Effects of mycotoxins on hormone production in primary Leydig cells isolated from pigs

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Mycotoxins are toxins produced by fungi in many agricultural products worldwide either pre- or post-harvesting. Fusarium species are among the most researched plant pathogenic fungi (1) that produce a number of mycotoxins including DON, NIV, T-2, HT-2, fumonisins, ZEN and its metabolites α- and β-zearalenol (2). Some fungi are able to produce more than one mycotoxin, but also multiple fungi can contaminate the same crop (3). Natural co-occurrence of Fusarium mycotoxins increases the concern on the exposure to mixtures of mycotoxins e.g. co-occurrence of DON/ ZEN/ T-2/ HT-2 (4,5).

Exposure to mycotoxins can result in a variety of health effects, ranging from acute toxic response to potential long-term carcinogenic and teratogenic effects (6). Effects of mycotoxins on reproduction in livestock have been reviewed extensively (7). ZEN ingestion in animals is associated with anestrus, abortion, increased embryonic and fetal death, increased stillbirths, reduced milk production, hyperestrogenism and poor quality semen (7,8) *In vitro,* ZEN increased the progesterone production in porcine granulosa cells (9). ZEN and α-ZOL have potent estrogenic effects and promote hormone production in H295R cells (10). DON, T-2 and HT-2 reduced cell viability, inhibit steroidogenesis and alter expression of steroidogenic genes in human adrenocarcinoma (H295R) cells (11)

Leydig cells are the testicular endocrine cells capable of producing steroid hormones. Pig Leydig cell culture is a good *in vitro* model to study steroidogenesis and screen effects of some chemicals (12,13). We hypothesize that *F. graminearum* culture extracts contain a mixture of naturally co-occurring mycotoxins that are able to cause deleterious effects *in vitro* in Leydig cells.

Methodology

Fusarium graminearum extracts: Fungal isolates from grain samples collected from different farms in Norway were cultured in the laboratory at the Norwegian Veterinary Institute. The fungal cultures were extracted in methanol: water, 9:1, microfiltered through hydrophobic filter (0.2 μm, Minisart) and aliquots stored at -20°C. Crude fungal extracts were used in cultured cells. The extracts were later screened for bioactivity and the bioactive components in the extracts identified (data not included here).

Primary Leydig cells were acquired from 8-10 day old piglets after routine castration. The testicles were kept in cold cell culture medium (DMEM/F12) with 2% Penicillin/Streptomycin/Neomycin (Invitrogen, Paisley, UK) and transported in a cool box from the farm to the laboratory (maximum 2h). Testicles were enzymatically digested by collagenase dispase and Leydig cells isolated as previously described (13).

A 1 ml suspension of cells at $3x10^5$ cells/ml concentrations was added into each well of 24-well cell culture plates and the plates incubated at 34°C, 5% CO_2 for 64 h for attachment. DMEM/F12 medium was supplemented with 1% ITS+ Premix (Invitrogen) 2.5% NuSerum (BD Bioscience) and 2% PSN (Invitrogen). The cells were exposed for 48 h in fresh medium with or without different concentrations of *F. graminearum* extracts (1/10,000,000-1/1,000) or solvent control, 0.1% MeOH. In LH stimulated cells, assay media was spiked with 0.5 ng/ml recombinant porcine luteinizing hormone (tuenre.pLH.ig; Tucker Endocrine Research Institute.

AlamarBlue™ assay was used to evaluate the cell viability in both unstimulated and LH stimulated Leydig cells.

Estradiol and testosterone hormones were quantified by solid phase radioimmunoassay Coat-a-Count RIA kits (Diagnostic Products Corporation, Los Angeles, USA) following manufacturer's instructions except for the standards that were prepared in cell culture medium used for the exposures.

Results

There was no significant effect in viability of unstimulated cells exposed to extract 087/2008 but a reduction was observed in the cells exposed to the highest concentration of extract 067/2007. In LH stimulated cells, 087/2008 showed a reduced viability in all the concentrations compared to the control. However the viability did not go below 90%. The highest concentration of 067/2007 reduced cell viability to 80% in the LH stimulated cells.

Exposure of unstimulated Leydig cells to extract 067/2007 did not affect hormone production. However, in LH stimulated cell, the extract was able to reduce both estradiol and testosterone levels significantly at the highest extract concentration (Fig 1). Extract 087/2008 increased testosterone production in unstimulated cells especially the two highest concentrations, but caused a reduction in estradiol with increasing extract concentration. In LH stimulated cell, highest concentration of 087/2008 extract reduced the levels of testosterone in media (Fig 2).

