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Toxic Effects of Maternal Zearalenone Exposure on Uterine Capacity and Fetal Development in Gestation Rats

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Abstract

The objectives of this study were to determine the effects of high-dose and early gestational exposure to zearalenone (ZEN) in female Sprague-Dawley (SD) rats, to correlate the maternal uterus with the fetus, and to explore the development and malformation of fetuses. Pregnant female SD rats were fed diets containing 0.3, 48.5, 97.6, or 146.0 mg/kg ZEN on gestational days (GDs) 0 through 7. All the females survived until GD 20, at which point a cesarean section was performed to harvest the organs, blood, and fetuses. The results indicated that exposure to ZEN during early gestation can impact the maternal reproductive capability. Delayed fetal development was directly linked to maternal toxicity. The toxic effects of ZEN caused early deaths more frequently than late deaths, and the deleterious effects lasted through the end of pregnancy.

Keywords

zearalenone, uterine capacity, developmental toxicity, teratogenesis, rat

Introduction

Zearalenone (ZEN), that is, 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcyclic acid lactone, is a nonsteroidal estrogenic mycotoxin that is produced as a secondary metabolite by a variety of *Fusarium* fungi that grow on cereal grains, including corn, rice, rye, and wheat.[1] Several in vivo studies have reported that ZEN exposure results in reduced feed intake, feed refusal, poor feed conversion, diminished body weight gain, and increased incidence of disease.[2] Zearalenone is particularly toxic to the reproductive system, resulting in uterine enlargement, alterations to the reproductive tract, reduced litter size, increased embryolethal resorption, decreased fertility, and changes in the progesterone (PRG) and estradiol (E2) plasma levels in laboratory animals.[3]

Zearalenone and its reduced metabolites have been shown to activate 17β-estradiol receptors (ERs).[4] Animal experiments have indicated that ZEN directly binds to ERs.[5] 17β-Estradiol receptors belong to the nuclear receptor superfamily and are ligand-induced intracellular transcription factors that mediate many of the biological effects of estrogens and mycotoxins.[6] Zearalenone has a resorcyclic acid lactone structure and can cross cell membranes to bind to cytosolic ERs to form a ZEN–ER complex. These estrogen-like effects regulate anabolic and reproductive activities.[7]

After drugs, mycotoxin, or xenobiotics enter the body through their receptors, adenosine triphosphate (ATP)-binding cassette (ABC) transporters play an important role in cancer drug resistance, protection against xenobiotics, and the general passage of drugs through cellular and tissue barriers.[8] Adenosine triphosphate-binding cassette transporters constitute a large and functionally diverse superfamily of membrane transporter proteins.[9] ATP-binding cassette drug transporters, which represent the body's own defense system, mediate ATP-dependent primary active transport, act as efflux pumps,[10] and mediate efflux across biological membranes.[11]

Many studies on laboratory and farm animals have commonly investigated the effects of long-term exposure to low ZEN concentrations. The aims of the present study were (1) to investigate whether rats exposed to high doses of ZEN during early gestation through their feed experienced estrogen responsiveness, modified physiological responses, and toxicological effects on the maternal organs, (2) to determine whether there is a correlation between the maternal uterus outcome and the fetus through maternal effect analysis, and (3) to evaluate the effect of ZEN exposure on the expression of ABC transporters and nuclear receptor in early pregnant rats. We

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Table 1. Zearalenone Concentrations in Feed and the Number of Treated Rats Per Concentration Group.

Experimental Group	Concentration of Zearalenone, mg/kg		Number of Rats
	Supplementation	Analyzed Concentration	
ZEN 0 (control)	0	0.3	12
ZEN 50	50	48.5	12
ZEN 100	100	97.6	12
ZEN 150	150	146.0	12

Abbreviation: ZEN, zearalenone.

also quantified the levels of ZEN and its major metabolites in rats at the end of the pregnancy.

Materials and Methods

Ethics Statement

This study was performed in strict accordance with the recommendations of the National Research Council Guide,[12] and all the animal experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang Province, China. All the surgeries were performed under anesthesia through the intraperitoneal injection of sodium pentobarbital, and every effort was made to minimize suffering.

Animals, Exposure to ZEN, and Experimental Design

Zearalenone was purchased from Fermentek Ltd (Jerusalem, Israel). The substance was reported to be stable for at least 8 months. Pregnant rats received diets containing different concentrations of ZEN from gestational days (GDs) 0 to 7. The rats were divided into 4 groups (ZEN 0, ZEN 50, ZEN 100, and ZEN 150) and received a daily feeding of 0.3, 48.5, 97.6, and 146.0 mg/kg (equal to 0, 4.5, 9, and 13.5 mg/kg bw/d) of ZEN, respectively. The animals were given drinking water and feed ad libitum.

