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EFFECTS OF THE MYCOTOXIN, DEOXYNIVALENOL, AND ITS MAJOR METABOLITE, DE-EPOXY DEOXYNIVALENOL, ON BOVINE REPRODUCTION

par

HILDA MORAYMA GUERRERO NETRO

Département de biomédecine vétérinaire

Faculté de médecine vétérinaire

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Abstract

Deoxynivalenol (DON) is a major mycotoxin found in animal feed and is known to reduce fertility in pigs by inhibiting progesterone secretion from granulosa cells. In cattle, it is metabolized to de-epoxy DON (DOM-1) in the rumen, and DOM-1 can reach high concentrations in blood and follicular fluid. One of the major pathways activated by DON is the ribotoxic stress response (RSR), which involves autophosphorylation of protein kinase R (PKR) and downstream activation of MAP kinases including MAPK3/1. It is not known if these mycotoxins affect bovine reproduction. The objectives of present thesis were (1) to determine how and at what doses DON affects ovarian granulosa cell function and to elucidate its mechanism of action; and (2) to determine how and at what doses major mycotoxin DON and its metabolite DOM-1 affect theca cell function in cattle. The results are separated into three articles. In the first article the effects of DON on granulosa cells were explored; treatment with DON resulted in a significant inhibition of estradiol and progesterone (P4) secretion, and an increase in the proportion of apoptotic cells after 4 days of treatment. Western blot demonstrated significant up-regulation of ERK1/2 and MAPK14 phosphorylation within 15-30 minutes of adding DON. We then determined the effect of DON on ERK1/2 target genes; *EGR1* and *FOS* mRNA levels were transiently stimulated with maximum levels at 1 h of adding DON, whereas *COX2* and *GADD45B* mRNA levels were upregulated but not until 24 h after DON treatment. In the second article, the effects of DON and DOM-1 on theca cells were assessed. Treatment with DOM-1 resulted in a dose-dependent inhibition of P4 and testosterone secretion, and an increase in the proportion of apoptotic cells, while DON inhibited P4 but did not alter testosterone secretion or the percentage of dead cells. Both

DON and its metabolite were maximally effective at concentrations of 1 ng/ml (in contrast, the effects of DON on occur at 100ng/ml). Western blot demonstrated rapid phosphorylation of MAPK3/1, PKR and of JUN kinase after addition of DOM-1 or DON. Interestingly, phosphorylation of MAPK14 was significantly increased by DOM-1 but decreased by DON. The addition of a PKR inhibitor abrogated the ability of DON and DOM-1 to increase phosphorylation of MAPK3/1, and partly abrogated the inhibitory effect of DON on MAPK14 phosphorylation, however, the PKR inhibitor further increased the phosphorylation of MAPK14 caused by DOM-1. Together, these results suggest that DON activates the RSR in bovine granulosa and theca cells, and that theca cells are more sensitive than granulosa cells to the effects of DON. The data also demonstrate for the first time in any cell type the ability of DOM-1 to affect cell function and health.

Key words: mycotoxin, deoxynivalenol, de-epoxy deoxynivalenol, granulosa cells, ribotoxic stress response.

Résumé

Le Deoxynivalenol (DON) est une mycotoxine majeure retrouvée dans l'alimentation animale et celle-ci est connue pour réduire la fertilité des truies en inhibant la sécrétion de progestérone par les cellules de granulosa. Chez le bétail, DON est métabolisée en de-epoxy DON (DOM-1) dans le rumen, et DOM-1 peut atteindre des concentrations élevées dans le sang et les liquides folliculaires. Une des voies majeures de signalisation activée par DON est le ribotoxic stress response (RSR), lequel induit une auto-phosphorylation de la protéine kinase R (PKR) et réduit l'activation des MAP kinases incluant la MAPK3/1. Il n'a pas encore été démontré que ces mycotoxines affectent la reproduction chez les bovins. Les objectifs de cette thèse sont (1) de déterminer comment et à quelles doses DON affecte la fonction des cellules de granulosa et d'élucider les mécanismes d'action entrant en jeu; et (2) déterminer comment et à quelles doses la mycotoxine majeure DON et son métabolite DOM-1, affectent la fonction des cellules de la thèque chez le bétail. Les résultats sont présentés dans trois articles distincts. Dans le premier article, nous explorons les effets de DON sur les cellules de granulosa bovines; les traitements avec DON résultant en une inhibition significative de la sécrétion d'œstradiol et de progestérone (P4), et en une augmentation de la proportion de cellules apoptotiques après 4 jours de traitement. Les expériences de Western-Blot démontrent une stimulation significative de la phosphorylation de ERK1/2 et de MAPK14 entre 15 et 30 minutes après le début du traitement des cellules par DON. Par la suite, nous avons déterminé les effets de DON sur les gènes cibles de ERK1/2. En effet, les niveaux d'ARNm de *EGR1* et *FOS* sont transitoirement augmentés avec des niveaux maximum à 1h de traitement par DON, tandis que les niveaux d'ARNm de

COX2 et *GADD45B* sont augmentés mais plus de 24h après le début du traitement par DON. Dans le second article, les effets de DON et DOM-1 sur les cellules de thèque ont été étudiés. Le traitement des cellules par DOM-1 résulte en une inhibition dose-dépendante de la sécrétion de P4 et de testostérone, et en une augmentation de la proportion de cellules apoptotiques, tandis que DON inhibe la sécrétion de P4 sans altérer celle de la testostérone ou bien le pourcentage de cellules mortes. Les deux mycotoxines sont effectives de manière maximale à des concentrations de 1 ng/ml (en revanche, DON affecte les cellules de granulosa à 100 ng/ml). Les résultats de Western-Blot démontrent la phosphorylation rapide de MAPK3/1, PKR et de JUN kinase après un traitement par DON ou DOM-1. En présence d'un inhibiteur spécifique de PKR, DON et DOM-1 sont incapables d'induire la phosphorylation de MAPK3/1, et l'effet inhibiteur de DON sur la phosphorylation de MAPK14 est en partie abrogé. Néanmoins, l'inhibiteur de PKR augmente davantage la phosphorylation de MAPK14 induite par DOM-1. Ensemble, ces résultats suggèrent que DON active le RSR dans les cellules de thèque et les cellules de granulosa bovines, et que les cellules de la thèque sont plus sensibles que les cellules de granulosa aux effets de DON. Ces données démontrent pour la première fois l'habilité de DOM-1 à affecter les fonctions et la survie cellulaires.

Mot clés: mycotoxine, deoxynivalenol, de-epoxy deoxynivalenol, cellule de la granulosa, ribotoxic stress response.

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List of abbreviations

3ß-HSD: 3ß-hydroxysteroid dehydrogenase

17ß-HSD: 17ß-hydroxysteroid dehydrogenase

AKT: protein kinase B

Atg: autophagy-related

BCL-2: B-cell lympoma 2

BID: interacting-domain death agonist

CGC: cumulus granulosa cells

COX: cyclooxygenase

CYP11A1: , cholesterol side-chain cleavage enzyme

CYP17A1: steroid 17 alpha-hydroxylase/17,20 lyase

CYP19A1: cytochrome P450 family 19 subfamily A member 1

DAS: diacetoxyscirpenol

DOM-1: de-epoxy deoxynivalenol

DON: deoxynivalenol

E2: estradiol

ECM: extracellular matrix

EGF: epidermal growth factor

eIF-2a: eukaryotic initiation factor 2 a -subunit

ERK: extracellular signal-regulated kinases

FASL: FAS ligand

FGF: fibroblast growth factors

FHB: fusarium head blight

FOXO: forkhead box O

FSH: follicle stimulating hormone

GC: granulosa cells

Hck: hematopoetic cell kinase

IgA: immonuglobulin A

IGF-1: insulin-like growth factor I

LC3: 1A/1B-light chain 3

LH: luteinizing hormone

LHGCR: luteinizing hormone/choriogonadotropin receptor

MAPK: mitogen-activated protein kinase

MGC: mural granulosa cells

mTOR: mammalian target of rapamycin

Nec-1: necrostatin-1

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NIV: nivalenol

OT: ochratoxins

OTA: ochratoxin A

P4: progesterone

P450c17: steroid 17 alpha-hydroxylase/17,20 lyase

P450scc: cholesterol side-chain cleavage enzyme

P53: tumor protein

PGC: primorial germ cells

PI3K: phosphatidylinositol 3-kinase

PKA: protein kinase A

PKC: protein kinase C

PP1: protein phosphatase 1

PP2: protein phosphatase 2

SMER: small-molecule enhancer of rapamycin effects

SRC: C-terminal Src kinase

RSR: ribotoxic stress response

StAR: steroidogenic acute regulatory protein

SMER: small-molecule enhancer of rapamycin effects

TC: theca cells

ULK-1: serine/threonine-protein kinase

ZAK: zipper sterile-alpha-motif kinase

ZAN: zearalanone

ZEA: zearalenone

α-ZAL: alpha zearalanol

α-ZEA: alpha zearalenol

β-ZAL: beta zearalanol

β-ZEA: beta zearalenol

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Introduction

In cattle, fertility is important for the maintenance of genetically superior dairy and beef herds, unfortunately, in the last decades there has been a well-documented decline in fertility in dairy cattle worldwide. At the same time fungal contamination has become a major problem around the world as it can lead to significant economic losses, The problem starts when animals ingest contaminated feed, leading to reduced productivity by diminution of weight gain, secretion of mycotoxins in milk and decreased fertility.

