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Effect of zearalenone on reproductive parameters and expression of selected testicular genes in mice

E. Zatecka[a], L. Ded[a], F. Elzeinova[a], A. Kubatova[a], A. Dorosh[a], H. Margaryan[a], P. Dostalova[a], V. Korenkova[b], K. Hoskova[c], J. Peknicova[a,*]

[a] Laboratory of Reproductive Biology, Institute of Biotechnology, Academy of Sciences of the Czech Republic, v. v. i., Prague, Czech Republic
[b] Laboratory of Gene Expression, Institute of Biotechnology, Academy of Sciences of the Czech Republic, v. v. i., Prague, Czech Republic
[c] Department of Veterinary Sciences, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences in Prague, Prague, Czech Republic

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ABSTRACT

We tested the effect of two different concentrations (150 μg/l and 0.15 μg/l) of mycotoxin zearalenone (ZEA) on the reproductive parameters and expression of testicular genes in male mice. In adult males, no reduction of body or reproductive organ weight was observed, and the seminiferous tubules were morphologically normal with ongoing spermatogenesis. However, we found decreased sperm concentration, increase of morphologically abnormal spermatozoa and increased binding of apoptotic marker annexin V. This study was also focused on the evaluation of gene expression profiles of 28 genes playing important roles during the processes occurring in the testicular tissue. We detected changes in the expression of genes important for proper spermatogenesis. Surprisingly, we observed a stronger effect after exposure to the lower dose of ZEA.

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1. Introduction

Zearalenone (ZEA, F-2 toxin) is a nonsteroidal oestrogenic mycotoxin produced by a variety of *Fusarium* fungi, which are common contaminants of cereal crops worldwide [1]. ZEA is commonly found in maize or corns with the highest concentration in wheat, bran, corn and their products (*e.g.* corn flakes). ZEA is mainly a field contaminant; however the toxin production can also occur during storage in poor conditions [2]. Also, it has been shown that ZEA is transported from the fields to the aquatic systems by rain water [3].

The concentrations in food and feed vary over a wide range, depending on climatic conditions. Considering the mean levels of ZEA in the principal foods and their consumption, the average human daily intakes of ZEA range from 2.4 to 29 ng/kg b.w./day in adults, while toddlers (12–36 months old) have the highest average daily intakes ranging from 9.3 to 100 ng/kg b.w./day [2]. It has been shown that ZEA can also be excreted into cow milk [4].

ZEA is rapidly absorbed after oral administration. Its uptake is estimated to be approximately 80–85%, but it is difficult to measure owing to extensive biliary excretion. ZEA and its derivatives are detected in blood about 30 min after oral administration bound to human globulins as reproductive hormones [5,6]. Studies with radiolabelled zearalenone in mice showed that it is distributed to oestrogen target tissues such as uterus, interstitial cells of the testes and ovarian follicles. Some radiolabels were also found in adipose tissues, indicating that storage in adipose tissue may take place [7].

The main effect of zearalenone results from its oestrogenic activity. ZEA and its derivatives – α-zearanenol (α-ZOL) and β-zearalenol (β-ZOL) – compete with 17β-estradiol (E2) for the specific binding sites of oestrogen receptors (ERs). Several investigations have demonstrated that binding of ZEA and its derivatives initiates a sequence of events known to follow oestrogen stimulation [7]. Efficiency of binding of ZEA to ER in target tissues is <1–10% than that of E2, whereas α-ZOL shows stronger binding and β-ZOL lower affinity to ER [8]. The specific manifestations of ZEA are dependent upon the species, relative dose, and life stage during which ZEA is consumed. The most sensitive species is the pig; however it has been shown that ZEA can also have adverse effects on other species including rodents.

A study by Yang et al. [9] has shown that ZEA and α-ZOL affect steroidogenesis in mature mouse Leyidig cells *in vitro*. During this study authors observed a decrease of testosterone production in cells co-treated with ZEA or α-ZOL and human chorionic gonadotropin (hCG). They also detected decreased

* Corresponding author at: Laboratory of Reproductive Biology, Institute of Biotechnology, Academy of Sciences of the Czech Republic, v. v. i., 142 20 Prague 4, Czech Republic. Tel.: +420 241 062 642; fax: +420 244 471 707.
E-mail address: jana.peknicova@img.cas.cz (J. Peknicova).

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expression of 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD-1), cytochrome P450 side chain cleavage enzyme (P450scc) and steroidogenic acute regulatory protein (StAR), which play a crucial role during steroidogenesis. In adult animals testosterone is critical for proper spermatogenesis and sperm maturation, and disruption of spermatogenesis can thus adversely affect male fertility.

The negative effect of ZEA on reproductive parameters can also be observed *in vivo*. In another study of Yang et al. [10], adult male mice were exposed to intraperitoneal injections of ZEA or α-ZOL at the concentration 0, 25, 50 or 75 mg/kg b.w. daily for 7 days. In all groups the authors observed a significantly increased number of abnormal spermatozoa and significantly decreased number of live spermatozoa. Testicular and cauda epididymal sperm counts were also reduced, as well as serum testosterone. These effects were observed in the treated males at all doses in a dose-dependent manner. Besides the decrease in sperm quality, a significantly low pregnancy rate was observed when untreated females were mated with the treated males. At high concentrations (50 and 75 mg/kg b.w.), authors noticed a decrease of b.w. and increase of relative seminal vesicle weight.