Sprague-Dawley (SD) rats were obtained from Jilin University Laboratory Animal Centre (Changchun, China) and were acclimated for 1 week prior to experimentation. The weights of the male rats ranged from 300 to 325 g, and the weights of the female rats ranged from 190 to 210 g (9 weeks). Male rats were used as sires only and were not treated. The doses of ZEN were selected based on the work of Collins et al[13] and Ueno et al.[14] The rats were maintained in plastic rat cages and had free access to food and water. The animals were maintained on a 12-hour light/12-hour dark cycle at 24 $\pm$ 2°C in the study room. The relative humidity was maintained at 47% to 55%.[13] Throughout the study, the male and female rats were fed experimental animal rations (University Laboratory Animal Centre, Changchun, China). The feed was prepared in an experimental feed mill using high-precision mixers, and all the feedstuffs were subjected to postprocessing analytical control. The concentrations of the feed composition were compared using validated analytical methods (National Standards of the People's Republic of China, GB/T 19540-2004). The findings are presented in Table 1.

Cohabitation began at approximately 4:30 PM on each mating day (2 females per male). The next morning, each female was examined for the presence of sperm in a vaginal lavage. A total of 96 sperm-positive females were presumed to be pregnant on GD 0, and a stratified random procedure was used to assign each animal to the control group or 1 of the 3 treatment groups.[13] Each pregnant rat was maintained individually in a polycarbonate metabolic cage (Tecniplast, Hohenpeißenberg, Germany).

Cesarean Section and Collection of Samples

The animals were killed under anesthesia through the intraperitoneal injection of sodium pentobarbital (0.5 mL/kg) on GD 20. For blood collection, microcentrifuge tubes were coated with 10 mL of heparin sodium salt (20 U/mL in phosphate-buffered saline). Whole blood was collected from the submandibular site as described previously and maintained on ice for 30 minutes. The samples were centrifuged at 3000*g* and 4°C for 10 minutes. The plasma layers were removed, placed into sterile microcentrifuge tubes, and stored at −40°C until assayed. The mesometrium was trimmed from the right and left uterine horns. Fragments of the maternal organs (placenta, ovary, and uterus) were quickly removed. Each placenta, ovary, uterus, fetal brain, and uterine horn with its fetuses was weighed using an electronic analytical balance. The lengths of each uterine horn, fetuses, placenta, fetal crown-rump length, fetal tail length, and individual available uterine space per fetus measured on a uterine horn were assessed using a dissecting microscope and micrometer lens (accuracy = 0.05 mm).[15] The fetuses were macroscopically assessed for the presence of congenital malformations. Afterward, the remaining reproductive organs were frozen in liquid nitrogen, stored at −80°C, and either subjected to RNA and quantitative reverse-transcription polymerase chain reaction (PCR) to analyze their gene expression or fixed for histopathology or high-performance liquid chromatography (HPLC) analysis.

Quantification of ZEN

The preliminary treatment was conducted according to the procedures outlined by Koraichi et al.[3] The concentrations

of ZEN were determined by HPLC according to the procedures outlined by Videmann et al[16] and Koraichi et al.[3]

Histopathology

The ovaries were fixed in 10% neutral-buffered formalin. The center of each ovary was sectioned (thickness, 5 μm) and stained with hematoxylin and eosin.[17] The percentage of the stained areas was measured using the AnalySIS-trame grabben CSIS system (AnalySIS 3.4, SIS, Münster, Germany) as previously described.[18,19] The slides were observed under 100× or 200× magnification using an optical microscope (Nikon, Japan).

Hormone Levels

The prolactin (PRO), follicle-stimulating hormone (FSH), luteinizing hormone (LH), E2, and PRG levels were determined using an enzyme-linked immunosorbent assay kit (R&D Systems, Inc, Minneapolis, Minnesota) according to the manufacturer's recommended protocol.

Quantitative Real-Time PCR

The total RNA was isolated from the organs using a reagent box (E.Z.N.A. Total RNA Kit; Omega Bio-tek, Inc, Doraville, Georgia) according to the manufacturer's recommended protocol. The concentration of RNA was estimated based on the absorbance at 260 nm, which was determined using a spectrophotometer. The RNA quality was determined by analyzing the RNA integrity through agarose gel electrophoresis and by confirming that the A260 nm/A280 nm absorbance ratio was between 1.8 and 2.0.

The ABCB1, ABCC1, ABCG2, ERs, and β-actin expression levels were determined through quantitative real-time PCR using an ABI PRISM 7500 SDS thermal cycler apparatus (Applied Biosystems, Foster City, California). The first strand was synthesized from the total DNase-treated RNA using random hexamers and murine Moloney leukemia virus reverse transcriptase according to the manufacturer's recommended instructions (high-capacity complementary DNA [cDNA] reverse transcription kits were obtained from Applied Biosystems, China). The primers were designed using the Primer Express software (Applied Biosystems) from the rat sequences for β-actin (GenBank accession number: NM031144, amplicon length: 207 bp, forward primer: 5′-ACCCGCGAGTACAACCTTC-3′, reverse primer: 5′-RP CCCATACCCACCATCACACC-3′), ABCB1 (GenBank accession number: NM133401, amplicon length: 194 bp, forward primer: 5′-AACGGAAGAGCAGACAAGAACT-3′, reverse primer: 5′-CCAAAGACCAGCATCATAAGTG-3′), ABCG2 (GenBank accession number: NM181381, amplicon length: 241 bp, forward primer: 5′-GCAGTTCAGGTTATGTGGTTCA-3′, reverse primer: 5′-TCCATTCCTATGCTTGTCCTTT-3′), ABCC1 (GenBank accession number: NM022281, amplicon length: 138 bp, forward primer: 5′-CAATGTCCTCTGAGATGGAGAC-3′, reverse primer: 5′-CTCTACACGGCCTGA ATGG G-3′),[20] and ERs (GenBank accession number: NM012689, amplicon length: 73 bp, forward primer: 5′-CCAAAGCCTCG-GAATGG-3′, reverse primer: 5′-AGCTG CGGGCGATTGAG-3′).[21] Briefly, the PCR reactions were performed starting with 50 ng of first-strand cDNA and 500 nmol/L of both sense and antisense primers in a final volume of 20 μL using SYBR Green I PCR core reagents (SYBR Real-Time PCR Kit were provided by TaKaRa BIO CATALOG, Da Lian, China). The expression values were calculated as $2^{-\Delta Ct}$.[22]

