# Effects of Zearalenone on Reproductive Performance and Placental Immune-Related Gene Expression in Sows (Postprint)

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## Abstract

This study aimed to investigate the effects of dietary zearalenone (ZEN) supplementation on reproductive performance and placental immune-related gene expression in sows. Forty Landrace × Large White crossbred sows with similar parity, body weight of 200 kg, and at day 30 of gestation were selected and randomly allocated into 2 groups, with 20 replicates per group and 1 sow per replicate. The control group was fed a basal diet, while the treatment group received the basal diet supplemented with 1.5 mg/kg ZEN. The experimental period was 74 days. The results showed that, compared with the control group: 1) dietary ZEN supplementation significantly increased the number of stillborn piglets and weak piglets in gestating sows ($P < 0.05$), and significantly decreased the total number of piglets born ($P < 0.05$); 2) dietary ZEN supplementation significantly increased serum progesterone content in gestating sows ($P < 0.05$); 3) dietary ZEN supplementation significantly increased the expression levels of Toll-like receptor-2 (TLR-2) and progesterone receptor (PGR) genes in the placenta of gestating sows ($P < 0.05$). In conclusion, dietary supplementation with 1.5 mg/kg ZEN during gestation significantly reduced the total number of piglets born and significantly increased the number of stillborn and weak piglets. Low levels of ZEN in the diet still exert adverse effects on sow reproductive performance.

## Full Text

### Effects of Zearalenone on Reproductive Performance and Placenta Immunity-Related Gene Expression in Sows

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**Abstract:** This experiment was conducted to investigate the effects of dietary zearalenone (ZEN) on reproductive performance and placenta immunity-related gene expression in sows. Forty Landrace × Large White crossbred sows on day 30 of gestation, with similar parity and body weight of 200 kg, were randomly allocated into two groups with 20 replicates per group and one sow per replicate. The control group was fed a basal diet, while the experimental group received the basal diet supplemented with 1.5 mg/kg ZEN. The trial lasted for 74 days. The results showed that, compared with the control group: (1) dietary ZEN supplementation significantly increased the number of stillborn and weak piglets ($P < 0.05$) and significantly decreased the total number of piglets born ($P < 0.05$); (2) dietary ZEN supplementation significantly increased serum progesterone content in pregnant sows ($P < 0.05$); (3) dietary ZEN supplementation significantly increased the expression of Toll-like receptor-2 (TLR-2) and progesterone receptor (PGR) genes in the placenta of pregnant sows ($P < 0.05$). In conclusion, supplementing 1.5 mg/kg ZEN in the diet of pregnant sows significantly reduced the total number of piglets born while significantly increasing the number of stillborn and weak piglets. Even low levels of dietary ZEN exert adverse effects on sow reproductive performance.

**Keywords:** zearalenone; sows; reproductive performance; reproductive hormones; immunoglobulin; gene expression

## Introduction

Zearalenone (ZEN) is one of the major mycotoxins causing global food safety and livestock feed safety concerns. Long-term consumption of ZEN-contaminated feed can induce hyperestrogenism syndrome, leading to pathological changes and functional disorders of reproductive organs. ZEN contamination is particularly widespread in grains such as corn and wheat, posing substantial hazards to pig production, especially regarding sow reproductive performance. While numerous studies have reported on ZEN's effects on sow reproductive performance, research on its impact on placenta immunity-related gene expression remains unexplored. Vlata et al. found that high levels of ZEN (30 μg/mL) inhibited the proliferation of human T cells and B lymphocytes. In recent years, the detection rate and concentration of ZEN in corn and compound feeds have been notably high. Therefore, this study aimed to investigate the effects of low dietary levels of ZEN on sow reproductive performance and placenta immunity-related gene

expression, providing theoretical reference for feed safety issues caused by ZEN contamination.

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## Materials and Methods

### 1.1 Experimental Design

This study employed a single-factor experimental design. Forty Landrace × Large White crossbred sows on day 30 of gestation, with similar parity and body weight of 200 kg, were randomly divided into two groups (control and ZEN groups) with 20 replicates per group and one sow per replicate. The experimental period lasted 74 days.

### 1.2 Dietary Treatments and Management

The basal diet was formulated according to NRC (2012) nutrient requirements for swine, with composition and nutrient levels shown in . The control group received the basal diet, while the ZEN group received the basal diet supplemented with 1.5 mg/kg ZEN (purchased from Sigma Company). The trial was conducted at Guangzhou Conghua Breeding Pig Farm. Sows were fed twice daily (07:00 and 17:00), and disinfection and vaccination procedures followed the farm' s standard protocols.

### 1.3 Measurements

**1.3.1 Reproductive Performance of Pregnant Sows** Within 12 hours of farrowing, the total number of piglets born, number of live-born piglets, stillbirths, and mummified fetuses were recorded for each sow in both groups. The total number of piglets born, number of stillborn piglets, and number of weak piglets were calculated, and piglet birth weight was measured after recording litter weight data.