Mycotoxins are low-molecular-weight secondary metabolites produced by natural metabolic processes in fungi. The main mycotoxin-producing fungi are Aspergillus spp., Fusarium spp., and Claviceps spp. Mold growth can occur at different stages in a variety of grain products and can be enhanced by humidity, temperature and availability of oxygen. All these factors curtail prevention of fungal contamination. The ingestion of contaminated grains can lead to 1) diseases caused by the growth of fungi on the animal host, also known as mycoses and 2) diseases produced by exposure to the toxic fungal metabolites, known as mycotoxicosis. Mycotoxicosis symptoms depend on a wide variety of factors; in order to classify a disease as a mycotoxicosis, it is necessary to demostrate a dose-response relationship between disease and mycotoxin (Peraica, Radic et al. 1999).

In Canada, the mycotoxins of major concern are deoxynivalenol (DON), T-2 toxin, zearalenone (ZEA), fumonisin B1 and Ochratoxin A. Their effects may vary but generally include growth retardation, immunocompromise, feed refusal, vomiting, reduced ovarian function and other reproductive disorders. Zearalenone has been widely studied even though it is not considered to be highly toxic. Despite its low levels of

toxicity, there are some interesting aspects to take into account: ZEA can be detected in milk (Prelusky, Scott et al. 1990), it is absorbed and rapidly spread through the organism, and has dose-dependent estrogenic effects (Zinedine, Soriano et al. 2007). These estrogenic effects are due to the resemblance of ZEA with E2, and ZEA acts as a competitive inhibitor, is capable of binding to estrogen receptors, affecting reproductive organs and their function. This makes ZEA a perfect example of a weakly toxic but biologically potent compound.

On the other hand, deoxynivalenol (DON), also known as vomitoxin, has been less studied; it can alter the serotonergic activity of the brain and immune function. Reproductive problems associated with DON contamination appear to be focused at the ovary and may involve the ovulatory process as DON affects oocyte maturation in pigs (Alm, Greising et al. 2002). In cattle its role in reproduction is completely unknown, mainly due to its conversion in the rumen to a less toxic metabolite de-epoxy-DON. The objectives of this project are to determine how and at what doses DON and its main metabolite de-epoxy-DON affect ovarian function in cattle. These studies will offer major insight into the effects of these mycotoxins, with the ultimate goal of improving bovine reproduction health.

Chapter 1:
Literature review

1. The bovine ovary

The ovaries are the female gonads found in pairs located in the pelvic area, and in the cow they have an almond shape with a size of 3.5 x 2.5 x 1.5 cm (Marieb Elain N 1993). The development of the ovary starts as a thickening of the coelomic epithelium that lines the body cavity in the ventral-medial surface of the mid-region on embryonic day 34. The initiating cause of the thickening is unknown and studies performed mainly in mice are focusing on finding the genes required for this process (Gospodarowicz, Jones et al. 1974). The ovary is considered to have two main functions in reproduction: the first is gametogenesis, leading to the production through meiosis of competent oocyte, while the second is secretion of female sexual hormones such as estrogen and progesterone that are required for follicular development, maintenance of estrous cyclicity and reproductive functions including preparation of the reproductive tract for fertilization and subsequent establishment of pregnancy (Marieb Elain N 1993). In terms of steroidogenic function, the ovaries are required to perform a highly coordinated series of complex events that will lead to follicular development (Gospodarowicz, Jones et al. 1974).

1.1 The follicle

The structure of the follicle changes during development and can be classified into three different groups according to size, complexity and responsiveness to circulating gonadotropins: preantral, antral and preovulatory follicles (McGee and Hsueh 2000). Preantral follicles start as primordial follicles that possess a single layer of squamous pre-granulosa cells surrounding the oocyte. The theca cell layer has yet to form and there is no vascular system. As they start to grow primordial follicles become primary follicles consisting of a single layer of cuboidal granulosa cells. Primary follicles develop into

secondary follicles, in which two or more layers of granulosa cells surround the oocyte but the theca layer and the antral cavity are absent (McGee and Hsueh 2000). As the follicle transforms into an antral follicle, extracellular fluid accumulates between the granulosa cells that will later merge to form a central liquid-filled cavity called the antrum. The zona pellucida forms at this stage and two or more granulosa cell layers surrounding the oocyte become the cumulus granulosa cells (CGC). The theca cell layer is now well formed. The proportion of primordial follicles that undergo folliculogenesis and reach the antral stage is very low as most of the follicles undergo regression and atresia (Marieb Elain N 1993). Each follicle consists of an oocyte surrounded by the zona pellucida, one or more layers of somatic cells referred to as cumulus cells, an antrum, and a basal lamina that separates mural granulosa cells (GC) from the theca cells (TC). Theca cells are considered to be the interstitial tissue of the follicle (Figure 1) (Gospodarowicz, Jones et al. 1974).

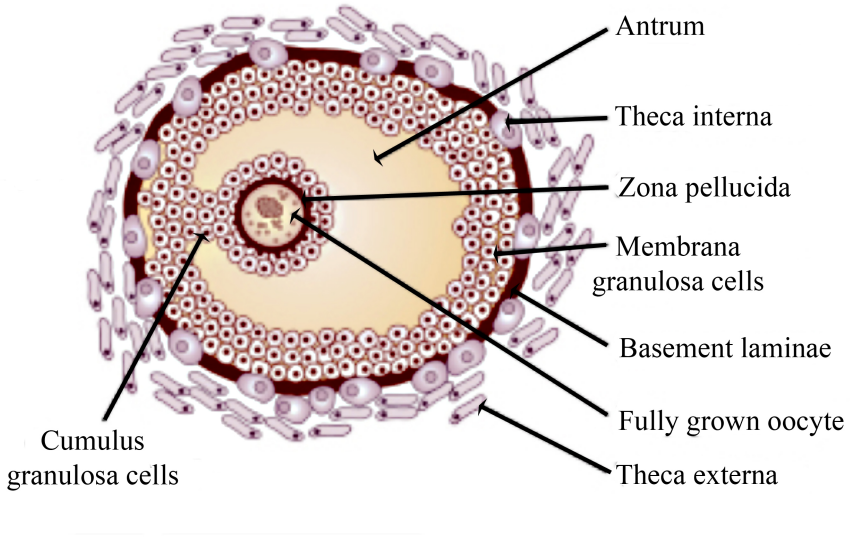

Figure 1: Schematic representation of a pre-ovulatory mammalian follicle. The cell types comprising the follicle are shown; the fully grown oocyte and cumulus granulosa cells. Also pointed out are theca cells, granulosa cells, extracellular matrix produced by the oocyte (zona pellucida) and antrum (Erickson, Magoffin et al. 1985).

1.1.1 Oocyte

The oocyte is the female germ cell prior to fertilization; the number of oocytes in the mammalian ovary is fixed early in life (Conner, Lefievre et al. 2005). The development of the oocyte starts with the primordial germ cells (PGC), which undergo meiosis to form an oogonium. Primordial germ cells have the ability to migrate extensively from their site of formation to the developing gonad. This process is regulated by somatic germ cell interactions and some additional factors including fibroblast growth factors (FGF); in mice FGF2 has been reported to be a mitogenic factor that affects motility of PGC by mediating activation of the MAP-kinase pathway, and FGF7 has also been found to have a role in regulating PGC numbers by activation of the receptor FGFRIIIb (Takeuchi, Molyneaux et al. 2005). Once the gonad is assembled from PGC, the cells start

differentiating and proliferating resulting in the formation of the oogonia. In many organisms the oogonia divide several times forming clusters of interconnected cells; after each division cytoplasmic bridges remain allowing continuous communication between cells and coordinated development. The oogonia differentiate by meiosis into primary oocytes that arrest in prophase and form the major reserve of oocytes in primordial follicles (Voronina and Wessel 2003). The best-documented stimulators of oocyte maturation are hormones and growth factors. Some mechanisms of maturation have been proposed include: 1) the production of a maturation-inducing substance by follicular cells that drives oocytes to mature, possibly involving activation of membrane receptors by steroid hormones; 2) inactivation of follicle-derived maturation inhibitor; and 3) inhibition of gap junction-mediated transport to prevent transfer of a follicle-derived inhibitor (Conner, Lefievre et al. 2005).

1.1.2 Granulosa cells

Granulosa cells are important for oocyte maturation as they provide nutrients that support further development (Albertini, Combelles et al. 2001). These authors indicate that, as follicles grow and the antral cavity is formed, the GC separates into two anatomically and phenotypically different subtypes: the CGC, which are in direct contact with the oocyte, have a high rate of proliferation, low steroidogenic capacity, low LH receptor (LHCGR) expression and high levels of insulin-like growth factor I (IGF-1); and the mural granulosa cells (MGC) that have a primarily endocrine function and support follicle growth, and which undergo terminal differentiation to luteal cells after ovulation. The interaction between oocytes and CGC is complex; CGC express characteristics distinct from those of the MGC, that are acquired under the influence of the oocyte and

that promote cell differentiation and development of the GC (Albertini, Combelles et al. 2001).

The oocyte achieves this by secreting labile paracrine signaling factors, and perturbation of these signaling results in the production of an oocyte unable to undergo normal maturation (Yeo, Gilchrist et al. 2009). It is possible that MGC are antagonist or insufficient for supporting the last stages of oocyte maturation (Eppig, Chesnel et al. 1997). GC lack a vascular supply, therefore they require contact with their neighboring cells via gap junctions; these gap junctions contain different connexins such as connexin 32, 43 and 45 (Johnson, Redmer et al. 1999). Connexin 43 has been studied widely in the mouse where it has been detected from the onset of folliculogenesis, just after birth, and persists through ovulation (Ackert, Gittens et al. 2001). It has been found that, in later stages, coupling between GC is mediated specifically by connexin 43 and is essential for continued follicular growth, expansion of the GC population during early stages of follicular development, and that mutations in this gap junction lead to a retarded oocyte growth, poor development of the zona pellucida of both granulosa cells and oocytes (Gospodarowicz, Jones et al. 1974, Ackert, Gittens et al. 2001).