Statistical Analysis

The statistical analysis was conducted using SPSS software (SPSS Inc, Chicago, Illinois), and all the data are expressed as the mean ± standard error of the mean. Because the data were normally distributed, all the indices were analyzed using analysis of variance and Duncan multiple range tests. $P < .05$ was considered statistically significant.

Results

Maternal Observations

The dose-related effects of ZEN on the health of the females in all the groups after cesarean section on GD 20 are shown in Table 2. The feed consumption and body weight gain were monitored approximately once a week. The feed consumption decreased over the course of treatment. This phenomenon appeared during the early stages of gestation but was less obvious during the late stages. The overall body weight gain during GDs 0 through 20 decreased significantly in a dose-dependent manner in all the groups, especially during the last period of gestation. The average weight of the animals in 4 groups was similar (205.6, 205.8, 204.9, and 205.3 g) at the beginning of the experiment. On GD 20, the average weight of the animals in each group had decreased significantly.

The weights and lengths of the reproductive organs of female rats treated with ZEN on GD 20 are presented in Table 3. An increase in the dose of ZEN resulted in an increase in the average weight and deepened the color of the ovaries. In contrast, the placental wet weights and the uterine wet weights significantly decreased in a dose-dependent manner. The weights and lengths of the reproductive organs were positively correlated. The embryonic growth and fetal size were determined by the uteroplacental unit, which supplies nutrients to the fetus. The number of fetuses, percentage of death, body weight, body length, and malformation are summarized in Table 4.

Fetal Observations

The reproductive performance, fetal development, and fetal malformations on GD 20 are displayed in Table 4. The average percentage of late deaths per litter was significantly higher and the fetal crown-rump length, weight of fetal brain, and available uterine space per fetus were significantly decreased in the treated animals on GD 20 compared to the animals in the ZEN 50 and control groups. The average percentage of early deaths was higher than the average percentage of late deaths per litter.

Table 2. Mean Feed consumption, Mean Body Weight Gain, and Mean Body Weight Per Pregnant Sprague-Dawley Rat (g).

	Groups			
Days or Parameter	Control	ZEN 50	ZEN 100	ZEN 150
Mean feed consumption				
0-7	120.72 ± 0.94[a]	118.16 ± 1.08[a]	114.44 ± 1.20[b]	108.85 ± 1.48[c]
8-14	119.63 ± 0.73[a]	117.63 ± 0.79[ab]	116.66 ± 1.15[b]	110.72 ± 1.02[c]
15-20	114.77 ± 1.45[a]	113.83 ± 1.32[a]	112.17 ± 0.89[a]	108.49 ± 0.85[b]
Mean body weight gain				
0-7	24.81 ± 1.12[a]	23.97 ± 1.09[a]	22.70 ± 1.32[ab]	20.02 ± 0.78[b]
8-14	37.97 ± 1.15[a]	34.68 ± 1.85[ab]	29.98 ± 1.60[b]	24.49 ± 1.94[c]
15-20	62.60 ± 2.19[a]	59.38 ± 1.40[ab]	55.05 ± 1.05[b]	47.76 ± 1.30[c]
Mean body weight in GD 20	280.33 ± 1.59[a]	276.50 ± 1.92[a]	266.15 ± 1.24[b]	257.68 ± 1.12[c]

Abbreviations: GD, gestational day; ZEN, zearalenone.
[a,b,c]Means within a row with no common superscripts differ significantly ($P < .05$).

Table 3. Maternal Reproductive Organs Parameters in Female SD Rats in GD 20.

	Groups			
Parameters	Control	ZEN 50	ZEN 100	ZEN 150
Ovarian wet weight, g	0.14 ± 0.0006[c]	0.15 ± 0.0009[b]	0.15 ± 0.0009[ab]	0.15 ± 0.0009[a]
Placental wet weights, g	0.80 ± 0.0041[a]	0.78 ± 0.0065[a]	0.73 ± 0.0129[b]	0.66 ± 0.0075[c]
Length of the maternal placenta, cm	1.51 ± 0.06[a]	1.48 ± 0.07[a]	1.32 ± 0.06[a]	1.06 ± 0.07[b]
Uterine wet weights, g[e]	6.07 ± 0.0229[a]	5.42 ± 0.0950[b]	4.24 ± 0.0210[c]	4.03 ± 0.0753[d]
Weight of uterine horn with fetuses, g	27.54 ± 0.14[a]	27.03 ± 0.15[b]	24.13 ± 0.19[c]	17.91 ± 0.15[d]
Weight of uterine horn with fetuses, g[e]	55.46 ± 0.43[a]	54.58 ± 0.36[a]	48.19 ± 0.34[b]	35.62 ± 0.30[c]
Length of uterine horn with fetuses, cm	18.91 ± 0.11[a]	18.30 ± 0.16[b]	15.34 ± 0.15[c]	10.11 ± 0.10[d]