**1.3.2 Serum Reproductive Hormones and Immune Indices** On day 104 of gestation, 10 mL of blood was collected via jugular vein puncture before morning feeding. After standing at room temperature for 30 minutes to allow serum separation, samples were centrifuged at 3,000 r/min for 10 minutes and stored at -80°C. Serum reproductive hormone indices included follicle-stimulating hormone (FSHB), progesterone (PG), and prolactin (PRL) contents, all measured using ultraviolet spectrophotometry or enzyme-linked immunosorbent assay according to the 2015 Chinese Pharmacopoeia. Kits were purchased from Beijing Aoke Dingsheng Biotechnology Co., Ltd. Serum immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) contents were determined by ultraviolet spectrophotometry, with all kits, low molecular weight protein markers, and microplate readers purchased from Beijing Aoke Dingsheng Biotechnology Co., Ltd.

**1.3.3 Placental Immunity-Related Gene Expression** Placental tissue extraction followed the product instructions. Appropriate amounts of fetal placental tissue from pregnant sows were placed in a mortar, ground to powder after adding liquid nitrogen, then transferred to a homogenizer. Approximately 2 mL of Trizol reagent was added and left at room temperature for 5 minutes. After centrifugation at 4°C and 12,000 r/min for 10 minutes, the supernatant was transferred to another centrifuge tube using a sterile pipette. The supernatant consisted of three parts: a bottom phenol-chloroform phase, an intermediate layer, and an upper aqueous layer, with RNA present exclusively in the aqueous layer. The supernatant was collected, repeated once with chloroform, and centrifuged for 15 minutes. For each 1 mL of supernatant, 75% ethanol was added (approximately 4 mL of 75% ethanol per 4 mL of Trizol reagent), and the tube was gently shaken. After suspending the precipitate, total RNA from sow fetal placental tissue was obtained. Specific immune and reproduction-related genes and primer sequences for sow fetal placental tissue are shown in .

The PCR reaction system was 20 µL: 0.8 µL of each primer and 8 µL of distilled water solution containing 200 ng DNA, with double-distilled water ($ddH_2O$) added to reach 20 µL. Reverse transcription PCR steps: extracted mRNA was first reverse-transcribed into cDNA, which was then amplified by PCR. To prevent RNA degradation and maintain RNA integrity, samples were pre-denatured at 42°C for 5 minutes, with appropriate $ddH_2O$ added to reach a total volume of 20 µL. After gentle mixing, the PCR program was set as follows: denaturation at 95°C for 10 seconds, 95°C for 5 seconds, and annealing at 60°C for 46 seconds. Amplification was performed for 40-60 cycles under appropriate temperature parameters: 95°C denaturation for 15 seconds, 60°C annealing for 60 seconds, and 95°C reaction for 15 seconds. PCR amplification kits, DNA markers, low molecular weight protein markers, diethyl pyrocarbonate (DEPC), RNA enzyme inhibitors, competent cell preparation kits, plasmid extraction kits, peptone, and DNA gel recovery kits were all purchased from Beijing Aoke Dingsheng Biotechnology Co., Ltd., with operations performed according to instructions.

### 1.4 Data Processing and Statistical Analysis

Data were analyzed using one-way ANOVA with SPSS 19.0 software, followed by Duncan' s multiple comparison test. All data are expressed as mean ± standard error. $P < 0.05$ was considered statistically significant.

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## Results

### 2.1 Effects of ZEN on Reproductive Performance of Pregnant Sows

As shown in , compared with the control group, dietary ZEN supplementation significantly increased the number of stillborn and weak piglets ($P < 0.05$) and

significantly decreased the total number of piglets born ($P < 0.05$). Although piglet birth weight showed a decreasing trend, the difference was not significant ($P > 0.05$).

### 2.2 Effects of ZEN on Serum Reproductive Hormone Content

As shown in , dietary ZEN supplementation significantly increased serum PG content in pregnant sows ($P < 0.05$), but no significant differences were observed in serum FSHB and PRL contents between the two groups ($P > 0.05$).

### 2.3 Effects of ZEN on Serum Immunoglobulin Content

As shown in , no significant differences were found in serum IgA, IgG, and IgM contents between the control and ZEN groups ($P > 0.05$).

### 2.4 Effects of ZEN on Placental Immunity-Related Gene Expression

As shown in , dietary ZEN supplementation significantly increased the expression of Toll-like receptor-2 (TLR-2) and progesterone receptor (PGR) genes in the placenta of pregnant sows ($P < 0.05$), while no significant differences were observed in other gene expressions between the two groups ($P > 0.05$).

