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Mycotoxins and female reproduction: *in vitro* approaches

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REVIEW PAPER

Abstract

Exposure to mycotoxins has been linked to adverse effects on female reproduction by interfering with the synthesis, metabolism or degradation of steroid hormones, interaction with steroid receptors or impairing oocyte maturation and competence. To assess such effects, many studies initially focussed on possible endocrine actions of mycotoxins using specific cell lines known to express key enzymes involved in the synthesis of steroid hormones. Using these models, zearalenone, deoxynivalenol, ochratoxin A, T-2 and HT-2 toxins, and aflatoxin B_1 were claimed to be endocrine active substances. As yet, zearalenone is the only mycotoxin for which a direct interaction with oestrogen receptors could be demonstrated, classifying this mycotoxin as an endocrine disruptor. Mycotoxin exposure of complex cell systems like ovarian follicles at the earliest (primordial) to most advanced (pre-ovulatory) stages can serve not only as the first indication of the potential of a mycotoxin to affect female reproduction, but also provides insight in specific mechanisms involved in such an effect and identifies vulnerable phases in follicle development. Zearalenone is the most widely studied mycotoxin regarding female reproduction, but effects on oocyte maturation have also been demonstrated for deoxynivalenol. Exposure to zearalenone impairs the formation of primordial, while its metabolite α-zearalenol is more harmful to fertilised oocytes than zearalenone itself. This short overview aims to provide an introduction into the different models, such as cell lines and oocytes, commonly used to assess the potential adverse effects of mycotoxins on female reproduction.

Keywords: oocytes, ovarian follicles, endocrine active mycotoxins

1. Introduction

Mycotoxins are fungal, low-molecular weight secondary metabolites that occur worldwide and adversely affect human and animal health and, in some cases, reproduction (Fink-Gremmels, 1999). One of the most prominent mycotoxins with a role in the reproductive status of humans and animals is zearalenone (ZEA), a mycotoxin with strong affinity to oestrogen receptors. ZEA has been described as endocrine disruptor, as it not only binds to both classes of oestrogen receptors, but also interacts with hydroxysteroid dehydrogenases (HSDs) involved in the synthesis of endogenous hormones (Fink-Gremmels and Malekinejad, 2007; Frizzell *et al.*, 2011; Minervini and Dell'Aquila, 2008; Schoevers *et al.*, 2012; Tiemann and Dänicke, 2007). According to the current definitions, an endocrine disruptor is a natural or synthetic chemical substance that exerts endocrine activity and for which a plausible link between endocrine activity and adverse effects have been demonstrated in animals and/or humans. In contrast, endocrine active substances are substances that may interact with any component of the endocrine system, as demonstrated in cells that are able to synthesise steroid hormones under culture conditions. However, such (temporary) changes do not necessarily indicate an adverse effect under *in vivo* conditions, where compensatory regulatory and feed-back mechanisms control the hormonal homeostasis (EFSA, 2013). In terms of risk identification and risk assessment these differences are of major importance in the translation of *in vitro* findings obtained in isolated cells or subcellular (enzyme) fractions to the potential risk of adverse effects in the living organism.

An alternative approach to assess potential adverse effects of mycotoxins on female reproduction is the use of oocytes, obtained from the ovaries of different animal species, in particular farm animals. Oocytes are the female gametes enclosed in primordial, primary and secondary follicles or in antral follicles, which can be cultured under *in vitro* conditions. Each follicle developmental stage is regulated by different autocrine, paracrine, juxtacrine and endocrine factors and any effect of mycotoxins on these developmental stages are indicative for follicle losses and infertility. Using hormone and growth factors enriched culture media, the growth of immature oocytes from antral follicles and oocyte maturation can be observed *in vitro*. Impairment of antral follicle development is likely to results in a subfertile status in mature animals. Finally, using *in vitro* fertilisation adverse effects of mycotoxin and other chemicals on oocyte and early embryonic development can be identified. The aim of this brief review is to provide a summary of the different models used in the assessment of potential adverse effects of mycotoxins on female reproduction. To facilitate the translation of the effect of mycotoxins on steroid receptors, steroid synthesis and oocyte maturation to reproductive phenotypes, a brief introduction on the organisation and hormone synthesis of the mammalian ovary is provided.