Abbreviations: GD, gestational day; SD, Sprague-Dawley; ZEN, zearalenone.
[a,b,c,d]Means within a group with different superscripts are significantly different at $P < .05$.
[e]Double sides of the uterine horn.

Table 4. Reproductive Performance, Fetal Development, and Fetal Malformations in Pregnant SD Rats on GD 20.

	Groups			
Parameters	Control	ZEN 50	ZEN 100	ZEN 150
Total number of fetuses	154	150	124	88
Number of implanted embryos per litter	12.83 ± 0.21[a]	12.79 ± 0.19[a]	10.99 ± 0.23[b]	8.31 ± 0.22[c]
Number of viable fetuses per litter	12.83 ± 0.21[a]	12.50 ± 0.20[a]	10.33 ± 0.14[b]	7.32 ± 0.24[c]
Average % early deaths	0.00 ± 0.00[c]	0.33 ± 0.02[c]	1.33 ± 0.03[b]	3.83 ± 0.31[a]
Average % late deaths	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.83 ± 0.04[a]	1.17 ± 0.03[a]
Average fetal body weight, g	3.76 ± 0.07[a]	3.71 ± 0.03[ab]	3.55 ± 0.03[b]	2.97 ± 0.08[c]
Average fetal body weight, g[e]	3.76 ± 0.07[a]	3.15 ± 0.14[b]	2.34 ± 0.10[c]	1.52 ± 0.09[d]
Fetal crown-rump length, cm	2.67 ± 0.10[a]	2.62 ± 0.13[a]	2.03 ± 0.11[b]	1.36 ± 0.10[c]
Fetal tail length, cm	0.73 ± 0.03[a]	0.64 ± 0.06[ab]	0.52 ± 0.04[b]	0.35 ± 0.04[c]
Available uterine space per fetus, cm	2.93 ± 0.09[a]	2.89 ± 0.07[a]	2.25 ± 0.07[b]	1.61 ± 0.08[c]
Weight of fetal brain, g	0.630 ± 0.008[a]	0.612 ± 0.006[a]	0.526 ± 0.008[b]	0.426 ± 0.010[c]
Exencephaly	0.00 ± 0.000[b]	0.00 ± 0.000[b]	0.04 ± 0.004[b]	0.17 ± 0.008[a]
Exophthalmos	0.00 ± 0.000[b]	0.00 ± 0.000[b]	0.04 ± 0.004[b]	0.21 ± 0.008[a]

Abbreviations: GD, gestational day; SD, Sprague-Dawley; ZEN, zearalenone.
[a,b,c,d]Means within a row with no common superscripts differ significantly ($P < .05$).
[e]Analysis adjusted for number of implanted embryos.

Table 5. Concentrations of FSH, LH, PRO, E2, and PRG in the Plasma in Female Rats on GD 20.

Parameters	Groups			
	Control	ZEN 50	ZEN 100	ZEN 150
FSH, IU/L	13.67 ± 0.12[c]	14.52 ± 0.20[b]	15.29 ± 0.23[a]	15.30 ± 0.17[a]
LH, ng/L	12.70 ± 0.15[c]	13.28 ± 0.09[c]	16.44 ± 0.18[b]	17.92 ± 0.24[a]
PRO, μg/L	18.46 ± 0.14[c]	20.40 ± 0.25[b]	21.51 ± 0.75[b]	24.67 ± 0.63[a]
E2, ng/L	68.91 ± 0.18[a]	65.27 ± 0.17[b]	64.84 ± 0.08[b]	59.83 ± 0.24[c]
PRG, pg/mL	744.77 ± 2.95[a]	726.10 ± 1.47[b]	712.78 ± 5.52[b]	681.84 ± 9.22[c]

Abbreviations: E2, estradiol; FSH, follicle-stimulating hormone; GD, gestational day; LH, luteinizing hormone; PRG, progesterone; PRO, prolactin; ZEN, zearalenone.
[a,b,c]Means within a line with different superscripts are significantly different at $P < .05$.

The average fetal body weights exhibited a marked decrease with an increase in the dosage. In the ZEN 150 group, the fetal malformations, exencephaly, and exophthalmos were found to be increased compared with those of the other groups.

Concentrations of FSH, LH, PRO, PRG, and E2 in the Plasma of Female Rats at GD 20

The gonadotropin (FSH, LH, and PRO) and sex steroid (PRG and E2) concentrations in the blood plasma obtained during cesarean section on GD 20 are analyzed in Table 5. The LH and FSH concentrations were increased in the ZEN 100 and ZEN 150 groups. The PRO level was significantly increased in a dose-dependent manner in the ZEN 50 through ZEN 150 groups. The PRG and E2 levels were significantly decreased in all the treatment groups.