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## Discussion

ZEN is a 2,4-dihydroxybenzoic acid lactone, and pigs are the most sensitive species to its effects. Glavits et al. reported that ZEN can cause vulvar swelling in sows, leading to poor reproductive performance, vulvar edema, abortion, mammary gland enlargement, and sometimes neonatal piglet death. Minervini et al. found that feeding diets containing 9 mg/kg ZEN to sows and piglets from day 32 of gestation induced false estrus in gilts, primarily due to ZEN interactions causing chromosomal abnormalities in oocytes. Jadamus et al. observed that feeding ZEN-contaminated diets (180 mg/kg) to sows increased the return-to-estrus rate in cycling sows and the abortion rate in normally pregnant sows, indicating reproductive system damage by ZEN, with some piglets from primiparous sows showing estrogen hyperactivity symptoms. In the present study, feeding pregnant sows a diet containing 1.5 mg/kg ZEN not only reduced the total number of piglets born and piglet birth weight but also significantly increased the rates of stillbirth and weak piglets.

ZEN exhibits estrogen-like effects and can competitively bind to estrogen receptors in animals, activating estrogen response signaling pathways and causing estrogen receptor dimerization, which triggers a series of estrogen-like responses and leads to hyperestrogenism syndrome in sows. Previous studies have shown that dietary ZEN supplementation causes ovarian atrophy, false estrus, prolonged estrus intervals, abortion, and the production of malformed and stillborn piglets. Experiments involving ZEN injection in neonatal female

rats and ZEN feeding in piglets have confirmed the reproductive toxicity and teratogenic effects of ZEN. These findings demonstrate that ZEN and its biotransformation products can cause hyperestrogenism syndrome in sows. The current study showed that serum PG content was significantly higher in sows fed ZEN-supplemented diets compared to the control group, indicating that feeding low-level ZEN-contaminated diets can cause precocious puberty, prolonged estrus cycles, abortion, return to estrus, and other reproductive abnormalities, as well as reduced fetal growth, infertility, and fetal malformations. Additionally, ingested ZEN is primarily metabolized in the liver and kidneys, where it can cause degenerative changes in these tissues.

Research has shown that ZEN not only induces apoptosis in normal cells but also stimulates proliferation in specific tumor cells, such as breast cancer cells, acting as a mitogenic factor. The effects of ZEN on cell growth are also observed in the immune system, as Vlata et al. found that high levels of ZEN (30 μg/mL) inhibited the proliferation of human T cells and B lymphocytes. However, in the present study, dietary ZEN supplementation did not significantly affect serum immunoglobulin content in pregnant sows.

The placenta of pregnant sows is a vital organ for embryonic growth and development, facilitating nutrient supply and waste excretion, and serving as an immune barrier against toxic substances and heavy metals from the uterine environment. The results indicate that dietary ZEN supplementation affected the expression of placental innate immune-related genes in the Toll-like receptor signaling pathway, thereby influencing the innate immune response in the placenta of pregnant sows. Placental innate immune genes, particularly Toll-like receptors, are important recognition receptors in innate immunity that play crucial roles in pathogen pattern recognition during innate immune responses, identifying microbial pathogens or viruses that affect placental development. Toll-like receptors are not only essential protein molecules involved in natural immunity but also bridge innate and adaptive immunity. In this study, the low expression levels of TLR-2 and Toll-like receptor-4 (TLR-4) genes in the ZEN group demonstrated ZEN' s ability to inhibit the innate immune response in sow placenta. Consequently, lower placental immunity is detrimental to normal fetal growth and development, significantly increasing the number of stillborn and weak piglets.

The high expression of the FSHB gene in the ZEN group indicates poor ovarian reserve function, which fails to protect hormone receptors within the ovary. The primary function of PG is to promote endometrial thickening and gland enlargement in preparation for embryo attachment. PG content levels can reflect ovarian follicle and corpus luteum secretion. The high expression of the PGR gene in the ZEN group suggests that ZEN and its metabolites disrupt the endocrine system, affecting ovulation and embryo implantation. PRL is a lactogenic hormone secreted by the adenohypophysis that primarily promotes milk secretion and is essential for mammary gland development. During mid-gestation, PRL works synergistically with estrogen, progesterone, and glucocorticoids, playing

important roles in ovarian steroid synthesis, corpus luteum formation, and luteolysis. The high expression of the prolactin receptor (PRL-R) gene in the ZEN group indicates that ZEN and its metabolites can cause anestrus, false estrus, and pseudopregnancy symptoms in sows. Therefore, even low levels of ZEN can cause embryonic developmental arrest, stillbirth, abortion, and abnormal reproductive system development.

In conclusion, supplementing 1.5 mg/kg ZEN in the diet of pregnant sows significantly reduced the total number of piglets born while significantly increasing the number of stillborn and weak piglets. Low dietary levels of ZEN still produce adverse effects on sow reproductive performance.

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