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The impact of Zearalenone on the meiotic progression and primordial follicle assembly during early oogenesis

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ABSTRACT

Zearalenone (ZEA) is a mycotoxin produced by fusarium graminearum. It can cause abnormal reproductive function by acting as an environmental estrogen. Research has traditionally focused on acute and chronic injury on mammalian reproductive capacity after ZEA treatment. Little research has been done studying the effects of ZEA exposure on early oogenesis. In this study, we investigate the effects of ZEA exposure on meiotic entry, DNA double-strand breaks (DSBs), and primordial follicle assembly during murine early oogenesis. The results show that ZEA exposure significantly decreased the percentage of diplotene stage germ cells, and made more germ cells remain at zygotene or pachytene stages. Moreover, the mRNA expression level of meiosis-related genes was significantly reduced after ZEA treatment. ZEA exposure significantly increased DNA-DSBs at the diplotene stage. Meanwhile, DNA damage repair genes such as *RAD51* and *BRCA1* were activated. Furthermore, maternal exposure to ZEA significantly decreased the number of primordial follicles in newborn mouse ovaries. In conclusion, ZEA exposure impairs mouse female germ cell meiotic progression, DNA-DSBs, and primordial follicle assembly.

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1. Introduction

Zearalenone (ZEA) is a mycotoxin that causes serious pollution and effects on both humans and animals (Minervini and Dell'Aquila, 2008). It is an estrogen-like substance present in the environment and can compete with estrogen in some species of animals. It also has been reported that ZEA exposure can cause abnormal reproductive function in animals, even inducing acute or chronic poisoning (Sambuu et al., 2011b). These effects represent significant economic loss to the agriculture industry. ZEA and its derivatives pollute crops, dairy, and meat leading to an increasing number of food safety issues and pose a serious threat to human health.

ZEA can give rise to estrogen hyperparathyroidism in livestock, fowl, and mice, which can affect female mammal mammoplasia, inhibit multiple ovulations, cause delayed lactation and vulvovaginitis, result in constant estrus, pseudopregnancy, abortions, birth defects, and infertility (Malekinejad et al., 2007; Schoevers et al., 2012). Pregnant animals, including pregnant women, exposed to ZEA could result in miscarriages, stillbirths, and fetal malformations. ZEA contaminated foods also lead to poisoning of the central nervous system (Zhu et al., 2012). ZEA changes the secretion of estrogen which promotes apoptosis and reduces oocyte numbers resulting in a dramatic effect to the reproductive lifespan of exposed individuals (Gajecka et al., 2013). As an estrogen-like compound, ZEA can inhibit the secretion of follicle-stimulating hormone (FSH) thus inhibiting ovarian follicle development and maturation during the preovulatory phase (Zwierzchowski et al., 2005). ZEA exposure results in elevated mRNA levels of the estradiol receptor β (ERβ), but the expression of other hormone-converting enzymes did not change (Hou et al., 2015). ZEA exposure decreased the number of normal follicles, and made the oocytes of adult sows exhaust prematurely (Malekinejad et al., 2007). ZEA can also induce sow pseudoestrus and affect sow oocyte maturation in vitro. ZEA exposure has also been shown to decrease mouse oocyte survival rates, and increase the abnormal morphology of the oocyte spindle (Hou et al., 2015). Existing research has focused on feed detection, adsorption mechanisms, and the effect of growth performance traits, but little has been investigated with regards to the development of the genital system, sex differentiation, gametogenesis, and maturation.

