Effects of Fusarium Toxins on Vulva, Reproductive Organ Indices, and Uterine Estrogen Receptor Distribution and Expression in Weaned Gilts (Postprint)

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Abstract

This experiment aimed to investigate the effects of Fusarium toxins in naturally moldy feed on the vulva, reproductive organ indices, and distribution and expression of estrogen receptors (ERs) in weaned gilts. Forty healthy three-way crossbred (Duroc × Landrace × Large White) weaned female piglets at 35 days of age with an average body weight of (8.45$±$0.94) kg were selected and randomly divided into 2 treatments, with 20 piglets per treatment. The control group was fed a basal diet, while the experimental group was fed a diet containing Fusarium toxins [zearalenone (ZEN) 0.90 mg/kg; deoxynivalenol (DON) 1.43 mg/kg; fumonisin (FUM) 5.85 mg/kg]. The pretrial period was 7 days, and the formal trial period was 35 days. The results showed that the vulva length, width, and area of piglets at 35 days and the reproductive organ indices of weaned gilts in the experimental group were significantly higher than those in the control group ($P<0.05$). Estrogen receptor α (ERα) immunopositive material was mainly observed in the cytoplasm of endometrial glandular epithelial cells and myometrial cells; estrogen receptor β (ERβ) immunopositive material was mainly observed in the cytoplasm of arterial wall smooth muscle and myometrial smooth muscle cells in the endometrium. Compared with the control group, the ERα immunopositive reaction in endometrial glandular epithelial cells of piglets in the experimental group was significantly enhanced, and the number of glandular acini was significantly increased; whereas the number of ERβ-positive cells in myometrial smooth muscle and arterial wall smooth muscle cells of piglets in the experimental group was significantly higher than that in the control group. The relative mRNA expression levels of ERα and ERβ in the uterus of weaned gilts in the experimental group were significantly higher than those in the control group ($P<0.05$). These results indicate that Fusarium

toxins can cause adverse effects on the reproductive system of weaned gilts, and such effects are achieved by regulating the relative expression levels of ERs in the uterus through transcription of ER genes, thereby altering the development of reproductive organs.

Full Text

Effects of Fusarium Toxins on Vulva Size, Reproductive Organ Index, Distribution and Expression of Estrogen Receptors in Uterus of Weaning Gilts

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Abstract

This study investigated the effects of Fusarium toxins in naturally moldy diets on vulva size, reproductive organ index, and the distribution and expression of estrogen receptors (ERs) in weaning gilts. Forty healthy weaning gilts (Duroc × Large White × Landrace) aged 35 days with an average body weight of (8.45$±$0.94) kg were randomly allocated into two treatments (n=20 per group). The control group received a basal diet, while the experimental group was fed a diet contaminated with Fusarium toxins [zearalenone (ZEN) 0.90 mg/kg; deoxynivalenol (DON) 1.43 mg/kg; fumonisin (FUM) 5.85 mg/kg]. After a 7-day adaptation period, the formal experimental period lasted 35 days. Results showed that the length, width, and area of vulva, as well as the reproductive organ index of gilts at 35 days, were significantly higher in the experimental group compared to the control group ($P<0.05$).

Immunopositive staining for estrogen receptor α (ERα) was predominantly observed in the cytoplasm of endometrial glandular epithelial cells and uterine myometrial cells. Estrogen receptor β (ERβ) immunopositive material was mainly localized in the cytoplasm of smooth muscle cells in the arterial intima and myometrial smooth muscle cells. Compared with the control group, the experimental group exhibited markedly enhanced ERα immunopositive reactions in endometrial glandular epithelial cells with significantly increased acinar numbers. Meanwhile, the number of ERβ-positive cells in uterine myometrial smooth muscle and arterial wall smooth muscle cells was significantly greater in the experimental group. The relative mRNA expression levels of both ERα and ERβ in the uterus of weaning gilts were significantly higher in the experimental group ($P<0.05$). These findings demonstrate that Fusarium toxins exert adverse effects on the reproductive system of weaning gilts by regulating the

relative expression of ERs in the uterus through transcriptional control of ER genes, thereby altering reproductive organ development.