2. Physiological traits of the mammalian ovary and synthesis of steroid hormones

The mammalian ovary has two major functions: the production of gametes (oocytes) and the synthesis of steroids and peptide growth factors (for review, see Edson *et al.*, 2009). The ovary contains thousands of oocytes enclosed in primordial follicles. In large mammals, these follicles are formed already during the early embryogenesis and represent the reserve of oocytes of the female individual throughout her entire reproductive life (Van den Hurk and Zhao, 2005).

Morphologically, ovarian follicles are classified as preantral or antral according to the absence or presence, respectively, of a follicular fluid-filled cavity denominated antrum (Figure 1). Preantral follicles are characterised by granulosa cells surrounding an immature oocyte and can be divided according to their developmental stage as primordial, primary or secondary follicles. Primordial follicles are defined as follicles containing an oocyte surrounded by one layer of flattened pre-granulosa cells. After activation of the arrested primordial follicles, i.e. transition from primordial to primary stage, the immature oocytes become surrounded by one layer of cuboidal granulosa cells. Sequestration of the theca cells from the surrounding stroma characterises the secondary follicle, the last stage of the preantral follicle growth. Impairment of this cascade of events by xenobiotics results in complete infertility.

Antral follicles are characterised by oocytes that are increasing in size and by the presence of a fluid-filled cavity (antrum) (Figure 1). Depending on enclosure of an immature or mature oocyte they are denominated as tertiary or pre-ovulatory follicles, respectively. The follicular fluid is derived from thecal blood vessels and contains metabolic products secreted by granulosa cells, for example the gonadotrophins (follicle-stimulating hormone and luteinizing hormone (LH)), steroids (progesterone, 17α-hydroxyprogesterone, androstenedione, and oestrogens such as estrone and oestradiol), together with various carbohydrates, mucopolysaccharides, lipids, proteins (albumin, α1-glycoprotein, α2-macroglobulin, b1A-globulin, immunoglobulin A (IgA), IgG, IgM and transferrin) and enzymes (hyaluronidases, endopeptidases and collagenases) (Edwards, 1974).

Many *in vitro* models focus particularly on the steroid synthesis in granulosa cells to identify endocrine active effects, which may result in suboptimal fertility. Under the influence of the hormones and substrates secreted by granulosa cells, oocytes grow to their maximum size and the surrounding connective tissue differentiates into a theca interna and externa. However, the oocyte remains immature, i.e. at the germinal vesicle stage with the nucleus arrested at prophase I of the first meiotic division. In response to the LH surge, a dynamic process involving oocyte cytoplasm and nuclear maturation is initiated and oocytes resume meiosis characterised by germinal vesicle breakdown and the assembly of the metaphase II spindle together with extrusion of the first polar body (for a detailed overview, see Li and Albertini, 2013). The following cytoplasmic maturation comprises cumulus expansion, cell organelle reorganisation, and storage of mRNA molecules and proteins that support fertilisation and early embryo

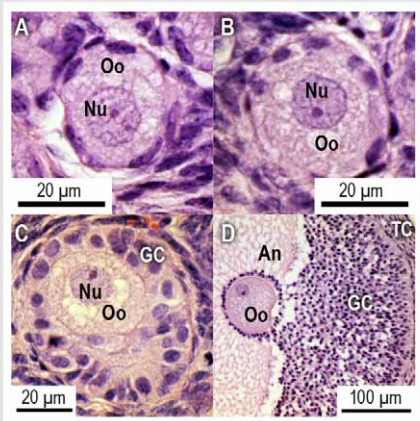

Figure 1. Representative images of (A) porcine primordial, (B) primary, (C) initial secondary and (D) antral follicles. Nu = nucleus; Oo = oocyte; CG = granulosa cells; TC = theca cells; An = antrum.

development (Ferreira *et al.*, 2009; Nagyova, 2012). At this stage, mycotoxin exposure can result in impairment of individual stages, as for example negatively affecting oocyte cumulus expansion, which leads to fertilisation failures or even direct embryo toxicity. Individual mycotoxins can impair one or more steps in follicle development, oocyte maturation, fertilisation and embryonic development.