1.1.3 Theca cells

Theca cells (TC) are endocrine cells that play essential roles within the ovary by producing androgens, under the control of luteinizing hormone (Adams, Jaiswal et al.), that are required for ovarian estrogen biosynthesis, and provide structural support for the growing follicle as it progresses through various developmental stages (Figure 2). The theca layer is highly vascularized and through this vascularization it provides the rest of the follicle with essential nutrients and endocrine hormones from the pituitary axis

(Magoffin 2005, Young and McNeilly 2010). It is thought that TC are recruited from surrounding stromal tissue; the current hypothesis of the origin of TC is that growing follicles secrete a series of signals that stimulates TC differentiation and some evidence suggests that these signals involve unknown small molecular-weight proteins secreted by GC (Magoffin 2005). During development, the majority of follicles undergo atresia, and the TC are often the final follicular cell type to die. For those follicles that ovulate, the TC then undergo hormone-dependent differentiation into luteinized TC of the corpus luteum (Young and McNeilly 2010).

1.2 Follicular growth and development

Folliculogenesis describes the formation of the primordial follicle and its progression through the successive stages of preantral, antral and finally preovulatory growth (Figure 3). The development from primordial follicle to preovulatory follicle is a time-consuming event, estimated in cows to take 180 days (Cahill and Mauleon 1981). It appears that follicle-stimulating hormone (FSH) plays a predominant role in follicle selection and final preovulatory growth. After the LH surge, a series of events that leads to ovulation; it is known that LH stimulates the expression of the progesterone (P4) receptor, the enzyme prostaglandin synthase 2 and the epidermal growth factor-like ligands, amphiregulin and epiregulin that induce changes in CGC (Shimada, Hernandez-Gonzalez et al. 2006).

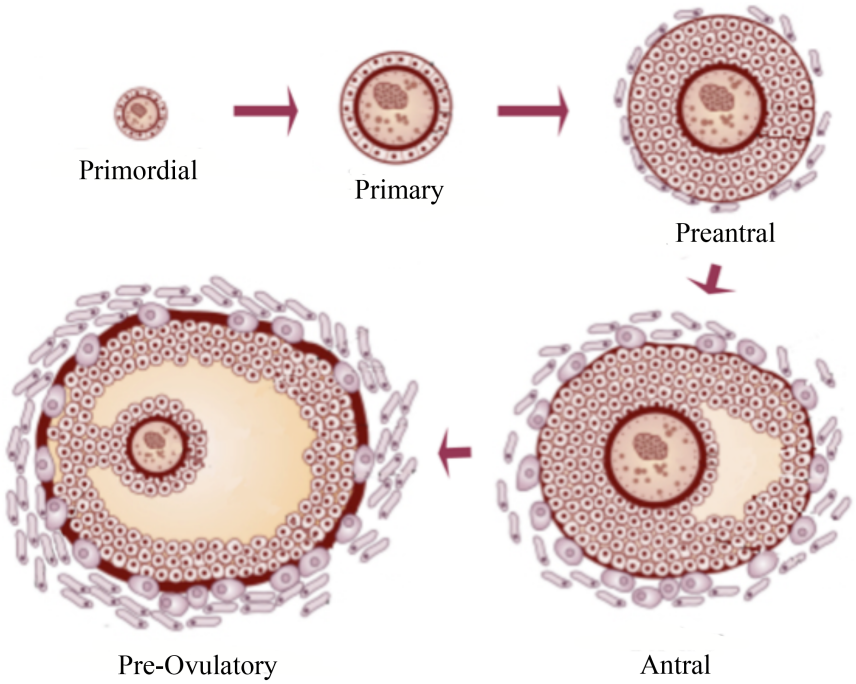

Figure 2: **Folliculogenesis.** Formation of the primordial follicle and its progression through different developmental stages until reaching a pre-ovulatory stage (Erickson, Magoffin et al. 1985, Young and McNeilly 2010).

In cattle a rise in blood FSH concentrations recruits a cohort of small antral follicles into a phase of growth (Adams, Matteri et al. 1992). The largest of these follicles becomes the 'dominant follicle', and secretes high levels of estrogen and inhibins, which then suppress pituitary FSH secretion, which in turn results in atresia in of the remaining follicles in the cohort (McGee and Hsueh 2000, Sisco, Hagemann et al. 2003). The dominant follicle also produces higher levels of autocrine and paracrine factors that stimulate FSH responsiveness of GC. One of these factors is IGF1, which serves to enhance GC responsiveness to FSH by increasing expression of the FSH receptor (McGee and Hsueh 2000, Albertini, Combelles et al. 2001).

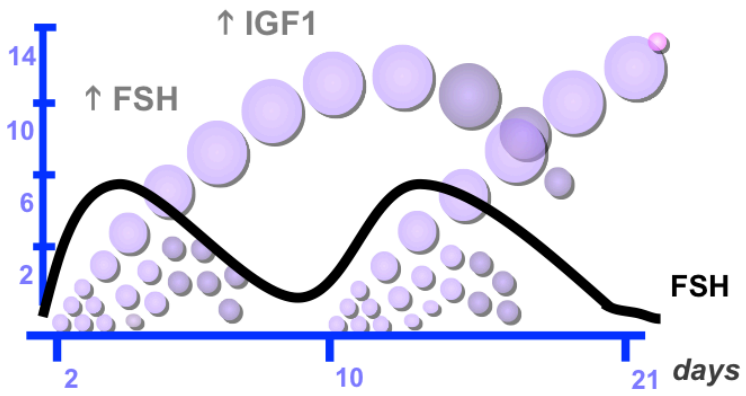

Figure 3: FSH regulation. Bovine cycle of 21 days with two follicular waves, and its relation to circulating FSH, each follicular recruitment is preceded by an increase in FSH and IGF1 blood levels. In the lack of an LH peak, follicles regressed and FSH levels rise again resulting in a new wave (Adams, Jaiswal et al. 2008).

Studies using ultrasonic imaging have documented that follicular growth in cattle occurs in a wave-like pattern and that the majority of estrous cycles in cattle consist of two or three waves (Ginther, Knopf et al. 1989). The first wave starts on the day after ovulation (Day 0), the second wave occurs around day 9 and, in the case of a third, it emerges around day 15 (Adams, Jaiswal et al. 2008). Each follicular wave is preceded by an increase in FSH that begins about day 2.5 before the wave emerge and starts to decrease about the time of the appearance of the cohort of follicles in the wave (Figure 3) (Bao and Garverick 1998). During follicular growth, three major events take place: recruitment, selection and dominance (Fortune 1994). The recruitment begins with the growth of 8-41 small follicles between 3-4 mm, which continue to grow at a similar rate for two days. After this period, one of the follicles is selected and continues growing until

it becomes dominant, the rest of the follicles become atretic and regress (Adams, Jaiswal et al. 2008).

1.2.1 Recruitment

Follicle activation or recruitment takes place in two phases: 1) an initial activation of the dormant primordial follicles into the growing follicle pool; and 2) a cyclical recruitment in response to FSH (Figure 4) (McGee and Hsueh 2000). It has been demonstrated that FSH can bind to GC of preantral follicles making them responsive to FSH, permitting them to follow a wave-like pattern in response to periodical endogenous surges of FSH (McGee and Hsueh 2000, Adams, Jaiswal et al. 2008).

Follicle recruitment is associated with initiation of simultaneous expression of steroidogenic enzymes, cholesterol side-chain cleavage enzyme (*CYP11A1*) and cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*) mRNA in GC of the recruited cohort of follicles, which are likely to be increased by circulating FSH (Bao and Garverick 1998). During growth of the cohort, follicles grow from 5 mm to 8-9 mm diameter, and the GC express *CYP19A1* and CYP11A1 mRNA, but not *3β-HSD* mRNA, and the TC express *LHCGR*, *CYP11A1*, steroid 17 alpha-hydroxylase/17,20 lyase (*CYP17A1*), 3ß-hydroxysteroid dehydrogenase *(3β-HSD)*, and steroidogenic acute regulatory protein (*StAR*) mRNA. This suggests that GC start to metabolize androgens coming from TC to estradiol (E2), and cholesterol to pregnenolone, but not pregnenolone to P4 because of the lack of *3β-HSD* (Bao and Garverick 1998). Follicles at this stage of development are all antral, and most will undergo atretic degeneration, leaving just the dominant follicle to reach the preovulatory stage (Kolpakova, Wiedlocha et al. 1998,

McGee and Hsueh 2000). This stage of follicular development is considered to be gonadotropin-dependent (Bao and Garverick 1998).