Keywords: Fusarium toxins; weaning gilts; vulva; uterus; estrogen receptors

Introduction

Fusarium toxins are secondary metabolites produced by toxigenic molds during growth and reproduction on substrates (grains, crops, and feed), representing the most economically damaging class of contaminating mycotoxins to global cereal production [1-2]. The Fusarium toxins most hazardous to animal health and production include zearalenone (ZEN), deoxynivalenol (DON), and fumonisin (FUM). Among these, ZEN poses the greatest threat to animal reproduction, inducing hyperestrogenism in sows characterized by vulvar swelling, rectal prolapse, and hyperplasia of the uterus and mammary glands [3]. Reports have confirmed that ZEN can alter ER mRNA expression levels in the uterus [4-5].

Most studies investigating the effects of Fusarium toxins on animal reproductive systems have used purified toxins, whereas in actual production settings, Fusarium molds typically produce diverse toxin mixtures [6]. Moreover, the effects of Fusarium toxins on the distribution of ERs within the uterine horns of weaning gilts have not been reported. Therefore, this study aimed to investigate the impacts of Fusarium toxins from naturally moldy corn and corn gluten meal on reproductive organ development, ER distribution, and expression in weaning gilts to provide a theoretical basis for healthy swine production.

Materials and Methods

1.1 Experimental Animals and Management

Forty crossbred (Duroc × Large White × Landrace) weaning female piglets aged 35 days with an average body weight of $(8.45\$\pm0.94)kgwererandomlydividedintotwotreatments(n = 20pergroup).Initialbodyweightdidnotdifersignificantlybetweentreatments(P > 0.05).TheexperimentwasconductedattheAnimalScienceandTechnologyParkofShandongAgriculturalUnive$ equipped with plastic slatted floors, nipple drinkers, and feed troughs, allowing ad libitum access to feed and water. The pig house was thoroughly cleaned and disinfected before the trial, with weekly disinfection during the experiment. Infrared heat lamps maintained ambient temperature at approximately 30°C during the first week, subsequently adjusted to 26–28°C, with relative humidity at approximately 65%.

1.2 Experimental Design and Diets

The basal diet for weaning gilts was formulated according to NRC (2012) [7] nutrient requirements, with composition and nutrient levels shown in Table 1 . A single-factor experimental design was employed: the control group received the basal diet, while the experimental group was fed a diet in which 50% naturally moldy corn and 50% moldy corn gluten meal replaced the corn and corn gluten

meal in the basal diet. The adaptation period lasted 7 days, followed by a 35-day formal experimental period.

1.3 Sample Collection and Analysis

1.3.1 Vulva Area Measurement During the experimental period, vulva length and width were measured every 3 days using vernier calipers to calculate vulva area. The vulva of piglets approximates a rhombus shape in top view; therefore, this study used the rhombus area formula (length × width)/2 to approximate vulva area (Figure 1 [Figure 1: see original paper]) for comparing vulvar enlargement effects among treatments.

1.3.2 Reproductive Organ Index At the end of the experiment, 10 piglets per treatment were randomly selected for slaughter. After electrical stunning and exsanguination, the thoracic and abdominal cavities were opened. Reproductive organs (ovaries + uterine horns + vaginal vestibule) were visually examined, lesions recorded, and weighed to calculate the reproductive organ index. Two uterine horn samples (approximately 1.5 cm × 1.5 cm) were rapidly collected: one fixed in Bouin' s solution for immunohistochemical analysis, and one snap-frozen in liquid nitrogen and stored at -80°C for determination of relative ER mRNA expression.

Reproductive organ index (g/kg) = reproductive organ weight (g) / live body weight (kg).

1.3.3 Immunohistochemistry (Strept Avidin-Biotin Complex, SABC Method) Tissue blocks fixed in Bouin' s solution were dehydrated through graded ethanol series, cleared with xylene, and embedded using a BMJ23 embedding machine. (1) Sections (5 m) were cut using a microtome (LEICA RM2135, Germany) and routinely deparaffinized and rehydrated. (2) Antigen retrieval was performed using citrate buffer (0.01 mol/L, pH 6.0), followed by three washes with phosphate-buffered saline (PBS) (0.01 mol/L, pH 7.2) for 5 min each. (3) Endogenous peroxidase was blocked by incubating with 3% H_2O_2 at room temperature for 30 min in the dark, followed by three PBS washes. (4) Sections were blocked with 10% fetal bovine serum at 37°C for 1 h. (5) Primary antibodies were applied: rabbit anti-estrogen receptor α (ERα) polyclonal antibody (1:150) (140113W, Beijing Biosynthesis Biotechnology Co., Ltd.) and rabbit anti-estrogen receptor β (ERβ) polyclonal antibody (1:150) (999882W, Beijing Biosynthesis Biotechnology Co., Ltd.), incubated overnight at 4°C, followed by three PBS washes. (6) Biotinylated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:200) (K132429E, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) was applied and incubated at 37°C for 1 h, followed by three PBS washes. (7) Horseradish peroxidase-streptavidin (1:150) was added and incubated at 37°C for 45 min, followed by three PBS washes. (8) Diaminobenzidine (DAB) was used for color development, with microscopic monitoring to control reaction time. (9) Sections were counterstained