3. Mycotoxins as endocrine active substances

Steroid hormones support the normal functioning of the ovary, including follicle development, oocyte maturation and ovulation. For a normal follicular recruitment and development, oestrogens, and their signalling through oestrogen receptors ERα and ERβ, are crucial, while progesterone and LH are involved in ovulation. This process of hormone synthesis predominantly takes place in the granulosa and theca cells (Figure 2). In brief, cholesterol is transported from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR), as the cytochrome P450 (CYP)11A, which is responsible for the conversion of cholesterol into pregnenolone, is located in the mitochondria. 3β-HSD, which is located in mitochondria and endoplasmic reticulum, converts pregnenolone into progesterone. Subsequently, the enzyme CYP17, located in the endoplasmic reticulum of theca cells, converts progesterone to dehydroepiandrosterone (DHEA) and 3β-HSD converts DHEA to androstenedione. Another aromatase, CYP19, converts androstenedione to estrone, which in turn will be converted to oestradiol by 17β-HSD (Jamnongjit and Hammes, 2006). Besides the activity of these enzymes, follicle development depends on the presence of various growth factors such as growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15, which are synthesised in the oocyte and act in the proliferation of granulosa cells. Conversely, kit ligand (KL) is secreted by the granulosa cells and promotes oocyte growth (Figure 2).

Various mycotoxins are known for their endocrine-like activity and have been identified as potential endocrine active substances. For example, aflatoxin B_1 (AFB_1) has been classified as a potential endocrine active substance, as it is metabolised into aflatoxicol in a human choriocarcinoma cell line (JEG-3 cells), resulting in the up-regulation of CYP19A1 expression after 96 h exposure to 1 μM AFB_1 (Storvik *et al.*, 2011). However, it remains unknown whether or not such an isolated effect on the activity of a single enzyme is able to adversely affect female reproduction. Other mycotoxins that may act as endocrine active substances are the trichothecenes deoxynivalenol (DON), T-2 and HT-2 toxins. Ndossi *et al.* (2012) have shown that exposure of human adrenocortical carcinoma cells (H295R) to these *Fusarium* toxins resulted in the up-regulation of CYP1A1 expression (DON, T-2 and HT-2 toxins), as well as that of CYP17, 3β-HSD, CYP11B1 and CYP11B2 (DON). However these changes seem to remain without any clinically significant hormonal imbalance.

In parallel, ochratoxin A (OTA) also interferes with steroidogenesis by increasing the oestradiol synthesis, as demonstrated in the cell line H295R (Frizzell *et al.*, 2013). By exposing JEG-3 cells to OTA, it has been demonstrated that this mycotoxin remarkably induces 3β-HSD (Woo *et al.*, 2013). Considering that placental transfer of OTA has already been observed in various animal species and in humans, these findings may have clinical relevance as they concern the exposure of early developmental stages of the ovary (Galtier, 1991; Minervini *et al.*, 2013; Woo *et al.*, 2012), while embryonic malformations have been