Histopathological Examination

The histopathological analysis revealed numerous ovarian follicles at different stages of development in the control samples (Figure 1A). In the ZEN 50 group, there were less functional follicles at different stages of development compared with the ZEN 0 group (Figure 1B), and there was a thickening and loosening of the granular layer of rounded ovarian follicles (Figure 1C). The phenomenon of ovarian follicular atresia increased (Figure 1D), the granular layer thickened, and the follicular antral fluid became uneven (Figure 1E). Abnormal ovarian follicles and nonfunctional follicles were frequently observed (Figure 1F). The granular layers disappeared and were replaced by connective tissue. The ovarian tissues exhibited obvious fibrotic changes (Figure 1G).

Concentrations of ZEN in Maternal Tissues and Fetal Brains

The quantification of the ZEN concentrations in the maternal tissues, namely, the uterus, ovary, and placenta, was performed. In all the tissues, the ZEN concentrations increased significantly in a dose-dependent manner, as shown in Table 6. Significant differences became apparent in a ZEN 100 group. The concentrations of ZEN in the fetal brains are not shown because it could not be detected.

Effects of ZEN Exposure on Messenger RNA Expression in the Tissues of Pregnant Rats and Fetal Brains

A significant upregulation of all the detected messenger RNA (mRNAs) was observed in the uterus, ovary, placenta, and fetal brain on GD 20, as shown in Table 7. The expression level of each mRNA was significantly different in the female organs compared with the fetal brains. The ZEN 150 group exhibited the highest expression of ABC transporters and nuclear receptors in the 3 maternal tissues and the fetal brains. These effects occurred in a dose-dependent manner.

Discussion

The early events of pregnancy are associated with rapid changes in the expression of the genes required for nutrient transport, cellular remodeling, angiogenesis, and relaxation of vascular tissues as well as cell proliferation and migration.[23] Embryonic loss is a significant problem in a number of species (rats, sheep, horses, pigs, and humans) due to the maternal diet during the early gestational period.[24] This phenomenon is due to not only nutrition intake but also mycotoxin ingestion. Malekine found an accelerated response (an increased incidence of oocytes with nuclear abnormalities and impairment of embryonic development) in oocytes exposed ex vivo to low concentrations of mycotoxin.[25] Early gestation is a key period in fetal development. In our work, the adverse effects of ZEN exposure during early gestation were obvious and lasted through the end of the gestation period. The average percentage of early deaths was higher than average percentage of late deaths, as shown in the data table. Early gestation ZEN exposure influenced the embryonic implantation, which was the reason for this phenomenon. The high-dose–response curves for ZEN reported in our study differed from those reported by Marmugi et al for very low doses.[26]

The administration of estrogens to pregnant animals has been found to exert detrimental effects during pregnancy. The exposure to *Fusarium*-contaminated feed reduced the conception rates and litter sizes and resulted in the enlargement of the