with hematoxylin, dehydrated, cleared, mounted, and observed under bright-field microscopy to determine immunopositive cell distribution patterns.

1.3.4 Determination of Relative mRNA Expression of ERs Specific primers for porcine ERα, ERβ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were designed using Primer 6.0 based on sequences reported in GenBank and synthesized by Shanghai Bioengineering Company (Table 2).

Uterine horn samples (50–100 mg) stored at -80°C were processed for total RNA extraction using the Trizol reagent kit (Invitrogen, USA) according to the manufacturer' s instructions. RNA quality and concentration were assessed using a UV spectrophotometer, with optical density (OD) values ranging from 1.8 to 2.0. Extracted RNA was immediately reverse-transcribed using the PrimeScript® RT Master Mix Perfect Real Time kit (TaKaRa Code: DDR036A, Lot: BK1302) in a 20 L reaction volume. The RT-PCR reaction system (20 L) was prepared according to the TaKaRa fluorescence quantitative kit instructions. Amplification conditions were: pre-denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 34 s.

1.4 Statistical Analysis

Fluorescence quantitative PCR data were processed using the 2^(-ΔΔCt) method to analyze relative mRNA expression of ERα and ERβ genes in the uterus. Data were analyzed using SAS 9.2 software with two-sample paired t-test for means. Differences were considered significant at $P<0.05$.

Results

2.1 Effects of Fusarium Toxins on Vulva Size and Reproductive Organ Index of Weaning Gilts

The effects of Fusarium toxins in naturally moldy diets on vulva size of weaning gilts are shown in Table 3 . At the start of the experiment (0 d), no significant differences were observed in vulva length, width, or area among treatments ($P>0.05$). At the end of the experiment (35 d), vulva length, width, and area were all significantly greater in the experimental group compared to the control group ($P<0.05$). The reproductive organ index of weaning gilts was also significantly elevated in the experimental group ($P<0.05$).

2.2 Effects of Fusarium Toxins on Distribution of ERα and ERβ in Uterus of Weaning Gilts

Immunohistochemical results revealed that immunopositive reactions for ERα and ERβ in gilt uteri appeared yellow, yellow-brown, or brown, while negative control tissues showed no staining, confirming the specificity of the SABC immunohistochemical method used in this study.

2.2.1 Effects of Fusarium Toxins on Distribution of ERα in Uterus of Weaning Gilts Observations revealed that ERα was primarily localized in endometrial glandular epithelial cells and uterine myometrial cells, with cytoplasmic staining, while no ERα distribution was detected in uterine luminal epithelium or vascular endothelial cells (Figure 2 [Figure 2: see original paper]-A, Figure 2-D). Compared with the control group (Figure 2-B, Figure 2-C), the experimental group showed markedly enhanced ERα immunopositive reactions in uterine glands with significantly increased acinar numbers (Figure 2-E, Figure 2-F). Most glandular epithelial cells in the experimental group displayed strong positive reactions (Figure 2-E, Figure 2-F), whereas control group glandular epithelial cells showed weak ERα immunoreactivity with some unstained cells present. However, no obvious difference was observed in the number of ERα-immunopositive cells in the endometrial stroma between the two groups.