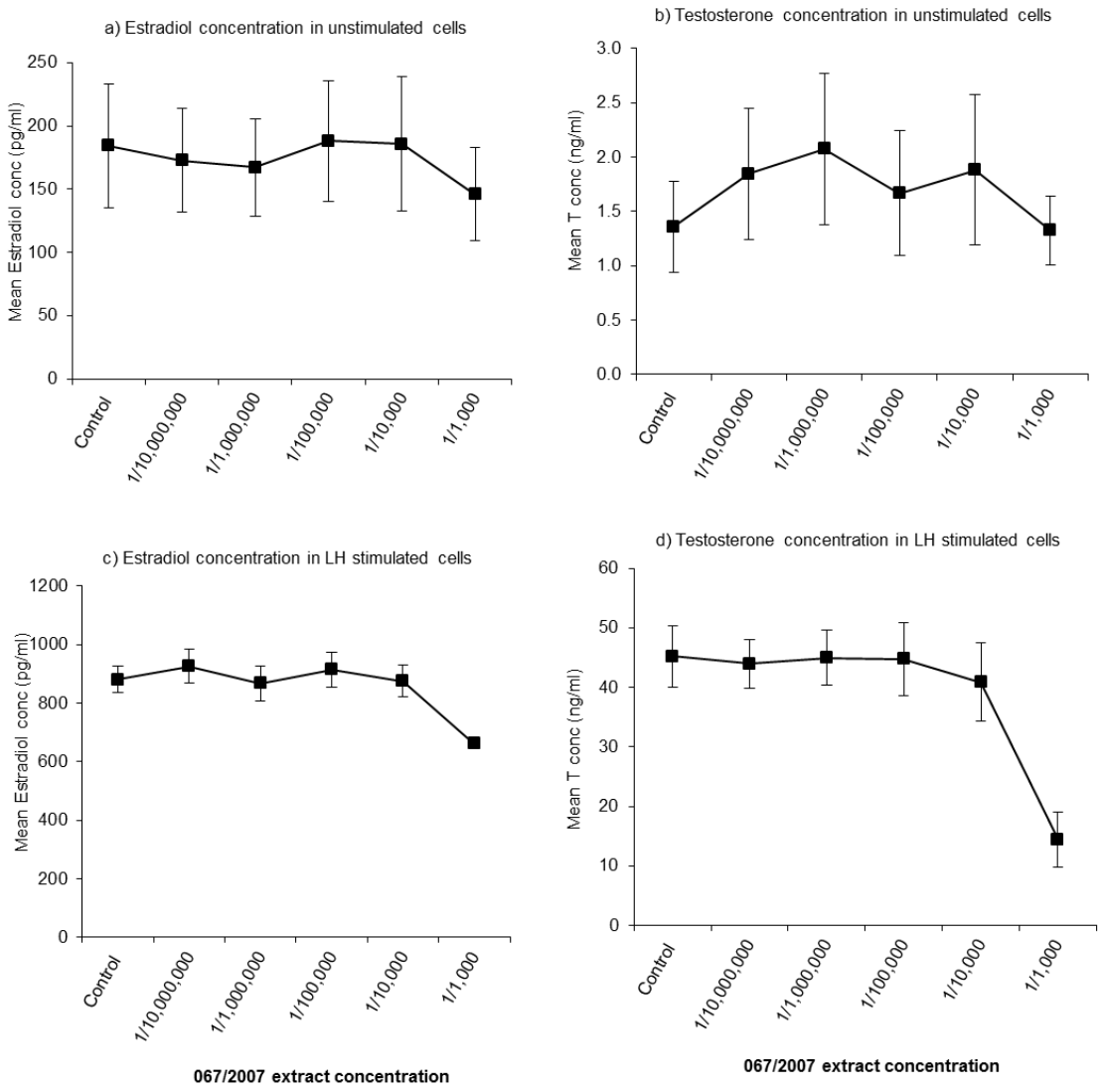

Fig. 1. Dose response of F. graminearum extract 067/2007 on estradiol production in unstimulated (a) and LH stimulated (c) and testosterone in unstimulated (b) and LH stimulated (d) Leydig cells. Values are means from three separate experiments (n = 9) ± SEM.