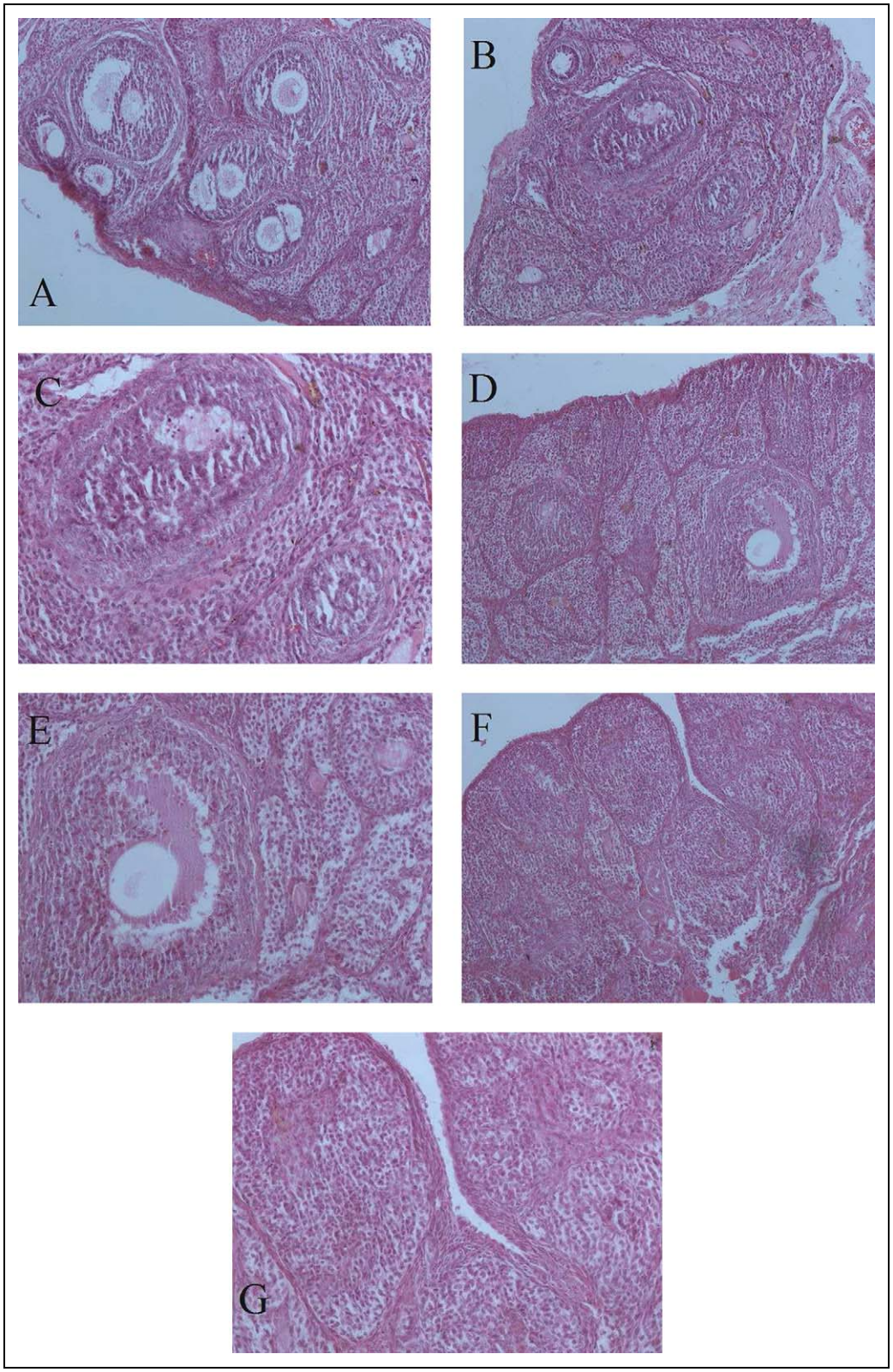

Figure 1. Histological analyses of ovarian structures (hematoxylin and eosin [H&E] stain). A, Ovarian follicles at different stages of development among control samples (ZEN 0). B, Decreasing numbers of follicle at different stages of development in ZEN 50. C, Thickening of the granular layer rounded ovarian follicle in ZEN 50. D, Increasing ovarian follicular atresia in ZEN 100. E, Thickening of granular layer and uneven follicular antral fluid in ZEN 100. F, Nonfunctional follicles were frequently observed in ZEN 150. G, Granular layers disappeared and were instead by connective tissue in ZEN 150. A, B, D, and F, The slides were observed under ×100 magnification. C, E, and G, The slides were observed under ×200 magnification. ZEN indicates zearalenone.

Table 6. Concentrations of ZEN and Its Metabolites on Matrix in GD 20 (ng/g).

Parameters	Groups			
	Control	ZEN 50	ZEN 100	ZEN 150
Ovary	0.000 ± 0.000[c]	0.011 ± 0.002[c]	0.754 ± 0.022[b]	3.460 ± 0.101[a]
Uterus	0.000 ± 0.000[d]	0.121 ± 0.015[c]	0.557 ± 0.012[b]	6.728 ± 0.110[a]
Placenta	0.000 ± 0.000[c]	0.190 ± 0.007[c]	3.542 ± 0.081[b]	8.488 ± 0.087[a]
Fetal brain	ND	ND	ND	ND

Abbreviations: GD, gestational day; ND, not detectable; ZEN, zearalenone.
[a,b,c,d]Means within a line with different superscripts are significantly different at $P < .05$.

Table 7. The mRNA Expression Levels in the Tissues of Pregnant Rats and Fetal Brains.

Parameters	Groups			
	ZEN 0	ZEN 50	ZEN 100	ZEN 150
Ovary				
ABCB1	0.687 ± 0.01[b]	0.701 ± 0.02[b]	0.761 ± 0.02[a]	0.770 ± 0.02[a]
ABCG2	1.07 ± 0.07[ab]	0.94 ± 0.02[b]	1.20 ± 0.12[a]	1.26 ± 0.02[a]
ABCC1	0.88 ± 0.03[b]	0.92 ± 0.03[b]	1.03 ± 0.07[b]	1.21 ± 0.07[a]
ERs1	2.26 ± 0.06[ab]	2.15 ± 0.06[b]	2.34 ± 0.05[a]	2.44 ± 0.07[a]
Uterus				
ABCB1	28.54 ± 0.50[b]	29.95 ± 0.82[ab]	31.69 ± 1.04[a]	32.17 ± 1.24[a]
ABCG2	18.95 ± 1.00[b]	19.50 ± 0.96[b]	21.52 ± 1.43[ab]	23.66 ± 0.60[a]
ABCC1	1.98 ± 0.08[b]	2.17 ± 0.07[ab]	2.35 ± 0.15[a]	2.41 ± 0.09[a]
ERs1	2.07 ± 0.11[b]	2.12 ± 0.10[b]	2.26 ± 0.13[ab]	2.54 ± 0.12[a]
Placenta				
ABCB1	1.07 ± 0.04[ab]	0.96 ± 0.03[b]	1.20 ± 0.06[a]	1.21 ± 0.04[a]
ABCG2	0.502 ± 0.004[b]	0.523 ± 0.007[ab]	0.524 ± 0.011[ab]	0.535 ± 0.008[a]
ABCC1	0.414 ± 0.007[bc]	0.395 ± 0.006[c]	0.435 ± 0.010[ab]	0.439 ± 0.009[a]
ERs1	0.253 ± 0.006[ab]	0.233 ± 0.006[b]	0.240 ± 0.009[ab]	0.260 ± 0.007[a]
Fetal brain				
ABCB1	0.900 ± 0.008[ab]	0.886 ± 0.023[ab]	0.869 ± 0.017[b]	0.933 ± 0.007[a]
ABCG2	0.082 ± 0.003	0.084 ± 0.001	0.080 ± 0.002	0.089 ± 0.004
ABCC1	0.037 ± 0.0005[b]	0.038 ± 0.0004[ab]	0.038 ± 0.0003[ab]	0.039 ± 0.0005[a]
ERs1	0.232 ± 0.003[ab]	0.229 ± 0.002[b]	0.237 ± 0.004[ab]	0.241 ± 0.002[a]

