100, and 1,000/1,000 ng/mL) for 24 hours of co-culture. They found that low doses of DON and ZEA enhanced superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities, whereas high-dose exposure reduced SOD and GPx activities and decreased total antioxidant capacity. Addition of ZEA (1.25, 5.00, 20.00, and 80.00 g/mL) to cultured rat ovarian granulosa cells for 48 hours resulted in significantly increased malondialdehyde (MDA) content in a dose-dependent manner [11]. These results demonstrate that ZEA and DON within certain concentration ranges can promote ROS generation and produce lipid peroxides (LPO) in porcine and rat ovarian granulosa cells. LPO can further decompose into various cytotoxic substances such as MDA, reduce antioxidant enzyme activities, cause cellular lipid peroxidation damage, and interfere with ovarian granulosa cell proliferation and apoptosis.

4 Interactive Effects of Fusarium Toxins ZEA and DON on Reproductive Toxicity

Mycotoxin contamination in grains, feed ingredients, and finished feed typically involves mixed contamination, primarily because a single mold species can produce multiple toxins and the conditions for various mycotoxin productions are similar. ZEA and DON are the most frequently detected Fusarium toxins, and their interactive effects represent a current research focus.

Mycotoxin interactive effects refer to the interrelationships exhibited when two or more mycotoxins are present simultaneously and their toxic responses in animals. Interactive effects of multiple mycotoxins can be categorized as additive, sub-additive, synergistic, potentiating, or antagonistic.

Feeding naturally contaminated diets containing ZEA and DON to sows (ZEA 0.088 mg/kg and DON 3.07 mg/kg for 5 weeks) and mice (ZEA 1,897 g/kg and DON 3,875 g/kg for 4 weeks) resulted in abnormal chromatin morphology during oocyte meiosis and inhibited oocyte maturation and activation rates, thereby affecting oocyte development. Fluorescence intensity analysis revealed increased total DNA methylation levels in mouse oocytes, along with increased H3K9me3 and H4K20me3 content [50-51]. These results indicate that ZEA and DON can reduce mouse oocyte developmental capacity through epigenetic modifications.

Zhang et al. [52] added ZEA and DON to mouse diets (2 and 8 mg/kg, respectively), which significantly increased the uterine index, with combined exposure exhibiting a potentiating effect. In contrast, addition of 3.1 mol/L -ZEA (or -ZEA) and 3.3 mol/L DON to cultured bovine ovarian granulosa cells for 2

days reduced granulosa cell numbers, inhibited proliferation, and significantly decreased progesterone content, with combined exposure showing a synergistic effect. Individual exposure experiments found that both -ZEA and -ZEA promoted estrogen secretion, whereas DON inhibited it. However, combined exposure experiments revealed that -ZEA and DON together promoted estrogen secretion, while -ZEA and DON together inhibited it [27,53]. This discrepancy may be due to the higher toxicity of -ZEA compared to -ZEA. These findings indicate that combined ZEA and DON exposure can synergistically increase the mouse uterine index, inhibit ovarian granulosa cell proliferation, and reduce progesterone content.

Both Fusarium toxins ZEA and DON can cause reproductive disorders in female animals, primarily affecting reproductive organ and embryo development, germ cell proliferation and viability, and sex hormone secretion through hormone-mediated effects, cell apoptosis, and oxidative stress. While the effects of ZEA on animal reproductive performance have attracted widespread attention, research on DON' s impact on animal reproductive performance remains limited, with mechanisms not fully elucidated, and the interactive effects of combined toxins require further investigation.

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