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Toxic effects of zearalenone and α-zearalenol on the regulation of steroidogenesis and testosterone production in mouse Leydig cells

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Abstract

Zearalenone (ZEA) and its derivative α-zearalenol (α-ZOL) are produced by fungi of the genus Fusarium and, after ingestion via contaminated cereals, may lead to animal fertility disturbances and other reproductive pathologies. The previous study demonstrated the toxic effects of ZEA and α-ZOL through disturbances in male fertility and other reproductive pathologies in mice. In this study, we further examined the direct biological effects of ZEA and α-ZOL on steroidogenesis production, primarily in Leydig cells of mice. Mature mouse Leydig cells were purified by Percoll gradient centrifugation and the cell purity was determined by 3β-hydroxysteroid dehydrogenase (3β-HSD) staining. To examine ZEA and α-ZOL-induced biological consequences, we measured testosterone secretion and transcription level of 3 key steroidogenic enzymes including 3β-HSD-1, P450scc and StAR, in ZEA and α-ZOL/human chorionicgonadotropin (hCG) co-treated cells. Our results showed that ZEA and α-ZOL (10^{-4} M, 10^{-6} M and 10^{-8} M) significantly suppressed hCG (10 ng/ml)-induced testosterone secretion. The suppressive effect is correlated with a decrease in the level of transcription of 3β-HSD-1, P450scc, and StAR ($P < 0.05$).

Keywords: ZEA; α-ZOL; 3β-HSD-1; p450scc; StAR; Mouse Leydig cells

1. Introduction

Zearalenone (ZEA) and its derivative, α-zearalenol (α-ZOL), are a family of phenolic compounds produced by several species of Fusarium (*F. graminearum*, *F. culmorum*, *F. crookwellense*, *F. sambucinum* and *F. equiseti*), which can infect many important crops such as corn, wheat, sorghum, barley, oats, sesame seed, hay and corn silage (Manka et al., 1985; D'Mello, 1997). Several studies carried out in Europe and a number of transcontinental countries have reported a high incidence of ZEA in cereals and in animal feed (Bottalico et al., 1989; Muller et al., 1998; Scudamore et al., 1998; Scudamore and Patel, 2000).

ZEA binds to estrogen receptors (ERs) in vitro, with similar affinity for both forms: ERa and ERβ (Kuiper et al., 1998). Despite having a lower affinity for estrogen receptors than 17β-estradiol (100–1000 times less), ZEA and α-ZOL act through ERs (Kiang et al., 1978; Nikov et al., 2000) to activate transcription of estrogen-responsive genes in vivo (Gray et al., 1985; Katzenellenbogen et al., 1979; Mehmood et al., 2000) and in vitro (Kuiper et al., 1998; Mayr, 1988), as well as promoting undesirable estrogenic effects.

These fungal toxins have been associated with hyperestrogenism and other reproductive disorders in swine. In sows, a series of reproductive disorders may occur at greater levels of ZEA in feed (50–100 mg/g feed), including the induction of vulvovaginitis, vaginal and rectal prolapses, delayed onset of the first estrus, infertility characterized by continuous estrus, pseudo-pregnancy, ovarian

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abnormalities and pregnancy loss (Osweiler, 1986). The fertility-inhibiting effect associated with long-term consumption of ZEA-infected maize, has been studied in adult male albino rats. The fertility rate was further reduced by 25–30% if the animals were kept on a contaminated diet up to 14 weeks (Ruzsas et al., 1979). There was a tendency for boars, fed 9 ppm dietary ZEA, to produce lower total and gel free volumes of semen, with lower total motile sperm (Young and King, 1986). ZOL may reduce aggressive behavior, testes growth, and sexual activity in farmed fallow bucks by ear implants at a dose of 36 mg at 90 d intervals (Wilson et al., 2002). However, these reproductive toxicities of ZEA and α-ZOL for testicular function have mostly relied on the in vivo approach of using animal models. Complications in pharmacokinetic distribution and secondary effects attributed to other unidentified factors may make it difficult to decipher the direct mechanistic toxicities of ZEA and α-ZOL to the cells. Therefore, it is necessary to adopt cell models to determine direct biological effects of ZEA and α-ZOL to validate the in vivo findings.