To show whether the action of ZEA includes induction of apoptosis of testicular cells, Kim et al. [11] performed an *in vivo* study in rats. During this study 10-week-old male rats were exposed to a single intraperitoneal dose of ZEA (5 mg/kg b.w.) and analyzed at 3, 6, 12, 24, or 48 h after exposure. Germ cell degeneration caused by apoptosis was observed at stages I–VI of spermatogenesis 12 h after the exposure. The frequency of TUNEL-labelled germ cells increased in a stage-specific manner, with gradually increasing frequency at stages I–VI of seminiferous tubules with the time after exposure. These results show that a single dose of ZEA induces testicular germ cell apoptosis in a time-dependent and stage-specific manner in the rat testis *in vivo*. The induction of apoptosis in testicular tissue after ZEA treatment was also shown by Yuan et al. [12]; these authors have additionally shown that traditional medicinal plant *Gynostemma pentaphyllum* protects against toxicity caused by ZEA through anti-oxidation and anti-apoptosis effects mediated by the regulation of Bax and Bcl-2 expression.

Filipiak et al. [13] performed an *in vivo* study of pubertal rats in which they investigated the effect of two xenoestrogens, diethylstilbestrol (DES) and ZEA, with comparison of their effect with natural oestrogen 17β-estradiol (E2). While E2 and DES significantly reduced the numbers of spermatogonia, spermatocytes and Sertoli cells, ZEA only reduced the numbers of spermatogonia and Sertoli cells. The authors also measured the testis weight and seminiferous tubule diameter and length, which were significantly decreased by all three substances. In general, ZEA appeared to be the weakest of the three oestrogenic substances.

The aim of the present study was to assess the effect of treatment with a low dose of mycotoxin zearalenone on the male gonadal pathology, sperm quality and expression of selected genes. We have analyzed a wide range of genes expressed in the testes. For this purpose we selected genes playing important roles during spermatogenesis, genes expressed in Sertoli cells, and genes playing a role in apoptosis and hormonal response. We assume that analysis of these genes can reveal how ZEA affects germ cell development and subsequently the semen quality in mice.

2. Materials and methods

2.1. Animals and treatment, number of progeny, sex ratio

For our experiment we used the CD1 outbred mice strain (An Lab, Prague, Czech Republic) with high heterozygosity and average litter size (12–13 pups/litter). Mice (experimental and control groups) were kept under standard experimental conditions (constant temperature and 12-h light regime) in the animal facility of the Institute of Molecular Genetics, v. v. i., Prague. Animals were fed on soy-free feed (Ssniff, Soest, Germany). The diet and water were administered *ad libitum* and all stress factors were reduced to a minimum. Experimental groups were treated with different concentrations of ZEA (Sigma, Prague, Czech Republic), which was dissolved in drinking water. In this *in vivo* experiment there were two experimental groups – a group exposed to higher concentration of ZEA (150 μg/l), and a second group exposed to lower concentration of ZEA (0.15 μg/l). Animals exposed to the low dose were exposed to an environmentally relevant concentration (around 25 ng/kg b.w.) and animals exposed to the high dose were exposed to 1000 times higher concentration. ZEA was administered starting from the first day of mothers' pregnancy, and the number of progeny and the sex ratio were evaluated. The born pups were exposed during gestation, lactation, pre-pubertal and pubertal period, and up to the age of 70 days, when they were sacrificed and subjected to analysis. In each group, 18 animals were analyzed.

2.2. Anogenital distance, body and organ weight

Animals in the control and experimental groups were killed at the same age of 70 days. Anogenital distance (AGD) and body weight were measured and subsequently the reproductive organs were dissected and weighed. Left and right testes, epididymis, seminal vesicles and prostate were separated, cleaned and weighed individually. Immediately after weighing the left testes were frozen in liquid nitrogen and stored at −70 °C for further analysis; the right testes were used for histological analysis and epidymides were placed into warmed (37 °C) PBS for sperm release (see below).

2.3. Preparation of cells

Mouse sperm were obtained from the cauda epididymis. Spermatozoa were left to release spontaneously into 1 ml of warmed PBS at 37 °C in a CO_2 incubator for 15 min. Then the cell suspension was transferred into a new tube and PBS was added to 1 ml final volume. The concentration of spermatozoa was evaluated in a haemocytometer chamber under 100× magnification. Part of the epididymal spermatozoa was used for assessing viability, morphology and apoptotic stage. The rest of the suspension was washed twice in PBS, centrifuged for 15 min at 200 × *g* and dropped onto glass slides for immunocytochemical analysis.

2.4. Sperm viability

To determine the viability of epididymal spermatozoa a Live/Dead sperm viability kit was used (Invitrogen, Eugene, USA); the laboratory manual had to be slightly modified to enable its use for mouse sperm. First, the cell suspension was centrifuged for 5 min at 200 × *g*, then 1 μl of cell pellet was mixed with 200 μl of HEPES buffer and 1 μl 50× diluted SYBR 14 (in HEPES), and the mixture was incubated for 5 min at 37 °C in the dark. Subsequently, the mixture was centrifuged for 5 min at 200 × *g*. The supernatant was removed and the cell pellet was mixed with 200 μl of HEPES buffer and 1 μl propidium iodide (PI) and centrifuged for 5 min at 200 × *g*. The supernatant was removed and 20 μl of PBS was added, then 10 μl of the suspension was placed onto a glass slide and evaluated under a Nikon Eclipse E400 fluorescence microscope using a 40× Nikon Plan Fluor 40/0.75 (Nikon, Prague, Czech Republic). Green (SYBR 14)-labelled spermatozoa were alive; orange-labelled (PI) spermatozoa were dead.

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