Abbreviations: ZEN, zearalenone.
[a,b,c]Means within a line with different superscripts are significantly different at $P < .05$.

ovaries and the swelling of the vulva in piglets.[27] We must give greater attention to the physiological influences that vary the fetal environment.[28] It has been recognized for many years that fetal growth is relatively more sensitive to the fetal environment.[29] Similar activity is attributed to phytoestrogens, that is, small doses of ZEN have a stimulatory effect, whereas higher doses stimulate proliferation.[30] In the group of rats exposed to high doses of ZEN in the present study, the uteruses were atrophic, and decreased ovary weights were observed.[31] The opposite tendencies were observed in our study because we added a high dose of ZEN to the feed to enhance the weight of the ovaries, and the uteruses developed more slowly with a decrease in the number of fetuses. Similar results were observed by Yang et al in minks, which exhibited increased uterine weights.[32] Pigs fed 0.35 mg ZEN/kg bw/d in the presence or absence of an inorganic adsorbent for 35 days and displayed no changes in their uterine weight in the absence of the adsorbent and increased uterine weights in the presence of the adsorbent.[33] The greatest decrease in the litter size was observed after the combined selection for indices of ovulation rate and uterine capacity. Towna et al reported that a compatible uterine length was necessary for fetal survival and development.[34] In our study, the uterine space was correlated to embryo survival and embryonic loss, which implies associated effects on fetal development. Our experiments suggest that the moderated uterine space due to ZEN exposure in early gestation affects placental and embryonic development.

In the toxicology field, the toxic effects on fetal development have usually been considered a consequence of fetal exposure to environmental contaminants through placental transfer.[35] The placental transfer of mycotoxins can indicate a potential risk of direct effects on the fetus.[32] In fact, the placenta is the key organ that regulates the exchange of nutrients, gasses, and waste, as well as endogenous and foreign molecules, between the maternal and the fetal circulations.[36] From our research, ZEN reduced the placental size, and this effect

will have negative consequences on fetal development. The blood from the maternal body flows through chorionic plate arteries into the placenta and is then perfused by the other pair of vessels, namely, the large central artery and intraplacental arteries,[37] which can be affected by ZEN.

During pregnancy, ZEN reduces embryonic survival when administered above a threshold and sometimes decreases fetal weight, especially during the early gestation period.[38] Pregnant rats fed 10 mg/kg ZEN delivered decreased numbers of viable offspring. The offspring that did survive had decreased skeletal ossification, but their soft-tissue development was not affected.[39] Breeding gilts that were fed 22.09 mg/kg ZEN experienced decreased reproductive performance, as evidenced by a decreased number of corpora lutea, a decreased number of live embryos, an increased number of dead-born piglets, and aborted fetuses. These effects were less pronounced in gilts fed mashes containing 2.2 mg/kg ZEN.[32] In an early study by Arora et al, single doses of 20 mg/kg ZEN resulted in 2 completely resorbed litters.[40] These results are in agreement with the data reported by Kordic et al[31] who observed a dose-dependent effect of ZEN on the reproductive performance of breeding gilts, which exhibited decreased numbers of live embryos and reproductive problems, including an increased number of stillborn piglets. Long and Diekman reported that gilts fed 5 to 30 mg/kg ZEN from days 2 to 15 postmating had normal embryonic development, but no fetuses were present on days 40 to 43 postmating in gilts fed 60 or 90 mg/kg ZEN.[41] In this respect, there was no difference between pigs and rats. Based on our research data, we concluded that the deleterious effects of ZEN are more hazardous to embryonic development during early gestation than to fetal development during late gestation. Various estrogenic effects, including decreased fertility, increased embryolethal resorptions, reduced litter size, altered weights of the adrenal, thyroid, and pituitary glands, and changes in the plasma levels of PRG and E2, have been observed, but no teratogenic effects have been found in mice, rats, guinea pigs, or rabbits.[42] However, exencephaly and exophthalmos were obviously detected in the ZEN 150 group. The examination of the fetuses for malformations indicated that the period of greatest susceptibility the teratogenic effects of high-dose ZEN is early gestation.[43]