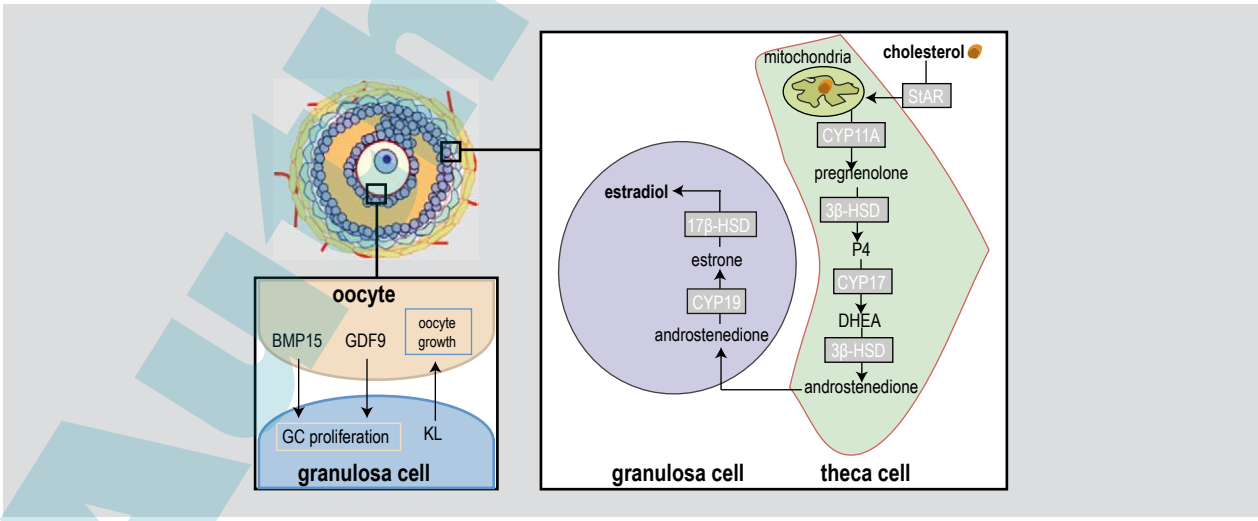

Figure 2. Factors that determine oocyte development and steroidogenesis in granulosa and theca cells. BMP15 = bone morphogenetic protein 15; CYP = cytochrome P450; DHEA = dehydroepiandrosterone; GC = granulosa cell; GDF9 = growth differentiation factor 9; HSD = hydroxysteroid dehydrogenases; KL = kit ligand; StAR = steroidogenic acute regulatory protein.

described only at higher concentrations (Napoletano *et al.*, 2010; Singh and Hood, 1985).

Also, alternariol (AOH) and alternariol monomethyl ether impair progesterone synthesis by granulosa cells (Tiemann *et al.*, 2009). As recently shown by Frizzell *et al.* (2013) when applying reporter gene assays, AOH has an antagonist effect on androgen, progestagen and glucocorticoid nuclear receptors, indicating the importance of further studies focussing on this mycotoxin. Further details on the individual experiments and the measured endpoints are presented in Table 1.

4. Mycotoxins as endocrine disruptors

As yet, only ZEA and some of its metabolites seem to fulfil the criteria of endocrine disruptors by acting as agonists/antagonists of oestrogen receptors and modulating the activity of enzymes involved in steroid synthesis. Already in 1978, Boyd and Wittliff described the binding characteristics of ZEA, α-zearalanol (α-ZOL) and α-zearalanal on free cytosolic receptors and nuclear oestrogen receptors. These data were confirmed and extended in recent experiments, in which ZEA showed a strong oestrogenic activity and activated ERα in human cervical epithelial cancer cells and human hepatocellular cancer cells (Li *et al.*, 2012). The same effect was demonstrated in Ishikawa human endometrial adenocarcinoma cells (Li *et al.*, 2012). The oestrogenic activity of ZEA, α-ZOL and β-zearalanol (β-ZOL) had previously been demonstrated in the human breast adenocarcinoma cell line and breast cancer cell line MDA-MB-231 (Malekinejad *et al.*, 2005; Minervini *et al.*, 2005). In addition, a deleterious effect of ZEA on equine granulosa cells has been described (Minervini *et al.*, 2006). ZEA is a substrate for 3α-HSD and 3β-HSD (Malekinejad *et al.*, 2005, 2006a,b; Olsen *et al.*, 1981). In turn, Frizzell *et al.* (2011) have demonstrated that ZEA and its metabolites can induce progesterone, oestradiol, testosterone, and cortisol production in the human adrenal gland-derived cell line H295R. This may be indirectly caused by an inhibition of apoptosis (Yu *et al.*, 2005) and therefore an increased cell proliferation (Khosrokhavar *et al.*, 2009).