2.2.2 Effects of Fusarium Toxins on Distribution of ERβ in Uterus of Weaning Gilts ERβ immunopositive material was predominantly localized in the cytoplasm of smooth muscle cells in the intimal arterial wall and myometrial smooth muscle cells (Figure 3 [Figure 3: see original paper]-A, Figure 3-D), showing strong positive reactions. In contrast, uterine glandular epithelial cells and stromal cells were mostly negative for ERβ (Figure 3-B, Figure 3-C, Figure 3-E, Figure 3-F), although occasional weak nuclear staining was observed in some glandular epithelial and stromal cells. Compared with the control group, the experimental group exhibited stronger ERβ staining with more numerous positive cells, primarily distributed in myometrial smooth muscle and arterial wall smooth muscle cells (Figure 3-E, Figure 3-F). A small number of stromal cells showed ERβ positivity in the control group (Figure 3-C). No obvious difference was observed in ERβ distribution in arterial wall smooth muscle between the two groups.

2.3 Effects of Fusarium Toxins on Relative mRNA Expression of ERs in Uterus of Weaning Gilts

The effects of Fusarium toxins on relative mRNA expression of ERs in weaning gilt uteri are shown in Figure 4 [Figure 4: see original paper]. Compared with the control group, the experimental group exhibited significantly elevated relative mRNA expression levels of both ERα and ERβ in the uterus ($P<0.05$).

Although high-quality ingredients were selected for the control diet, regrettably, various toxin levels were still detected, further demonstrating the ubiquity of Fusarium toxin contamination in China and the urgency of this research. The toxin contents in the control diet were below Chinese feed hygiene standards (ZEN <0.5 mg/kg, GB13078.2-2006; DON <1 mg/kg, GB13078.3-2007; no standard established for FUM) and EU maximum limits for piglet diets (ZEN, DON, and FUM <0.1, 0.9, and 5 mg/kg, respectively) [8]. In contrast, the experimental diet exceeded both Chinese and EU standards for ZEN, DON, and FUM, ensuring that toxin levels in the control group did not confound interpretation

of experimental results. Under the conditions of this study, Fusarium toxins significantly reduced average daily feed intake and average daily gain while significantly increasing feed-to-gain ratio in weaning gilts [9].

Discussion

3.1 Effects of Fusarium Toxins on Reproductive Organs of Weaning Gilts

Among Fusarium toxins, ZEN poses the greatest hazard to the reproductive system. ZEN exhibits estrogenic effects by binding to ERs in animals [10], inducing hyperestrogenism in sows with typical symptoms including vulvar redness and swelling, vaginal and rectal prolapse, uterine hyperplasia, and mammary gland enlargement [3]. Rainey et al. [11] observed vulvar swelling in sows within 7 days of consuming diets containing 1.5 mg/kg ZEN. James and Smith [10] reported that piglets consuming ZEN-contaminated diets (3.61 and 4.33 mg/kg) showed nearly doubled uterine weights. Chen et al. [12] found that low-dose ZEN (1.1–3.2 mg/kg) significantly increased reproductive organ index and vulva area in weaning gilts in a dose-dependent manner. In the present study, experimental group piglets exhibited obvious vulvar redness and swelling, with significantly greater vulva length, width, and area compared to controls, indicating that Fusarium toxins (0.90 mg/kg ZEN) in naturally moldy diets exerted toxic effects on reproductive organs, consistent with previous findings. However, the experimental diet also contained DON (1.43 mg/kg) and FUM (5.85 mg/kg). In vitro studies have confirmed that ZEN metabolites α-zearalenol (7.5 μmol/L) and β-zearalenol (30.0 μmol/L), as well as DON (1.88 μmol/L), significantly inhibit oocyte nuclear maturation, though the mechanisms remain unclear [13]. Both ZEN and DON (3.12 μmol/L each) can induce spindle structure abnormalities, leading to reduced oocyte numbers and embryonic abnormalities, but no synergistic effects were observed [14]. No reports have documented DON effects on vulva size or reproductive organ index, nor FUM hazards to animal reproductive systems. Moreover, Fusarium toxin metabolism in animals is complex, and in vitro studies are insufficient for definitive conclusions. The toxic effects of Fusarium toxins on gilt reproductive organs under our experimental conditions are unequivocal. Further studies by our research group are ongoing to elucidate the interactions among ZEN, DON, and FUM regarding their effects on weaning gilt reproductive organs.