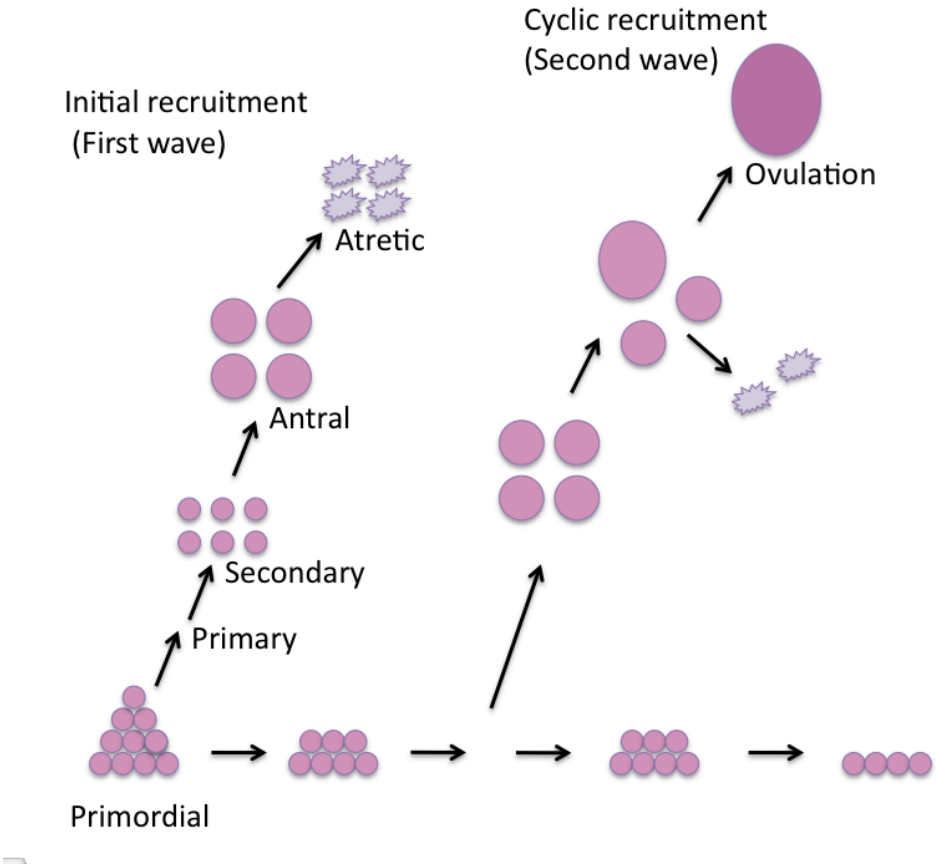

Figure 4: Recruitment of bovine ovarian follicles. Bovine follicle recruitment in a two-wave pattern. Initial recruitment or first wave, primordial follicleas are recruited and reach antral stagen, in the absence of LH follicles become atretic. Cyclic recruitment, in the absence of ovulation an increase in FSH levels lead to a new follicular wave (McGee and Hsueh 2000).

1.2.2 Selection and dominance

In monovulatory species, selection is the process whereby a single follicle is chosen from the cohort of medium-sized growing follicles for further development while the rest become atretic (Fortune, Rivera et al. 2004). In polyovulatory species, multiple follicles are selected and grow synchronously until ovulation. The exact process of follicle selection remains unknown, although it has been suggested that the selected follicle shows increased expression of FSHR, LHCGR and 3β-HSD in GC, permitting it to be responsive to LH and continue developing in the face of lowered FSH concentrations (Aerts and Bols 2010). It has also been proposed that the increased follicular growth rate is due to an increase in IGF1 bioavailability in the dominant follicle (Lucy 2007). It has been established that the development of one antral follicle until it becomes dominant requires 42 days in the cow, or the equivalent of two estrous cycles (Aerts and Bols 2010). A dominant follicle has higher concentrations of E2 in follicular fluid, higher LHCGR mRNA levels in TC and GC, higher levels of CYP17A1 and CYP19A1 in GC compared with non-dominant growing follicles (Fortune, Rivera et al. 2004). Another characteristic of dominant follicles is the high expression of StAR mRNA in TC, which may assure enough cholesterol transport to the mitochondria for androgen production (Bao and Garverick 1998). If the dominant follicle becomes the preovulatory follicle, a cascade of events started by the preovulatory LH surge results in ovulation. LH increases the synthesis of progesterone receptors, prostaglandins and epidermal growth factor (EGF)-like factors in GC, and induces the primary oocyte to complete meiosis I (Hsieh, Lee et al. 2007). There is also an up-regulation of the expression of proteases thought to play critical roles in follicular rupture (Russell and Robker 2007).

1.2.3 Atresia and autophagy

Follicular atresia occurs by a hormonally controlled programmed cell death process known as apoptosis. During apoptosis the nucleus disrupts into spherical dense fragments known as piknotic nuclei and DNA cleavage forming the DNA ladder, marker of cell death (Wyllie, Kerr et al. 1980). Within the ovary, there are two forms of cell degeneration: 1) attrition: pre-antral degeneration of germ cells, normally regulated by basic fibriblat growth factor (FGF) (Pesce, Farrace et al. 1993) and; 2) atresia: follicle degeneration hormonally controlled, the main characteristic of which is that follicles entering atresia can be rescued by FSH (Hsueh, Billig et al. 1994). Follicular atresia has been associated with a suppression of E2 secretion and CYP19A1 expression (Bao and Garverick 1998), therefore in the absence of survival factors, endogenous apoptotic pathways become activated. In GC, FSH activates phosphatidylinositol 3-kinase (PI3K) - protein kinase B (AKT) signaling, when this is downregulated, genes such as forkhead box O (*FOXO*) are de-phosphorylated and transferred to the nucleus where they activate other apoptotic factors (Brunet, Bonni et al. 1999) such as FAS ligand (*FASL*). *FASL* mRNA levels can be reduced by FSH (Lin and Rui 2010). B-cell lymphoma 2 (*BCL-2)*, an anti-apoptotic agent that works by isolating caspases (pro-apoptotic) or preventing other apoptotic factors from being released from the mitochondria (Tsujimoto 1998). Apoptosis is a complicated process, involving different pathways that might play a role in other cellular processes such as autophagy. The processes of apoptosis and autophagy are intimately related.

Autophagy or autophagocytosis can be simply defined as cellular “self-eating”. During this process, degradation of cytoplasmic constituents and organelles takes place in the lysosome, especially under conditions of stress or starvation (Cuervo 2004). There are

three types of autophagy: macrophagy, microphagy and chaperone-mediated autophagy (Cuervo 2004, Mizushima, Yoshimori et al. 2010). Macroautophagy is mediated by a unique organelle, the phagophore or isolation membrane (Mizushima 2007). The phagophore has the function of enclosing a portion of cytoplasm and organelles forming what is called the autophagosome (Shibutani and Yoshimori 2014). The membrane of the autophagosome then fuses with the endosome and lysosome to degrade the isolated material, and this is believed to be a selective process to deplete specific organelles from the cell (Mizushima 2007). Microautophagy is mediated directly by the lysosome in a non-selective process that takes place when cytoplasmic material is trapped in the lysosome by invagination, and chaperone-mediated autophagy is a selective system to detect and degrade soluble cystosolic proteins (Marzella, Ahlberg et al. 1981). Autophagy is a complicated process, as effects differ by cell type; one of the main pathways involved in autophagy is mammalian target of rapamycin (mTOR), a regulator of nutrient signaling. In brain cells from Huntington's mouse and fly models, rapamycin, an inhibitor of mTOR, can induce autophagy and decrease cell death by reducing levels of the toxic mutant protein (Ravikumar, Vacher et al. 2004). However, in other studies, small-molecule enhancer of rapamycin effects (*SMER*), a small-molecule enhancer of autophagy, could induce autophagy, independently of the mechanistic target of rapamycin (mTOR) pathway (Sarkar, Perlstein et al. 2007).

Autophagy-related (Atg) proteins are a large family of 31 proteins localized in a restricted region called the phagophore assembly site or pre-autophagosomal structure (Yang, Kozopas et al.). In mammals, some of these, such as serine/threonine-protein kinase *(ULK1/2), Atg13, FIP200, Atg101, Beclin 1, Atg14,* 1A/1B-light chain 3 *(LC3),*

Atg12 and *Atg16L1*, were observed on isolation membranes and received the name of "AP-Atg proteins" because of their involvement in autophagosome formation by interacting with each other for recruitment of the pre-autophagosomal structure (Suzuki and Ohsumi 2007). Of these, only *LC3*, the mammalian homolog of Atg8 in yeast, is known to be present in the autophagosomes, which makes *LC3* the best marker for autophagy (Mizushima, Yoshimori et al. 2010). *LC3* has three different isoforms: *LC3A*, *LC3B*, and *LC3C*. The main isoform is *LC3B*, which is transformed by *Atg4* to *LC3B-I*, which is later conjugated with phosphatidylethanolamine to become *LC3B-II*. *Beclin-1* was originally identified as a partner of *Bcl-2*, an antiapoptotic protein. The processes of apoptosis and autophagy are intimately related, and the *beclin-1* and *Bcl-2* interaction is mediated by interacting-domain death agonist *(BID)* domain by interacting with *Bcl-1*. During stress this domain is down-regulated allowing *Beclin-1* to induce autophagy. Not only *Bcl-2* is related with apoptosis and autophagy; caspase essential during apoptosis, necrostatin-1 (Nec-1) necrosis blocker, tumor protein (P53) regulator of cell cycle specially as a tumor suppressor and, PI3K/ AKT /mTOR are common pathways for both, therefore further investigation is required to fully understand how the cell can go either way (OuYang, Wang et al. 2013).

1.3 Steroidogenesis

Steroid hormones are derivatives of cholesterol and can be classified into five categories: glucocorticoids (cortisol), mineralocorticoids (aldosterone), androgens (testosterone, androstenedione), estrogens (estradiol and estrone) and progestins (progesterone). In the bovine follicle five enzymes are required for the production of estradiol (Conley and Bird 1997). Steroidogenesis starts with the internalization of blood-

borne low-density lipoproteins, and once inside the cell cholesterol is maintained as liquid droplets (cholesterol esters), which are converted to free cholesterol by the enzyme cholesterol ester hydrolase. Free cholesterol is then mobilized to the mitochondria by *StAR* where it is converted to pregnenolone by the enzyme *CYP11A1* cleavage (Clark, Wells et al. 1994). Pregnenolone can follow two different routes: 1) conversion to progesterone by the enzyme *3β-HSD* or 2) conversion to 17α-hydroxypregnenolone by the enzyme *CYP11A1*. 17α-hydroxypregnenolone can be converted to androstenedione by CYP11A1 and 3ß-HSD (Schwartz and Roy 2000). Androstenedione is converted into testosterone by 17ß-hydroxyosteroidehydrogenase (*17β-HSD*); TC secrete androstenedione and testosterone and GC can convert androstenedione to estradiol by *17β-HSD* and testosterone to estrone by *CYP19A1*. Progesterone can be mobilized directly from TC to GC (Figure 5) (Miller and Auchus 2011).