Recently, a large number of proteomic changes associated with steroidogenesis have been identified (Busk *et al.*, 2011), including the impairment of the cellular phosphorylation pathways and mitochondrial dysfunction. Interestingly, when protein regulation by ZEA, α-ZOL and β-ZOL was compared, no common pattern of protein deregulation was observed, indicating that these three mycotoxins present different biological activity, as also indicated by the differences in binding activity and activation of oestrogen receptors (Busk *et al.*, 2012).

The endocrine disrupting capacity of ZEA is associated with clinical signs of an impaired reproduction in animals and humans. For example, pigs have been identified as the most sensitive animal species, and the oestrogen-like effects have been described in detail for the different age groups (for review, see Fink-Gremmels, 2008; Minervini and Dell'Aquila, 2008; Zinedine *et al.*, 2007). In humans, ZEA exposure has been linked to precocious puberty in girls (Massart and Saggese, 2010; Massart *et al.*, 2008), correlating with unusual high oestrogen levels. For example, Massart *et al.* (2008) have shown that pre-pubertal girls (<8 years old) exposed to ZEA presented oestradiol (E2) levels of 25 pg/ml.

5. Mycotoxins and oocyte maturation

One of the most prominent *ex vivo* models to study the potential effects of mycotoxins on female reproduction are oocytes. They can be cultured *in vitro* and their development can be followed closely in the presence and absence of mycotoxins, as mentioned above. Exposure to individual mycotoxins may affect follicular development by different and specific pathways and during different developmental stages, i.e. from their quiescent stage at the prepubertal age until maturation.

For example, oocyte quality was negatively affected when gilts (young sows) were fed ZEA-contaminated feed (Alm *et al.*, 2006). α-ZOL also affected embryo development (Alm *et al.*, 2002; Wang *et al.*, 2012) *in vitro* and is indeed suggested to be more harmful than the parent molecule ZEA. *In vitro* exposure of fertilised oocytes to 10 μM α-ZOL led to a decrease in cleavage rate, while blastocyst development was impaired after exposure to α-ZOL at a concentration of 15 μM (Alm *et al.*, 2002) or 30 μM (Wang *et al.*, 2012), showing that early embryos seem to be more sensitive to α-ZOL than blastocysts. Under *in vivo* conditions, porcine hepatic and granulosa cells convert ZEA predominantly into α-ZOL (Malekinejad *et al.*, 2006a,b), which may explain the clinically known sensitivity of pigs to ZEA exposure (Osweiler *et al.*, 1990). In a recent trans-generational study in pigs, we have not observed significant anatomical changes, but a decrease in the primordial follicle population in piglets exposed to ZEA during foetal and neonatal life (Schoevers *et al.*, 2012). Follicular degeneration was characterised by the occurrence of vacuoles in the oocytes. However, ZEA did not reduce the percentages of maturing oocytes or developed embryos (Schoevers *et al.*, 2012). Furthermore, mRNA expression of growth factors such as GDF9 as well as the steroidogenic enzymes 3β-HSD, CYP11A, CYP19A, ERα and StAR was unchanged, but an up-regulation of ERβ mRNA expression was observed.

In addition to the experiment in pigs, Minervini *et al.* (2001) have shown that ZEA and its derivatives have adverse effects on bovine oocyte maturation, while Sambuu *et al.* (2011a) observed no adverse effect of ZEA on oocyte maturation and fertilisation. In the study by Minervini *et al.* (2001),

Table 1. Overview of the enzymes and hormones affected by mycotoxins based on studies using different cell lines as models.