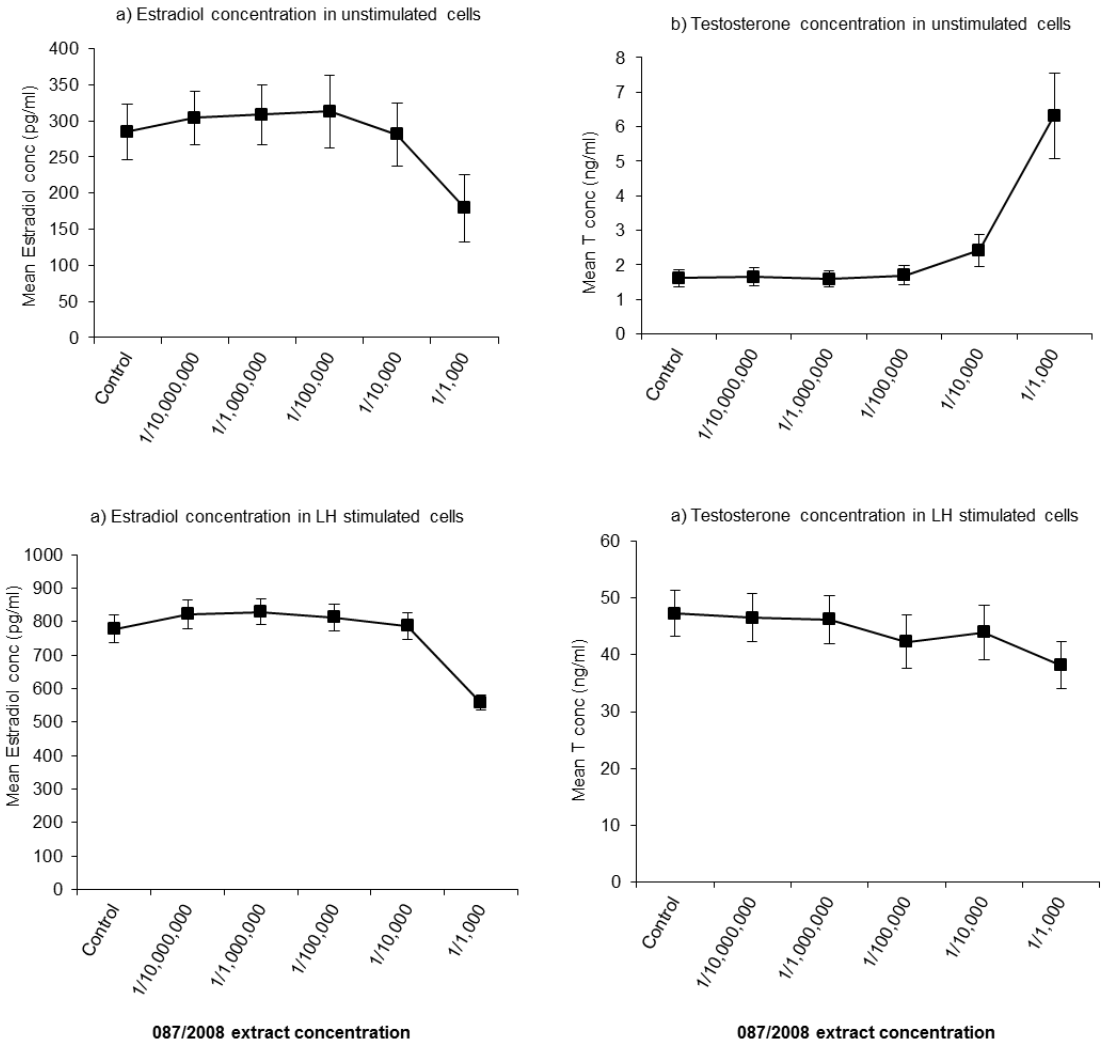

Fig. 2. Dose response of F. graminearum extract 087/2008 on estradiol production in unstimulated (a) and LH stimulated (c) and testosterone in unstimulated (b) and LH stimulated (d) Leydig cells. Values are means from three separate experiments (n = 9) ± SEM.

Discussion

Occurrence of natural mixtures of mycotoxins is common and increases a concern on the exposure to mixtures of mycotoxins since combinations of toxins consumed in food/ feeds could either have antagonistic, additive or synergistic effects. Understanding how mycotoxins affect normal cellular processes will theoretically predict what combinations of mycotoxins would have the greater chances in causing adverse effects on an individual (14).

Leydig cells are the main producers of androgens and estrogen in male individuals. Androgens produced by fetal testes induce the development of male genital tract. Estrogens are equally important in males since they play some important roles including a negative feedback

regulation on FSH secretion (15), masculinization of the brain and maintenance of male sexual behaviour in adults (16).

In the present study, highest concentration of *F. graminearum* extract 067/2007 had strong cytotoxic effect on the cells where it also caused a significant hormone reduction in LH stimulated cells but not in unstimulated cells. *F. graminearum* extract 087/2008 on the other hand did not affect viability of the cells but caused increasing testosterone and reduced estradiol production in unstimulated cells at the highest extract concentration. In LH stimulated cells, both hormones were reduced in cells exposed to highest concentration of 087/2008 extract. In man, estrogen deficiency leads to hypergonadotropism and increased testosterone levels (17). Pathological increase in estrogen in males can affect spermatogenesis (18).

Exposure to mixtures of mycotoxins has been reported to have a greater negative impact than the effect of single mycotoxins on livestock health and productivity (19).Tajima et al. (2002) studied the joint effect of Fusarium mycotoxins (T-2, DON, NIV, ZEN and FB1) and observed that at the highest doses, the mixture had a less than additive effect of the mycotoxins compared to the effects of the individual compounds. At lower dose levels however the mycotoxins mixture effect was additive. The authors concluded after a series of tiered bioassays, that several classes of mycotoxins when present simultaneously in a mixture might show interaction (20).

At some stage, producers or grain brokers blend mycotoxin-contaminated grain with clean grain in proportions that the animals would consume without obvious adverse effects on growth or reproduction (7). However, low concentration of several mycotoxins may interact and cause effects at concentrations below the detection limit (8). In vivo, the toxicokinetic behavior, metabolism and the toxicodynamic factors influence the final outcome of exposure to mixture of mycotoxins (21).

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