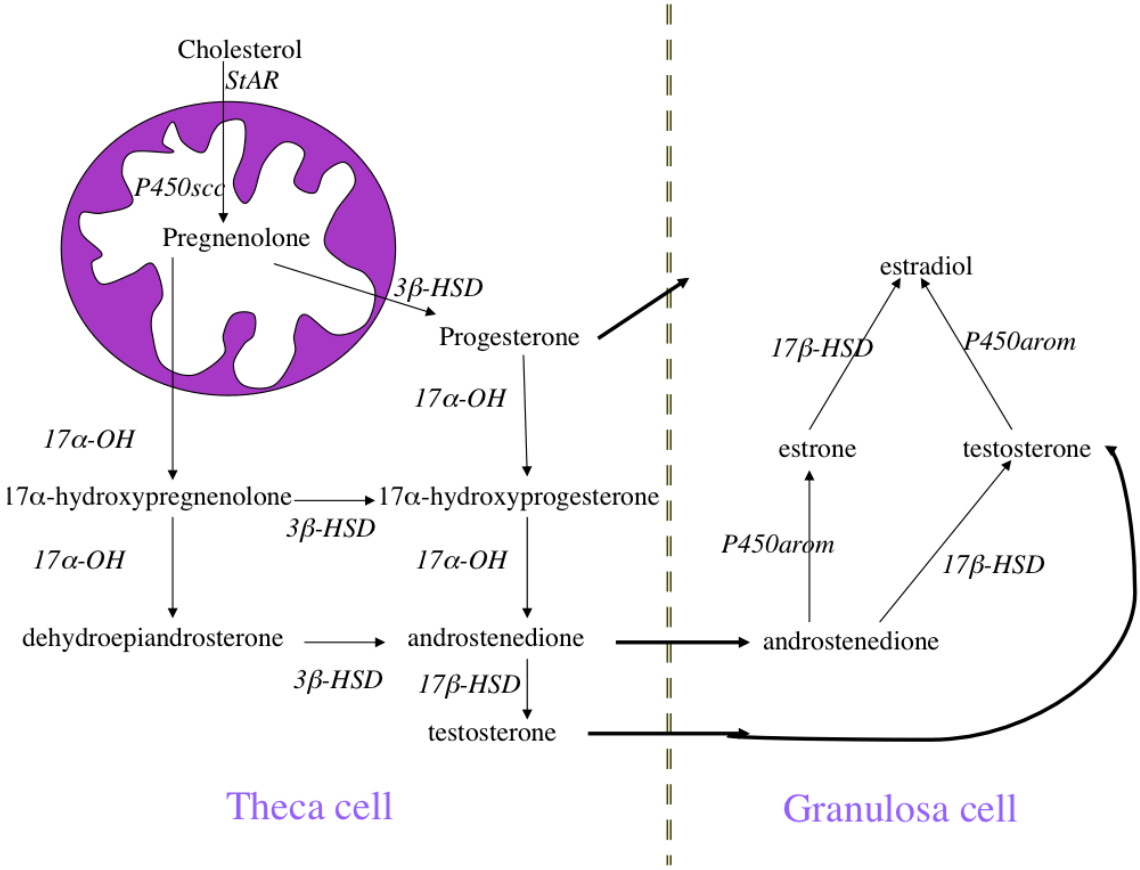

Figure 5: Steroidogenesis. Steroidogenic pathway diagram in ruminants. Cholesterol enters the mitochondira by the action of StAR enzyme. In theca cells take place the formation of main hormones progesterone and testosterone. On the other hand, only estrogens are produced by granulosa cells (Miller and Auchus 2011).

2. Mycotoxins

Mycotoxins are low molecular-weight secondary metabolites produced by natural metabolic processes in fungi. The main mycotoxin-producing fungi are *Aspergillus spp*. in tropical and subtropical regions, *Fusarium spp*., and *Claviceps spp* in North America and Europe (Kanora and Maes 2009). Mold growth can occur at different stages on a variety of grains including maize, wheat, barley, millet, peanuts, peas and oily feedstuffs,

making forages and cereals the most important source of mycotoxins to animals. Fungal contamination can be enhanced by: 1) humidity, although some molds can grow under low humidity; 2) temperature, fungi grows at temperatures between 20-30°C, however once present in the grain some mycotoxins can resist up to 350°C; and 3) availability of oxygen, mold are aerobic organisms and require oxygen for normal growth, however some fungi can grow in environments containing as low as 0.5% of oxygen. All these factors curtail prevention of fungal contamination. There are two main forms of grain contamination: 1) field fungi, including *Claviceps, Fusarium, Cladosporium, Diplodia, Gibbrella* and *Helminthosporium,* that invade the crops while they are still in the field under high humidity conditions (20%); and 2) storage fungi, including *Aspergillus* and *Penicillium* that require less humidity to develop (13%). Therefore one of the main methods of preventing mycotoxin contamination is the control of humidity (Santino, Poltronieri et al. 2005); unfortunately, this is not always possible under field conditions. Mycotoxins are produced by different fungal species over various agricultural stages, and some strains of fungi are capable of producing more than one mycotoxin even in conditions of low humidity, and can be enhanced by plant stress that predisposes to infestation. During storage, the presence of rodents and insects can damage the physical structure of grain, hence enhancing mold growth. Moreover, moisture migration, vapor condensation or water leaks can lead to mycotoxin contamination. The Canadian Food Inspection Agency proposes management practices to reduce the risk of mycotoxin contamination, such as: 1) limiting bird and insect damage, 2) harvesting grain as early as possible, 3) adequate drying and storage of grain, 4) proper ensiling conditions, 5) crop rotation and proper planning of the planting.

The ingestion of contaminated grains can lead to 1) diseases caused by growth of fungi on animal host, known as mycoses and 2) diseases produced by exposure to the toxic fungal metabolites, known as mycotoxicosis (Bennett and Klich 2003). Mycotoxicosis symptoms depend on the toxicity of the mycotoxin, level and time of exposure, animal species, age, sex, environment, nutritional status and possible synergistic effects of other chemicals. As a result, the diagnosis of mycotoxicosis is often very difficult. In order to classify a disease as a mycotoxicosis it is necessary to prove a dose-response relationship between disease and mycotoxin (Peraica, Radic et al. 1999, Bennett and Klich 2003, Kanora and Maes 2009). Mycotoxins can produce different effects according to the doses and time of exposition; high doses can cause acute illness or death and low doses can cause chronic toxicity (Abramson, Mills et al. 1997)

2.1 Major mycotoxins in Canada

Since the discovery of Aflatoxins in 1960, regulations have been established to protect consumers from the harmful effects of mycotoxins (Tabata, Kamimura et al. 1993). It has been estimated that around the world, approximately 25% of crops are contaminated by some mycotoxin, and some 99 countries have mycotoxin regulations (Figure 6) (FAO 2004). In temperate climes, such as Canada and U.S.A., the most common mycotoxins are zearalenone, fumonisins, ochratoxins, ergot and trichothecenes such as deoxynivalenol, nivalenol, T-2 toxin and HT-2 toxin (FAO 2004). They are considered important because they can negatively affect animal productivity, human health and international trade; it was estimated that mycotoxins lead to losses of $5 billion a year in Canada and the United States (Schmale III 2016).

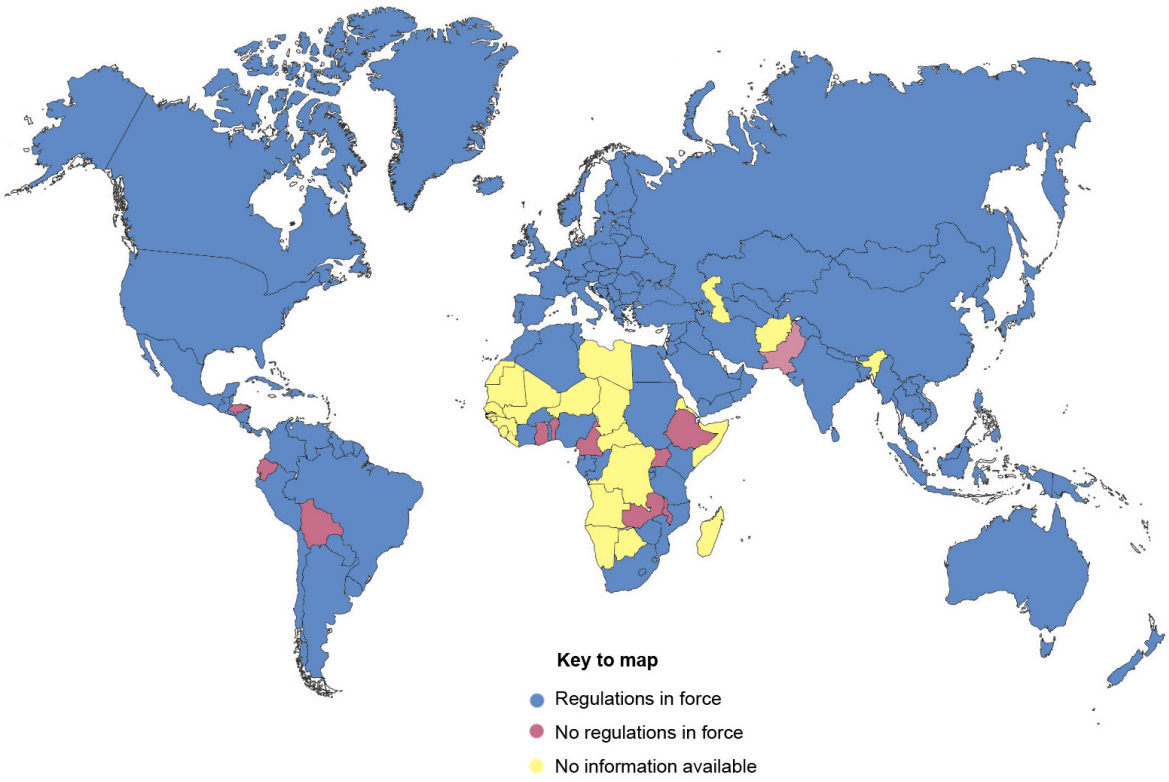

Figure 6: Countries with mycotoxin regulations. In blue, countries with mycotoxin regulation, the regulations may vary according to the region. In red, countries with no mycotoxin regulations. In yellow, countries that have no information available (FAO 2004).