3.2 Effects of Fusarium Toxins on Distribution of ERα and ERβ in Uterus of Weaning Gilts

In mammals, ERs consist primarily of ERα and ERβ subtypes, both nuclear receptors, though recent studies have revealed their widespread distribution in cell membranes and cytoplasm [15]. ERα and ERβ are distributed in various tissues but with distinct patterns: ERβ is concentrated in reproductive, cardiovascular, and central nervous systems, brain, and bone, whereas ERα is mainly distributed in reproductive tissues and mammary glands [16]. Both subtypes

exhibit similar binding affinity for estrogen, but ERα demonstrates greater transcriptional activity than ERβ, suggesting that estrogenic effects are primarily mediated through ERα [17].

Xu [18] reported that ERα is mainly expressed in Beagle dog endometrial gland cells, while ERβ is expressed in both nucleus and cytoplasm of endometrial gland cells, as well as in vascular endothelial and smooth muscle cell cytoplasm. ZEN can bind to ERs in animals, stimulating ER-mediated signal transduction. Different ER subtypes show varying affinity for ZEN, with Mueller et al. [19] reporting that ERα exhibits greater affinity than ERβ. Studies suggest ZEN may increase ER numbers in ovariectomized rat uteri by influencing estradiol-ER binding [20]. No reports have addressed DON or FUM effects on ER distribution and expression in weaning gilt uteri. Our immunohistochemical results demonstrated that all uterine cell components expressed ERα and ERβ. ERα was predominantly distributed in uterine glandular epithelial cells, while ERβ was mainly localized in arterial wall smooth muscle cells, myometrial smooth muscle cells, glandular epithelial cells, and stromal cells. Compared with controls, the experimental group showed widespread ERα distribution in uterine glands with glandular hyperplasia, consistent with significantly increased ERα expression. ERβ in the experimental group was extensively distributed not only in arterial wall and myometrial smooth muscle cells but also in glandular epithelial cells. Our findings revealed widespread ERβ distribution in vascular endothelium, myometrial smooth muscle, and stromal cells, potentially causing uterine smooth muscle cell hyperplasia and uterine wall thickening, consistent with increased reproductive organ index. However, whether ERβ can enter the bloodstream through vascular endothelium and whether DON and FUM affect ER distribution and expression in gilt uteri require further investigation.

3.3 Effects of Fusarium Toxins on Relative mRNA Expression of ERα and ERβ in Uterus of Weaning Gilts

In normal rat uteri, both ERα and ERβ mRNA are expressed, but ERα expression predominates [21]. Studies have shown that ERα mRNA is mainly distributed in the uterus, whereas ERβ mRNA is primarily expressed in the ovary [22-23]. Kuiper et al. [24] demonstrated that ZEN and its derivatives require longer transit time from cytoplasm to nucleus after binding to ERs compared to estrogen, thereby increasing RNA and RNA polymerase activities. Zhang et al. [4] observed dose-dependent relationships between ZEN content and ER expression in uterus and ovary of pregnant mice. Wang et al. [5] reported that dietary ZEN supplementation at 0.5 and 2.0 mg/kg significantly increased ERα mRNA expression in gilt uteri by 15% and 38%, respectively, while significantly decreasing ERβ mRNA expression. Piglets consuming 1.5 mg/kg ZEN-contaminated diets for 28 days showed no significant change in uterine ERα mRNA expression, but ERβ mRNA expression doubled [25]. No reports have addressed whether DON and FUM affect ERα and ERβ mRNA expression in weaning gilt uteri. Our study demonstrated that feeding naturally

moldy diets for 35 days significantly upregulated relative mRNA expression of both ERα and ERβ in weaning gilt uteri, consistent with ER distribution patterns. However, interactions among multiple toxins (ZEN, DON, and FUM) require further verification.

Conclusion

This study demonstrates that Fusarium toxins, particularly ZEN, affect vulvar swelling and reproductive organ index in weaning gilts by altering ER distribution and expression levels in the uterus. Under our experimental conditions, Fusarium toxins (ZEN 0.90 mg/kg, DON 1.43 mg/kg, FUM 5.85 mg/kg) significantly increased vulva size and reproductive organ index while enhancing ERα and ERβ distribution and expression in the uterus. Therefore, Fusarium toxins, especially ZEN, regulate reproductive organ development in weaning gilts by enhancing ERα and ERβ distribution and expression in the uterus. However, the interactions among ZEN, DON, and FUM regarding their effects on weaning gilt reproductive organs require further experimental verification.

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