It is well known that Leydig cells play a crucial role in synthesizing testosterone and regulating the process of spermatogenesis. Alteration of Leydig cell function can lead to adverse effects on testicular functions. In this study, the aim was to elucidate the effects of ZEA and α-ZOL on the process of steroidogenesis and testosterone secretion in Leydig cells of mice. To determine the mechanistic activities, ZEA or α-ZOL-stimulated mRNA of the key steroidogenic enzymes and the level of testosterone secretion were measured.

2. Materials and methods

All experimental animal use and experimental design for this study was approved by the Chinese Association for Laboratory Animal Sciences.

2.1. Cell culture

Leydig cells were isolated from testes of 60- to 90-day old Kunming mice. The cells were cultured for 2 days according to Biegel et al. (1995). The testes were decapsulated, digested in Erlenmyer flasks within an oscillating incubator (100 r.p.m and 34 °C) for 15 min by M199 medium containing 0.05% collagenase and 1% BSA. The suspension cells were transferred to a 50 ml tube and kept on ice for 2 min to allow the tubules to settle. The supernatant containing Leydig cells was filtered through a 70 μm nylon cell strainer (BD Biosciences). The cells were centrifuged at 350*g* for 20 min at 4 °C. The pellet was re-suspended in 10 ml M199 and loaded onto the top of Percoll gradient (5%, 30%, 58% and 70%) (Sigma) and centrifuged at 800*g* for 30 min at 4 °C. The cells in the third layer were collected, washed with M199 twice, and were re-suspended in phenol red-free DMEM/F12 (1:1) containing 10% charcoal stripped fetal calf serum (GeminiBio-Products, Woodland, CA, USA), 50 U/ml penicillin and 50 μg/ml streptomycin (GIBCO/BRL, Carlsbad, CA, USA). The cells were plated at a density of 10^5 cells/cm^2 in 24-well plates (Nunc, Nalge Nunc International, Rochester, NY, USA) at 0.5 ml/well and maintained at 37 ° C with 5% CO_2.

2.2. Histochemical staining of 3β-HSD and testosterone induction assay

Following 2 days of incubation, the purity of Leydig cells was examined by histochemical staining for 3β-hydroxysteroid dehydrogenase, according to the histochemical method of Mendelson et al with some modifications (Mendelson et al., 1975). In brief, Leydig cells were incubated in a 24-well plate with 0.4 ml/well staining solution containing 0.05 M PBS, pH 7.4 supplemented with 0.2 mg/ml nitro-blue tetrazolium (Sigma Chemical Co.), 1 mg/ml NAD and 0.12 mg/ml dehydroepiandrosterone (Sigma Chemical Co) for 90 min at 34 °C. The positive cells were stained a dark blue color and the purity of the Leydig cells was observed to be over 90%. Secondly, in the testosterone induction assay, Leydig cells were exposed to 10 ng/ml human chorionic gonadotrop (hCG) (Sigma Chemical Co) for 24 h.

2.3. Cell treatment

Two-day cultured Leydig cells grown in phenol red-free DMEM/F12 medium supplemented with 10% charcoal stripped fetal calf serum and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) were washed three times in 0.05 M PBS pH 7.4. The conditioned media (phenol red-free DMEM/F12 medium supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin and the corresponding leveled-drugs and 10 ng/ml hCG) were added to the 24-well plates at 0.3 ml/well. The Leydig cells were exposed for 24 h to one of the following four treatments: (i) 10 ng/ml hCG (Sigma) + dimethylsulfoxide (DMSO) (Sigma) solvent control; (ii) 10 ng/ml hCG + 10^{-4} M ZEA (Sigma, St. Louis, Mo.); (iii) 10 ng/ml hCG + 10^{-6} M ZEA; (iv) 10 ng/ml hCG + 10^{-8} M ZEA. Administration of α-ZOL was treated in the same manner. Cell viability was determined by the trypan blue dye-exclusion test, according to Kellokumpu (1987). The viability of the control and treated cells was over 90%. At the end of the incubation, the conditioned media were collected and stored at −20 °C until determination of testosterone concentration. The treated cells were used for the measurement of mRNA for steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage enzyme (P450scc) and 3β-HSD-1.

2.4. Total RNA extraction and semi-quantitative reverse transcription-polymerase chain reaction (PCR)

Total RNA was extracted from different treatment groups using Trizol reagent (Invitrogen, Carlsbad, CA, USA) followed by deoxyribonuclease I (Life Technologies, Inc.) treatment to remove DNA contamination. One