In Canada, small grains such as wheat, barley, oat and triticale are commonly affected by Fusarium, especially F. graminearum, F. avenaceum and F. culmorum, which are responsible for the production of deoxynivalenol. Wheat can also be affected by F. sporotrichioides, F. poae, F. acaminatum, F. sporotrichioides that produce T-2 and HT-2 toxins. Additionally, oats can be contaminated with metabolites of the saprophyte Alternaria aternata, but little is known about these toxins (Richardson 2013). Canada has established regulations for aflatoxins and guidelines for deoxynivalenol and HT-2 toxin (Table 1). The Canadian grain commission had issued an official grain grading guide that

contains the procedure for grain inspection. The major methods of mycotoxin detection are enzyme-linked immunosorbent assays, thin-layer chromatography, high-performance liquid chromatography, gas chromatography, liquid chromatography, mass spectrometry and flow-injection liposome immunoanalysis (Cigic and Prosen 2009). The Association of Official Analytical Chemist and the European standardization committee has approximately 40 standardized methods of mycotoxin analysis.

Table 1: Permitted levels of mycotoxins in Canada. Legislated maximum tolerated levels of two different mycotoxins, deoxynivalenol and HT-2 toxin in Canada (FAO 2004).

Mycotoxin	Commodity	Mg/Kg
Deoxynivalenol	Unclean soft wheat for human consumption	2
	Diets for cattle and poultry	5
	Diets for swine, young claves and lactating dairy animals	1
HT-2 toxin	Diets for cattle and poultry	0.1
	Diets for daily animals	0.025

Ochratoxins (OT) are produced by Aspergillus ochraceus and A. niger in countries with tropical and sub-tropical weather, and by some Penicillium species such as P. verrucosum and P. carbonarius in countries with temperate climates such as the found in Canada (Milani 2013). P. ochraceus is favored by high temperatures (13ºC to 37ºC) while P. verrucosum can grow at 0ºC (Pitt 1987). OT are pentaketides made of dehydro-isocoumarin linked to β-phenylalanine (el Khoury and Atoui 2010). There are five different OT: A, B, C, α and β. OTA is the most prevalent, present in coffee, wheat, maize, barley, beans, potatoes, bread, dried fruit, wine, beer, cocoa, peas, rice and even meat, especially pork and poultry. In the 1970s, an evaluation in Canada showed that 56% of animal feed was contaminated with OTA in a range between 27-30 mg/kg (Pfohl-Leszkowicz A. 2007). OTA is nephrotoxic, hepatotoxic, teratogenic, genotoxic and immunotoxic (el Khoury and Atoui 2010). OTA in combination with the mycotoxin citrinin is believed to be one of the etiologic agents having caused the Balkan endemic nephropathy, as levels of OTA were higher in patients with Balkan nephropathy and with tumors in the urinary tract, but this has not been fully confirmed (Petkova-Bocharova and Castegnaro 1991). A possible way of combatting the effects of OTA has been studied in hens. Low levels of OTA (0.5ppm) produce intestinal damage and kidney lesions in chickens, while higher doses (2ppm) lead to weight loss, decreased egg production, increased water intake, diarrhea and renal disorder (Prior, O'Neil et al. 1980). A way to reduce some of OTA effects is the addition of ascorbic acid to the diet (Haazele, Guenter et al. 1993).

Ergot alkaloids are produced by Claviceps purpurea, C. paspalli and C. Fusiformis. They are also secondary metabolites of some strains of Penicillium, Aspergillus and

Rhizopus. Ergot alkaloids can affect a variety of grains and grass but rye is especially vulnerable. Ergot toxins are classified as alkaloids, due to the presence of a tetracyclic ergoline ring system (Schardl, Panaccione et al. 2006). The ingestion of feed contaminated with ergotamine-ergocristine alkaloids from Claviceps purpurea produces ergotism in two forms: 1) the convulsive form, affecting the central nervous system leading to dizziness, headaches, depression, drowsiness, unconsciousness, panic, hallucinations, delusions and psychosis; and 2) the gangrenous form, also known as ignis sacer, which affects blood supply to the extremities, producing burning sensation, hallucinations, dry gangrene and, finally, loss of the affected part (Peraica, Radic et al. 1999, Bennett and Klich 2003).

In the digestive system ergot produces diarrhea, nausea and vomiting. Ergot effects had a major impact on human history, since during the Middle Ages ingestion of contaminated grains resulted in an increase in gangrenous ergotism known as St Anthony's fire (due to the burning sensation at the extremities), which was cured by visiting St Anthony's church that was located in an Ergot free area (Alm and Elvevag 2013). Historians also believe that during the Salem witch hunt, women presenting symptoms of ergot consumption were charged as witches and burned at the stake. Despite the negative effects of consuming ergot, the fact that it is a strong vasoconstrictor makes it useful in the reproductive field as treatment of excessive bleeding during menstrual cycles, the start of menopause, after a miscarriage and during childbirth to help to expel the placenta and contract the uterus. But ergot may also exert negative effects on the reproductive system since it can suppress fertility, induce abortion and stillbirth, agalactia or hypo agalactia and is secreted in milk (Alm and Elvevag 2013).

Fumonisins were first described in 1988 and are produced by F. verticilliodes (D'Mello, Placinta et al. 1988), F. proliferatum and F. nygamai, and they are thought to be synthesized by condensation of the ammonic acid alanine into an acetate-derived precursor (Bennett and Klich 2003). At least 15 different fumonisins have been reported, plus some minor metabolites, but only two are considered toxic and have thus received more attention: B1 and B2. After ingestion, fumonisins can affect the brain, liver, lungs, pancreas, testes, thymus, gastrointestinal tract, blood cells and cause some fetal malformations including neural tube defects. Horses are the most susceptible species to this toxin and their recovery after its consumption is rare (Bennett and Klich 2003). Fumonisin B1 is the most common and is associated with equine leukoencephalomalacia even in doses as low as 8ppm (Ross, Rice et al. 1991). In stallions, doses between 7.5 (5,4 ppm) and 15 μM (10.8 ppm) of B1 can reduce sperm motility (Minervini, Lacalandra et al. 2010). In the case of ruminants, cattle appear to be the least susceptible but after exposure to high doses, B1 can act as an inhibitor of dihydroceramide synthase, a regulator of heat shock on oocytes in vitro (Kalo and Roth 2011). Fumonisin B2 has received less attention, and is usually studied together with B1. Interestingly B2 contamination is increasing widely, being present even in some wines, which makes it a potential risk for human health (Logrieco, Ferracane et al. 2010).

2.2 Fusarium toxins

Fusarium toxins are produced by over 50 species of Fusarium. Widely distributed large filamentous fungi, they are commonly found in cereals, particularly in wheat, barley and maize (Adeyeye S. 2016). They grow in temperate regions and a high percentage of infection takes place during preharvest, particularly during flowering, most likely in the month of July, leading to a disease known as fusarium head blight (FHB) or scab (Edwards, Pirgozliev et al. 2001). FHB affects mainly durum, spring wheat and barley, resulting in decreased yield, grade and end-use quality. One of the main characteristics of fusarium resistance is the ability of the spores and mycelium to remain in seeds, crop residues and roots of other crops, this characteristic complicating prevention (Schmale III 2016). The main strategies to avoid FHB are: 1) the use of "high" quality seed tested to determine the percentage of contamination and the Fusarium species present in the seed before planting, thus avoiding the introduction of a new Fusarium into a field, 2) crop rotation, avoiding cereal crops for at least one year, 3) selection of cereals with high resistance to FHB and 4) good residue management (Richardson 2013). Fusarium toxins include a range of mycotoxins including fumonisin and trichothecenes (Table 2).

Table 2: Main Fusarium species with associated toxins. Different species of Fusarium produce a wide range of toxins with various degrees of pathogenicty. Ex. Deoxynivalenol can be produced by *F.gramineaurum, F.culmorum, F.psedograminearum* and *F.crookwellense* (Richardson 2013).

Species	Pathogenicity	Toxins
F. graminearum	High	3ADON, 15ADON, DON, NIV, ZEA
F. culmorum	High	3ADON, 15ADON, DON, NIV, ZEA
F. avenaceum	High	Moniliformin, enniatins
F. sporotrichioides	Low, but common	T-2, HT-2, beauvericin
F. psedograminearum	Crown rot fungus that can spread to the head	DON
F. poae	Low	DAS
F. acuminatum	Low	T-2, enniatins
F. crookwellense	Low	DON, NIV

Trichothecenes are a class of mycotoxins that comprises a very large family of related chemicals produced by taxonomically unrelated fungal genera such as Fusarium, Mycothecium, Trichoderma, Trichothecium, Stachybotrys, Verticimonosporium and Cephalosporidium. Over 150 trichothecenes and trichothecene derivatives have been isolated and characterized; they are all non-volatile, low-molecular weight sesquiterpene

epoxides and many of them are toxic for eukaryotic cells (Rocha, Ansari et al. 2005). They are divided into four groups (A-D), but only the type A and B, produced by Fusaria, are sufficiently widespread and toxic to warrant attention. All trichothecenes have a tricyclic nucleus, and they owe their toxicity to an epoxide at C-12 and C-13, and some of them to a double bond at C-9 and C-10 (Rocha, Ansari et al. 2005). The toxic effect of trichothecenes is mainly attributed to their ability to bind to the 60S ribosomal subunit of eukaryotes, resulting in altered protein synthesis and apoptosis (Rocha, Ansari et al. 2005). The major products of the trichothecene biosynthetic pathway (Table 3) are in group A, T-2 toxin and diacetoxyscirpenol (DAS), and in group B, deoxynivalenol (DON) and nivalenol (NIV). The major effects of trichothecenes on animals include growth retardation, immunocompromise, feed refusal, vomiting, and reproductive disorders such as reduced ovarian function (Gutleb, Morrison et al. 2002, Rocha, Ansari et al. 2005).