Mycotoxin	Cell line	Test performed	Target enzyme, protein or steroid	Observed effect
AFB_1	JEG-3 cells[1]	aromatase activity[1] qRT-PCR[1]	CYP19A1[1]	⇧CYP19A1[1] no (ant)agonist activity
DON	H295R[2a] RGA[2b]	hormone quantification[2a] endocrine disrupting potential[2b] qRT-PCR[2a]	E2, P4, T and cortisol[2a,b] M2CR, HMGR, StAR, CYP11A, CYP11B1, CYP11B2, CYP17, CYP21, 3β-HSD, 17β-HSD, CYP19, CYP1A1, NR5A1, NR0B1, EPHX[2a]	⇩E2, T and cortisol; ⇧ P4 [2a] (1000 ng/ml) ⇧CYP11B1, CYP11B2, CYP17, CYP21, 3β-HSD, CYP1A1[2a] ⇩HMGR, CYP19, NR5A1[2a] no (ant)agonist activity[2b]
T-2	H295R[2a] RGA[2b]	hormone quantification[2a] endocrine disrupting potential[2b] qRT-PCR[2a]	E2, P4, T and cortisol[2a,b] M2CR, HMGR, StAR, CYP11A, CYP11B1, CYP11B2, CYP17, CYP21, 3β-HSD, 17β-HSD, CYP19, CYP1A1, NR5A1, NR0B1, EPHX[2a]	⇩E2, T and cortisol; ⇧ P4 [2a] (5 ng/ml) ⇧CYP1A1[2a] ⇩HMGR, CYP19, NR0B1[2a] no (ant)agonist activity[2b]
HT-2	H295R[2a] RGA[2b]	hormone quantification[2a] endocrine disrupting potential[2b] qRT-PCR[2a]	E2, P4, T and cortisol[2a,b] M2CR, HMGR, StAR, CYP11A, CYP11B1, CYP11B2, CYP17, CYP21, 3β-HSD, 17β-HSD, CYP19, CYP1A1, NR5A1, NR0B1, EPHX[2a]	⇩E2, T and cortisol [2a] (50 ng/ml) ⇧CYP21, 17β-HSD, CYP1A1, EPHX[2] ⇩HMGR, CYP11A, CYP19[2a] no (ant)agonist activity[2b]
ZEA	H295R[3,4a,5] RGA[4b] HeLa, HepG2 and Ishikawa[6] MCF7 cells[7,8] T47Dcells[9]	proteomic analysis[3,5] hormone quantification[4a] endocrine disrupting potential[4b] luciferase assay[6] qRT-PCR[6] Western blot[6] cell proliferation (5 days)[7] oestrogenic activity[7] Flow cytometry[8] qRT-PCR[8] Western blot[8] cell proliferation (7 days)[9]	protein network analysis [3,5] E2, P4, T and cortisol[4a,b] ERα and ERβ[6] ERα and ERβ[7] no target[8] no target[9]	21 regulated proteins connected to the signalling cascade NF-κB and the oncogene ERBB2[3] ⇧E2, T, P4, cortisol[4a] (10 μM) no agonist activity[4b]; weak antagonist activity[4b] 9 regulated proteins in the cytosol: ANKRD27, RFX7, HSP90B1, HDGF, LRMP, C6orf138, SCARB1, BTF3L4 [5] 1 nM ZEA: HeLa and Ishikawa ⇧ ERα and HepG2 ⇩ERα[6] induces the p44/42 MAPK pathway[6] induces mitosis[7] ZEA as an oestrogenic compound[7] inhibits apoptosis[8] induces mitosis[9]
α-ZOL	H295R[4ab,5] MCF7 cells[7] T47Dcells[9]	hormone quantification[4a] endocrine disrupting potential[4b] proteomic analysis[5] cell proliferation (5 days)[7] oestrogenic activity[7] cell proliferation (7 days)[9]	E2, P4, T and cortisol[4ab] protein network analysis[5] ERα and ERβ[7] no target[9]	⇧E2, T, P4, cortisol[4a] (10 μM) no agonist activity[4b]; weak antagonist activity[4b] 14 regulated proteins in the cytosol: ADD1, NUDT21, DSP, MCM3, HSP90B1, GRP94c, HNRNPD, MDH2, C6orf138, PLEK, PDIA3, P4HB, SFRS1, SFRS2[5] induces mitosis[7]; oestrogenic compound[7] induces mitosis[9]

Table 1. Continued.