Dietary substances capable of altering hormone levels are of concern during pregnancy, which is a hormone-sensitive period for both the mother and the fetus.[44] Similar to the observations of Collins et al, we observed that gonadotropins (FSH, LH, and PRO) were significantly increased and that sex steroids (PRG and E2) were markedly decreased after ZEN exposure.[13] Follicle-stimulating hormone and LH act synergistically to promote follicular development and to determine follicular rupture and ovulation.[45] Follicle-stimulating hormone promotes follicular growth and induces LH receptors on the granulosa cell membrane.[46] These hormones play a key role as substrates necessary for the consequent E2 secretion that occurs in the granulosa cells under the influence of FSH.[47] The increased weights of ovaries were associated with hormone stimulation. Zearalenone may affect the uterus by decreasing PRG secretion and by altering the morphology of uterine tissues to lead to uterine atrophy.[48] Guibourdenche et al reported that villous trophoblasts proliferate and differentiate by fusion to form a continuous, uninterrupted, multinucleated layer that regulates the exchange of substrates, gasses, and other factors between the maternal and fetal circulations and synthesizes and secretes steroid hormones (PRG and estrogens) and many protein hormones.[49]

As a primary functional organ of the reproductive system, the ovary is responsible for oocyte and follicle development and steroid hormone production.[30] An increase in the concentration of ZEN resulted in ovarian tissues fibrosis and obvious retrogressive changes, as shown in Figure 1. These phenomena were the same as those reported by Alonso-Pozos et al.[50] The endocrine function changed as a result of the structural changes in the ovaries. In response to ZEN stimulation, the normal ovarian tissues were replaced by connective tissue, which led to an intensification of the atresia of ovarian follicle granulosa cells, an increase in weight, and a decreased production of E2 and PRG. These conclusions were consistent with the results of previous studies in gilts.[30,51]

Zearalenone is known to be rapidly absorbed after oral administration. The risk assessment, which is based on the exposure and hazard evaluation, needs to take into account the transfer of ZEN in the organism and should evaluate all contamination sources.[52] Because the plasma concentrations of the parent mycotoxin and their metabolites cannot be used as biomarkers for efficacy testing,[53] our work focused on the measurement of the ZEN concentrations in various organs. The amount of ZEN found in the reproductive organs increased with the administration of ZEN in the 3 maternal treatment groups. The deleterious effects of ZEN exposure occurred in a dose-dependent manner, as shown in the tables. However, ZEN was not found in the fetal brains.

To date, studies on the environmental induction of changes in gene expression and regulation related to disease etiology have mainly focused on specific genes or on the genetic pathways related to particular diseases.[54] Adenosine triphosphate-binding cassette transporters expressed in the placenta, which have been relatively well defined with respect to their roles in the transport of clinically and toxicologically important drugs/xenobiotics, include P-glycoprotein (ABCB1), the breast cancer resistance protein (ABCG2), and (to a lesser extent) the multidrug resistance proteins 1 to 3 and 5 (ABCC1-3 and 5).[55-57] Adenosine triphosphate-binding cassette B1 plays an important role in limiting fetal exposure to various potentially harmful drugs and xenobiotics.[58] This result is in agreement with in vitro studies conducted in the Caco-2 cell line.[16,59] The induction of ZEN detoxification has consequences not only for ZEN elimination but also for the concomitant exposure of animals (or humans) to ZEN. This is also valid for frequently associated toxins, such as trichothecenes, the uptake of which is closely related to the ABCB1 concentrations.[60] Zearalenone exposure also modulated the level of ABCG2 mRNA in the uterus, ovaries, and placenta. Adenosine triphosphate-binding cassette G2 expression is generally very high in the human placenta,[61] but

only low levels were observed in our study. These low levels may have been a result of species-related differences in the toxic effects of ZEN. Adenosine triphosphate-binding cassette C1 is present on the apical membrane of placental syncytiotrophoblasts[62] and may transport toxins and xenobiotics through the membrane into the extracellular matrix. The in vitro affinity of ZEN for uterine and oviduct estrogen receptors is greater in pigs than it is in rats and chickens.[63] Moreover, recent studies suggest that ZEN is capable of interfering with other receptor systems and signaling cascades that are known to display cross-talk with the ERs pathway.[64] The most widely acknowledged feature of ZEN is its ability to bind to the ERs and induce the expression of estrogen-responsive genes.[65] Grünfeld and Bonefeld-Jorgensen suggested that changes in the expression levels of ERs can significantly impact the signal amplification mediated through the ERs and thus disrupt the functions regulated by ERs.[66] Previous work has indicated that ERs are stimulated by ZEN in vivo. The mRNA expression level increased after exposure to increasing concentrations of ZEN, and the protective effects of ABC transporters were also increased, which is contrary to the conclusions reported by Koraichi et al.[3]

Conclusions

Our results provide the first demonstration that exposure to high doses of ZEN during early gestation lead to a relationship between uterus capacity, ovary morphology, and fetuses, exert a teratogenic effect on the fetus, and modulate the mRNA levels of ABC transporters in the maternal reproductive tissues. The nature and magnitude of this effect was both tissue and dose dependent. The administration of ZEN to rats during the early stages of pregnancy was found to cause early deaths more frequently than late deaths and induce teratogenesis in the fetuses.

Declaration of Conflicting Interests

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