Table 3: Major products of the trichothecene biosynthetic pathway. Principal classification of trichotecene products. Trichothecenes can be classify into two pincripal groups A and B (Rocha, Ansari et al. 2005).

Trichothecenes	
Group A	**Group B**
T-2 toxin and HT-2	Nivalenol
Diacetoxyscirpenol	Deoxynivalenol

2.2.1 Zearalenone

Zearalenone (ZEA) also known as F-2 toxin, is a non-steroidal estrogenic mycotoxin biosynthesized by a variety of Fusarium fungi, including F. graminearum, F. culmorum, F. cerealis, F. equiseti, F. crookwellense and F. semitectum (Bennett and Klich 2003). In Canada, corn is one of the grains most susceptible to ZEA, but it can also contaminate wheat, barley and soybeans, and cereal products such as flour, malt, soya beans and beer (Scott 1997). After absorption, ZEA can be metabolized into alpha zearalenol (α-ZEA), beta zearalenol (β-ZEA), alpha zearalanol (α-ZAL), beta zearalanol (β-ZAL) and zearalanone (Razani, Mohraz et al.) (Zinedine, Soriano et al. 2007). After absorption two major biotransformations take place: 1) hydroxylation through 3α- and 3β-HSD that results in α-ZEA and β-ZEA, and 2) conjugation of ZEA and its metabolites with glucuronic acid (Malekinejad, Agh et al. 2012). Studies using different species such as rat, mouse, pig, cow and hamster have shown that two different types of reductase are needed to convert ZEA into α-ZEA and β-ZEA, and that these reductases act according to tissue pH. However, there are also species differences, as pigs convert ZEA into α-ZEA, a more estrogenic metabolite, whereas cattle convert ZEA into β-ZEA (Olsen, Pettersson et al. 1981).

The effects of ZEA have been extensively studied. Despite its low level of toxicity, there are some interesting facts to take into account when animals are exposed to high doses of ZEA. At around 6,000 mg/l (6ppm) ZEA can be detected in milk (Prelusky, Scott et al. 1990), which suggests rapid distribution throught the organism probably due to the fast absorption of ZEA after ingestion. The percentage of intestinal absorbance depends on the species, pigs being one of the most sensitive, reaching an absorbance between 80-85% (Biehl, Prelusky et al. 1993).Experiments wherein Wistar rats were fed

with doses between 0.1 and 3 mg/kg, showed an increase in liver and uterine weights and also increased trabeculation of the femur (Becci, Voss et al. 1982). Doses ranging from 25 to 50 mg/kg administered to rats resulted in weight loss in a dose-dependent manner, followed by inflammation of the prostate gland, testicular atrophy, and formation of cysts in the mammary gland of males. Studies in rats and mice with doses ranging from 50 to 100 mg/kg showed liver lesions and subsequent development of hepatocarcinoma and pituitary carcinomas in both males and females. In females, dose-dependent estrogenic effects were seen in the uterus where ZEA led to fibrosis and also produced cystic ducts in mammary glands (Zinedine, Soriano et al. 2007). These estrogenic effects are caused by the resemblance of ZEA to E2 (Figure 7), allowing ZEA binding to estrogen receptors, a characteristic that classifies ZEA as a mycoestrogen with the capacity to affect reproductive organs and their function (Minervini and Dell'Aquila 2008).

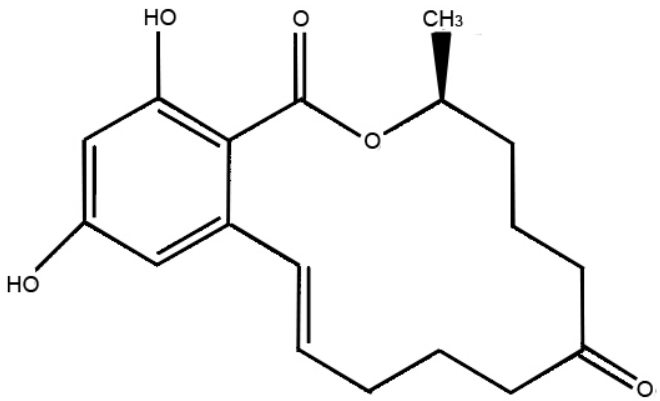

Figure 7: Zearalenone structure. Zearalenone molecular formula is $C_{18}H_{22}O_5$**.** (Maresca 2013).

In rats and mice, ZEA is able to decrease fertility and change the weight of adrenal, thyroid and pituitary glands (Zinedine, Soriano et al. 2007). In humans, ZEA was detected in hyperplastic and neoplastic endometrium suggesting its importance in endometrial cell proliferation and in carcinogenesis (Tomaszewski, Miturski et al. 1998). Low doses (3 ppm) in gilts produced inflamed and hyperestrogenized ovaries (Kanora and Maes 2009). Sows fed with 20 ppm presented an alteration in cycling, and intoxication with ZEA during pregnancy resulted in decrease of fetal weight and reduction of fetal survival (Zinedine, Soriano et al. 2007). It has been reported that ZEA has the capacity of reducing LH and P4 secretion (Tiemann and Danicke 2007). In males, ZEA can also affect the reproductive system; in pigs, ZEA can depress testosterone levels, the weight of testes and spermatogenesis (Zatecka, Ded et al. 2014). In cattle, hyperestrogenism has been associated with ZEA (Richard 2007).

However, these toxic effects have not prevented the use of ZEA as a treatment. A synthetic commercial formulation called zeranol (Ralgro) has been used to produce weight gain in sheep and cattle. This product has been banned in the European Union since 1989 but is still in use in Canada as implants of 3 pellets with 12 mg each resulting in a final dose of 36 mg of zeranol (Geldard and Wellington 1981). In humans, ZEA has been used to treat postmenopausal symptoms and, both ZEA and β-ZAL are oral contraceptives (Bennett and Klich 2003). ZEA is one of the best examples of a lowly toxic but biologically potent compound; in rats, a lethal dose is near 10,000 mg/kg while in pigs, the most sensitive species, it is around 5,000 mg/kg but, a dose as low as 1μg/kg can alter the reproductive system (Bennett and Klich 2003).

2.2.2 T-2 and HT-2

T-2 and HT-2 are produced by F. acaminatum, F. equiseti, F. poae and F. sporotrichoides, and can be produced at low temperatures ranging from 8 to 15°C. HT-2 is the major metabolite of T-2 and they are commonly found together in different cereals such as wheat, corn, barley, oats and rye. T-2 is one of the most toxic trichothecenes, but this depend on the species; in swine, chronic exposure to small doses of T-2 (12-16 ppm) results in weight loss, feed refusal, vomiting, small litters and small piglets, but weight loss can be observed with as low as 1ppm in chronic exposure (Weaver, Kurtz et al. 1978). In mice, T-2 can induce fetal toxicity, as trichothecenes cross the placental barrier, and contribute to thymic atrophy and underdevelopment of fetal liver (Holladay, Blaylock et al. 1993). To illustrate the mechanism of placenta transfer of T-2, BeWo cells, an immortalized cell line from a human placental choriocarcinoma, were used to demonstrate an active transport mechanism for T-2 uptake while HT-2 crossed the placenta by passive diffusion (Wang, Wang et al. 2014). Specifically, in the ovary, studies in swine have shown that T-2 reduced proliferation of GC in a dose-dependent manner. Moreover, T-2 alone and in combination with HT-2 also had an effect on the steroidogenic pathway by decreasing P4 production by GC (Maruniakova, Kadasi et al. 2014). As with other trichothecenes, T-2 has the ability to reduce protein and RNA synthesis (Murthy, Radouco-Thomas et al. 1985). It has also been suggested that T-2 interacts with cell membranes and alters calcium efflux, rubidium uptake and lactate dehydrogenase activity, at least in myoblasts (Bunner and Morris 1988).

2.2.3 Diacetoxyscirpenol (DAS)

DAS, also called anguidine, is a potent mycotoxin produced by F. poae, F. acuminatum, F. sambucinum and F. sporotrichoides in unharvested corn, silage, soybeans and some processed products from corn (Wyatt 1989). DAS can cause a variety of gastrointestinal, dermatological and neurological symptoms. For example, DAS has been associated with the alimentary toxic aleukia disease, the main symptoms of which are inflammation of the skin, vomiting, damage to hematopoietic tissues and, in acute cases, necrosis in the oral cavity, bleeding from the nose, mouth and vagina, and central nervous system disorders (Bennett and Klich 2003). In domestic animals, low doses (1-3.5 ppm) of DAS can cause symptoms such as feed refusal and decreased weight, diarrhea, lethargy and abdominal pain; an acute exposure to DAS results in severe inflammation of the gastrointestinal tract along with hemorrhage, edema, degeneration of bone marrow and death. In the reproductive field, DAS can lead to abortion and sterility in pigs (Jacobsen, Robert et al. 2007). One of the most important characteristics of DAS is the ability to be immunosuppressive; this leads to a decrease in resistance to infectious microbes, by inducing apoptosis and cell cycle arrest in lymphocytes. Induction of apoptosis by DAS involves DNA fragmentation along with caspase 8, 9 and 3 activation, with or without necrosis (Jun, Kim et al. 2007). Cell cycle arrest by DAS can be a consequence of downregulating Cdk4, important for cell cycle G1 phase progression, and cyclin B1, a regulatory protein involved in mitosis that interacts with Cdk1 to form a maturation-promoting factor; both are express predominantly during G2 of the cell cycle (Jun, Kim et al. 2007).