During mouse embryo development, female germ cells initiate meiosis at 13.5 days post coitum (dpc), then they reach to diplotene and

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arrested at this stage (McLaren and Southee, 1997). In this period, chromosomes experience pairing, synapsis and homologous recombination, which has been linked to the activities of a meiosis-specific supramolecular proteinaceous structure, the synaptonemal complex (SC) (Yuan et al., 2000). SCs can be detectable by silver staining or immunofluorescence staining of synaptonemal complex proteins 3 (SCP3). At leptotene, oocyte with SYCP3 stretches and aggregates appear throughout the nucleus (Roig et al., 2004). In zygotene nuclei, longer stretches of SYCP3 are present. At the pachytene stage, all homolog pairs are tightly connected by the SCs. SCP3 positive threads become thicker and less at this stage than that at zygotene. At diplotene, some chiasmas appeared along SCP3 positive threads (Pawlowski and Cande, 2005). Any abnormalities appeared during these important periods will result in infertility, abortion and birth-defect (Hunt and Hassold, 2008). At the early meiotic stage, DNA double-strand breaks (DSBs) are activated, and chromosomes are further guided to generate homologous recombination. γ-H2AX, the signal of DNA-DSBs, is regarded as the marker of meiosis because of its expression in early meiosis (Blanco-Rodriguez, 2009). The efficient repair of DNA-DSBs is critical for the maintenance of genome stability (Tarsounas et al., 2004). MLH1, a mismatch repair protein is necessary for meiotic recombination in mice, and MLH1 foci have been proposed to mark crossover sites on meiotic chromosomes (Baker et al., 1996; Anderson et al., 1999). Before entering into meiosis, oogonia proliferated by mitosis. But during the process, cytokinesis was incomplete so oogonia remained connected in germ cell cysts (Dutta et al., 2016). In mice, at 13.5 dpc and the following days, when female germ cells initiated meiosis then arrested at diplotene of meiosis prophase I, somatic cells of ovaries invaded these female germ cells and then the female germ cell cyst breakdown, and individual oocytes became surrounded by granulosa cells forming primordial follicles (Gondos et al., 1986; Motta et al., 1997; Cohen and Holloway, 2010). The primordial follicle pool is the foundation of follicular development and reproductive capacity.

In this study, we utilized mouse as a model and studied the toxic effects of ZEA exposure on pregnant mice. Particular focus was placed on the effects of ZEA exposure on female germ cell meiotic entry, DNA-DSBs, and primordial follicle assembly during early oogenesis.

2. Materials and methods

2.1. Animals and experimental design

CD1 mice from Vital River Laboratory Animal Technology Co. LTD (Beijing, China) were used in this experiment. All mice were maintained at 21–22 °C and a 12:12-h light/dark cycle with free access of food (No. 11002900002503, production license number: SCXK (Beijing): 2009–0012, Keaoxieli, Beijing, China) and water (Zhang et al., 2015). The appearance of a vaginal plug was defined as 0.5 dpc. All procedures were reviewed and approved by the Ethical Committee of Qingdao Agricultural University.

In this study, a total of 114 pregnant mice of 12.5 dpc were used. The number of mice used in each endpoint was clearly shown in each Figure and in Table S1. The mice were orally administered a different dosage of ZEA (Sigma-Aldrich, Z2152, USA) for 4 days (from 12.5 to 15.5 dpc, for analysis of SCs), 6 days (from 12.5 to 17.5 dpc, for analysis of SCs, DNA-DSBs and repair) and 7 days (from 12.5 to 18.5 dpc, for analysis of germ cell cyst breakdown and primordial follicle formation). After the last treatment at 15.5 dpc and 17.5 dpc in the morning, the mice treated for analysis of SCs, DNA-DSBs and repair were euthanized in the afternoon at the same day. And the mice treated for analysis of germ cell cyst breakdown and primordial follicle formation were exposed to ZEA for 7 days (from 12.5 to 18.5 dpc). After the last treatment, the mice were divided into 2 groups and maintained normally to 3 days postpartum (dpp) or 21 dpp respectively. Three different ZEA dosages of 0, 20, 40 μg/kg body weight (BW)/day were used. The PMTDI (provisional maximum tolerable daily intake) of 0.5 μg/kg BW/day for ZEA is now established, based on the effect (No observed effect level) of 40 μg/kg BW/day in pigs (Creppy, 2002). And in general, both rat and mouse have stronger tolerance than pig (Zinedine et al., 2007; Schoevers et al., 2012). That's why the maximum concentration of 40 μg/kg BW/day was selected in our study. Before use, ZEA was dissolved in 1% DMSO (dimethyl sulfoxide), then diluted it to the required concentration using normal saline.