Mycotoxin	Cell line	Test performed	Target enzyme, protein or steroid	Observed effect
β-ZOL	H295R[4ab,5] MCF7 cells[7]	hormone quantification[4a] endocrine disrupting potential[4b] proteomic analysis[5] cell proliferation (5 days)[7] oestrogenic activity[7]	E2, P4, T and cortisol[4ab] protein network[5] ERα and ERβ[7]	⇧E2, T, P4, cortisol[4a] (10 μM) no agonist activity[4b]; weak antagonist activity[4b] 5 regulated proteins in the cytosol: GRP94c, CIT, HSPA9, C6orf138, PLEK[5] induces mitosis[7]; oestrogenic compound[7]
OTA	H295R[10] JEG-3[11]	Western blot: aromatase protein amount[10] hormone quantification [10,11] qRT-PCR[11]	E2, P4 and T[10] P4[11] 3β-HSD[11]	no effect on aromatase amount ⇧E2[10] ⇧P4[11] ⇧3β-HSD[11] no (ant)agonist acitivity
AOH	granulosa cells[12] H295R[10a] RGA[10b]	hormone quantification[12,13a] qRT-PCR[12,10a] endocrine disrupting potential[10b]	P4[12]; E2, P4, T and cortisol[10a] M2CR, HMGR, StAR, CYP11A, CYP11B1, CYP11B2, CYP17, CYP21, 3β-HSD, 17β-HSD, CYP19, CYP1A1, NR5A1, NR0B1, EPHX[10a]	⇩P4[12] (0.8 μM); ⇧P4, E2[10a] (1 μg/ml) ⇧M2CR, CYP11B2, CYP17, CYP21, 3β-HSD, CYP10, CYP1A1[10a] ⇩NR0B1[10a] no agonist activity; antagonist effect on androgen, progestagen and glucocorticoid nuclear receptors[10b] (1 μg/ml)
AME	granulosa cells[12]	hormone quantification[12] qRT-PCR[12]	P4[12] 3β-HSD and CYP11A1[12]	⇩P4[12]

Superscript numbers and letters in the table refer to the same reference and cell line used, respectively. References used: [1] Storvik *et al.*, 2011; [2] Ndossi *et al.*, 2012; [3] Busk *et al.*, 2011; [4] Frizzell *et al.*, 2011; [5] Busk *et al.*, 2012; [6] Li *et al.*, 2012; [7] Minervini *et al.*, 2005; [8] Yu *et al.*, 2005; [9] Khosrokhavar *et al.*, 2009; [10] Frizzell *et al.*, 2013; [11] Woo *et al.*, 2013; [12] Tiemann *et al.*, 2009.
Abbreviations used: ADD1 = alpha-adducin; AFB_1 = aflatoxin B_1; AME = alternariol methyl ether; AOH = alternariol; ANKRD27 = ankyrin repeat domain-containing protein 27; BTF3L4 = transcription factor BTF3 homolog 4; C6orf138 = patched domain-containing protein C6orf138; CIT = putative uncharacterised protein CIT; CYP = cytochrome P450; DON = deoxynivalenol; DSP = desmoplakin; E2 = oestradiol; EPHX = (microsomal) epoxide hydrolase; ER = oestrogen receptor; ERBB2 = erythroblastic leukemia viral oncogene homolog 2; GRP94c = heat shock protein 94c; H295R cells = human adrenocortical carcinoma cells; HDGF = hepatoma-derived growth factor; HeLa = human cervical epithelial cancer cells; HepG2 = human hepatocellular cancer; HMGR = hydroxymethylglutaryl-CoA reductase; HNRNPD = heterogeneous nuclear ribonucleoprotein D0; HSD = hydroxylsteroid dehydrogenase; HSP = heat shock protein; HT-2 = HT-2 toxin; JEG-3 cells = human choriocarcinoma cell line; LRMP = lymphoid-restricted membrane protein; M2CR = melanocortin 2 receptor (adenocorticotropin hormone); MAPK = mitogen-activated protein kinases; MCF7 cells = human breast adenocarcinoma cell line; MCM3 = DNA replication licensing factor MCM3; MDH2 = malate dehydrogenase, mitochondrial; NF-Kb = nuclear factor kappa-light-chain-enhancer of activated B cells; NR0B1 = nuclear receptor subfamily 0, group B, member 1; NR5A1 = nuclear receptor subfamily 5, group A, member 1; NUDT21 = cleavage and polyadenylation specificity factor subunit 5; OTA = ochratoxin A; P4 = progesterone; P4HB = prolyl 4-hydroxylase, beta polypeptide; PDIA3 = protein disulfide-isomerase A3; PLEK = pleckstrin; RFX7 = DNA-binding protein RFX7; RGA = reporter gene assays; SCARB1 = scavenger receptor class B member 1; SFRS = splicing factor, arginine/serine-rich; StAR = steroidogenic acute regulatory protein; T = testosterone; T-2 = T-2 toxin; ZEA = zearalenone; ZOL = zearalenol.