2.2. Immunofluorescence staining of synaptonemal complexes

Fetal ovaries of 15.5 dpc and 17.5 dpc were collected mechanically from female fetal mice, and were used for immunofluorescence. The samples were immersed in HEB (hypo extraction buffer, 500 μl 600 mM Tris, 1 ml 500 mM sucrose, 1 ml 170 mM citric acid, 100 μl 500 mM EDTA, 50 μl 500 mM DTT, 100 μl 100 mM PMSF, dissolved in 7.25 ml ddH_2O) for 2 h. 0.1 M sucrose solution was dropped on the slides and the fetal ovaries were cut into small pieces to release the oocytes in it. The samples were fixed in 1% paraformaldehyde overnight. After dried, the slides were blocked in ADB (goat serum 0.1 ml, BSA 0.03 g, Triton-X-100 0.5 μl, dissolved in 10 ml TBS) for 30 min at 37 °C and incubated with rabbit anti-SCP3 polyclonal antibody (NOVUS, NB300–232, Colorado, USA) at a dilution of 1:150 in ADB at 37 °C for 6–8 h. The next day, the slides were rinsed with TBS and incubated with Cy3-labeled goat anti-rabbit secondary antibody at a dilution of 1:50 (Beyotime, A0516, Nantong, China) in ADB for 2 h at 37 °C, followed by incubation with 1 μg/ml Hoechst33342 (Beyotime) for 5 min at 37 °C. Finally, Vectashield (H-1000; Vector, Shanghai, China) was used to seal the covers (Zhang et al., 2012). After staining, the percentages of leptotene, zygotene, pachytene and diplotene stages were calculated to determine the effect of ZEA exposure on meiosis prophase I using the inverted fluorescence microscope. Total 500 germ cells were visually inspected to analyze the different period of chromosomes in meiosis prophase I.

2.3. DNA double-strand breaks and repair

Experimental procedure was same to immunofluorescence staining of SCs. The distributions of γ-H2AX, RAD51 and MLH1 on chromosome were performed by co-localization using the according primary antibodies (γ-H2AX: Abcam, ab26350; RAD51: Abcam, ab133534; MLH1: Abcam, ab92312) respectively with the SCP3 polyclonal antibody to reveal DNA-DSBs and repair. FITC-labeled goat anti-rabbit (Beyotime, A0562) and FITC-labeled goat anti-mouse (Beyotime, A056) were used as secondary antibody. The counting method was same with that of the SC analysis.

2.4. RNA extraction and quantitative RT-PCR

Total RNA was extracted from fetal ovaries at 15.5 dpc and 17.5 dpc using the RNAprep pure Micro Kit (Aidlab, RN28, Beijing, China) according to the manufacturer's instructions. Reverse transcription was performed using the TransScript® One-Step gDNA Removal Kit and cDNA Synthesis Kit (TransGen Biotech, AT311, Beijing, China). Quantitative real-time PCR (qRT-PCR) was carried out using the Light-Cycler® 480 SYBR Green I Master Kit (Roche, 04887352001) with a Roche real time PCR instrument (Roche LC480, Germany) according to the manufacturer's instructions (Li et al., 2016). Primers sequences used for qRT-PCR were listed in Table 1. PCR reactions were initiated at 95 °C for 10 min, followed by denaturing at 95 °C for 10 s, annealing at 60 °C for 30 s and 72 °C for 10 s. After 40 cycles there was a cooling step at 4 °C. Mouse vasa homolog (*Mvh*) was used to normalize the gene expression levels (Chen et al., 2013). Relative transcript level of target gene expression was calculated using the $2^{-\Delta CT}$ method (Zhang et al., 2014). Data represented the mean ± SD (standard deviation) of at least three independent experiments.

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