the tested ZEA concentration was high (30 µg/ml), while Sambuu *et al.* (2011a) used a maximal concentration of 1 µg/ml, as this concentration had been measured in follicle fluid (Takagi *et al.*, 2008). Such a low concentration was also detected in sows (Sambuu *et al.*, 2011b), without reducing oocyte developmental competence.

When immature oocytes from gilts were exposed to DON, their quality was impaired (Alm *et al.*, 2006) as well. Similarly, porcine cumulus-oocyte-complexes exhibited reduced cumulus expansion, impaired resumption of meiosis, disorganised chromatin and aberrant metaphase II structures, and consequently reduced developmental competence (Schoevers *et al.*, 2010). Exposure of oocytes to DON during *in vitro* maturation caused aneuploidy and abnormal embryo development in pigs (Malekinjead *et al.*, 2007; Schoevers *et al.*, 2010). Possibly, DON also affect follicles at the preantral stages, as this mycotoxin can pass the placental barrier (Dänicke *et al.*, 2007; Goyarts *et al.*, 2010).

Both *in vitro* and *in vivo* studies with mice have demonstrated that citrinin, a mycotoxin produced by *Penicillium* and *Monascus* species, negatively affects oocyte maturation, fertilisation, as well as embryo and foetal development (Chan, 2008; Chan and Shiao, 2007). However, only apoptosis was evaluated and no endocrine activity has been demonstrated up to now. Although Liu *et al.* (2012) claimed that in male rats citrinin leads to down-regulation of StAR, CYP11A, 3β-HSD activity and testosterone levels in Leydig cells, the up-regulation of apoptotic markers suggested that these effects were a consequence of cell death and not of endocrine activity.

5. Final considerations

Potential effects of mycotoxins on female maturation have been assessed by the use of different *in vitro* models addressing entirely different endpoints and hence varying in their predictive value for clinically relevant changes. Cell lines and subcellular fractions or receptor binding assays are powerful tools to study specific pathways of steroidogenesis, including genomic and proteomic analyses. The response, however, may be cell-type dependent (Li *et al.*, 2012) and the results obtained, often at high experimental concentrations of mycotoxins, are difficult to relate to realistic exposure scenarios and endocrine homeostasis *in vivo*.

In vitro models of oocyte maturation, fertilisation or activation, and embryo development using for example porcine oocytes are well developed and cost-effective and have been widely applied in endocrine disruptor studies (Bøgh *et al.*, 2001). Therefore, studies using different developmental stages from quiescent to competent oocytes are also recommended in the assessment of the effects of mycotoxins. Again, it needs to be emphasised that in many experimental approaches high, non-realistic concentrations of mycotoxins have been used. While this is helpful to identify mechanisms and vulnerable phases in oocyte maturation, confirmation is needed by *in vivo* studies or epidemiological surveys relating mycotoxin exposure to adverse effects in human and animals.

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