2.2.4 Nivalenol

NIV is produced by F. cerealis (also known as F. crookwellence) and F. poae, and is abundant in cereal crops such as wheat, corn, barley oats and rye, and in processed grains products including malt, beer and bread. It is considered one of the most toxic trichothecenes, ranking higher than fumonisin B1, and is approximately four times more toxic than DON in human blood cells (Minervini, Fornelli et al. 2004). In pigs, where 11-40% of ingested NIV can be absorbed in less than 8 hours, doses of 2.5 to 5 ppm led to vomiting and feed refusal, and at doses higher than 6 ppm additional signs of nephropathy, alterations in the gastrointestinal tract and increased levels of immunoglobulin A (Hedman, Pettersson et al. 1997, Murphy, MacKeigan et al.). In mice, peritoneal injection of high doses of NIV causes intrauterine growth retardation (Rocha, Ansari et al. 2005) and stillbirths due to severe damage to the placenta (Ito, Ohtsubo et al. 1986)NIV usually occurs with other mycotoxins (Kongkapan, Poapolathep et al. 2016), therefore most studies focus on the interaction between mycotoxins, especially with DON. In swine jejunal epithelial cells, NIV alone had the ability to reduce cell viability, which was enhanced in the presence of DON in a dose-dependent manner (Lam, Businelle et al. 2013). In a similar manner, experiments performed in mice showed the ability of NIV to reduce DNA synthesis up to 50% when it was combined with DON (Eriksen, Bogh et al. 2004). NIV is also considered an RNA and protein synthesis inhibitor, as it binds to the 60S subunit of eukaryotic ribosomes, disrupts the activity of peptidyl transferase and induces the ribotoxic stress response (RSR) (Lam, Businelle et al. 2013)

2.2.5 Deoxynivalenol

DON is also known by the colloquial name of vomitoxin because its marked ability to induce vomiting in different species by stimulating serotonergic activity in the central nervous system (Rocha, Ansari et al. 2005).It was first isolated (and named Rd toxin) by Japanese workers from barley infected with Fusarium in 1972 (Chiba, Nakano et al. 1972). DON is produced by F. graminearum and F. culmorum and is frequently found in grain, particularly in wheat and barley grown in North America (Abouzied, Azcona et al. 1991). DON is one of the less toxic trichothecenes but its importance lies in its prevalence in grain and its resistance to high temperatures (up to 350°C), making it stable during processing and cooking (Kushiro 2008). DON is usually presented along with two acetylated derivatives, 3ADON and 15ADON (Figure 8) (Maresca 2013). In large animals, DON is rapidly absorbed in the stomach and intestine, and intestinal epithelium is a target of DON toxicity, 15ADON is the most toxic of these derivatives, and inhibits intestinal epithelial cell proliferation and differentiation, disrupts barrier function and at the molecular level activates the RSR (Pinton, Tsybulskyy et al. 2012).Low doses of DON can cause anorexia while high doses induce emesis and long-term changes in feeding behavior; Swine are more sensitive to DON than are mice, poultry and ruminants, with males being more sensitive than females. Pigs can absorb up to 50% of ingested DON quite rapidly and peak plasma DON concentrations of DON are achieved four hours post ingestion, thus impacting organs such as lungs, heart, kidneys and brain(Prelusky, Scott et al. 1990, Goyarts and Danicke 2006)

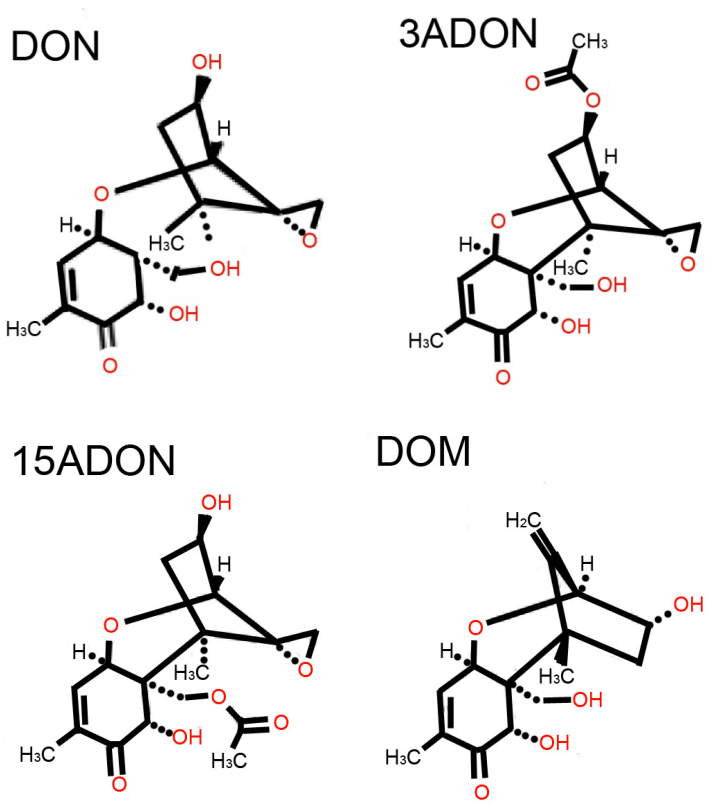

Figure 8: Chemical structure of deoxynivalenol and its major metabolites.

Deoxynivalenol molecular formula $C_{15}H_{20}O_6$, compared to its main metabolite formula, de-epoxy deoxynivalenol $C_{15}H_{20}O_5$ (Maresca 2013).

One of the main effects of DON is to stimulate or repress immune function, depending on dose and duration of exposure (Sobrova, Adam et al. 2010). In mice, DON induces autoimmune-like effects similar to human IgA nephropathy (Dong, Sell et al. 1991, Rotter, Prelusky et al. 1996). It has been proven that the increase in IgA induced by DON occurs through an increase in cytokine production by macrophages and T cells (Pestka, 2003 #48). Moreover, there is migration of cells secreting IgA into the systemic compartment that favors a change from IgG to IgA (Pestka, 2003 #48). Another important component of DON toxicity is the up-regulation of proinflammatory mediators

including metabolites of arachidonic acid. The metabolism of arachidonic acid is catalyzed by cyclooxygenase (PTGS), a rate-limiting enzyme that has two isoforms: PTGS1 that is constitutively expressed at low levels, and PTGS2 that is highly induced by mitogenic and proinflammatory stimuli and can be regulated at transcriptional and post-transcriptional levels (Dixon, Kaplan et al. 2000, Wadleigh, Reddy et al. 2000). An early study with mice lymphocytes showed that 12 hours of treatment with DON increased PTGS2 mRNA levels and apoptosis, suggesting a possible role of PTGS2 in apoptosis (Islam, Moon et al. 2002).

Studies have been performed on the effects of DON on the reproductive system. In mice, one of the first articles published reports that low doses of DON (0.37, 0.75 and 1.5 mg per kilogram of body weight) had no significant effect on mating, pregnancy rates or fetal malformations (Khera, Arnold et al. 1984). Nevertheless, a more recent study showed that DON can increase postnatal mortality in mice (Rocha, Ansari et al. 2005). In pigs, experiments performed to evaluate the effects of DON during in vitro oocyte maturation demonstrated that DON could significantly affect oocyte maturation by preventing oocytes from reaching metaphase II and inhibiting cumulus expansion at a dose of 1.88μM (Alm, Greising et al. 2002, Malekinejad, Schoevers et al. 2007, Schoevers, Fink-Gremmels et al. 2010)

Gastrointestinal bacteria play an important role in the effects of DON and its metabolites, and it is believed that differences in absorption rates between species depends on the ability of bacteria to transform DON into de-epoxy-DON (DOM-1) before it can be absorbed by the intestine (Worrell, Mallett et al. 1989). In ruminants, approximately 95-98% of DON is metabolized into DOM-1 but, until now, no negative

effects have been attributed to DOM-1 (Winkler, Kersten et al. 2015).

3. Ribotoxic stress response

The RSR can be simply defined as the process whereby translational inhibitors bind to ribosomes, rapidly activate mitogen-activated protein kinases (Moon and Pestka 2002) and induce apoptosis. This process is activated after exposure to fungi, bacteria and some plants, and DON is one of the most used activators for the study of RSR (Pan, Whitten et al. 2013). Upon mitogen-activated protein kinase (MAPK) activation, a variety of processes can take place, including cell growth, differentiation and apoptosis (Shifrin and Anderson 1999). The process through which DON triggers RSR has been well documented in different cell types, and 4 different ways to inhibit protein synthesis have been documented: 1) DON interferes with the peptidyl transferase function on the ribosome that leads to inhibition of protein synthesis(Ehrlich, Mangir et al. 1987); 2) DON causes degradation of 18S and 28S rRNA through apoptotic pathways(He, Zhou et al. 2012); 3) DON induces activation of protein kinase R (PKR) (He, Zhou et al. 2012), which phosphorylates eukaryotic initiation factor 2 a -subunit (eIF2α), resulting in inhibition of protein synthesis (Zhou, Lau et al. 2003); and 4) DON up-regulates microRNAs, which potentially target mRNAs for translation inhibition, especially for ribosomal proteins (He, Fan et al. 2010).

However, new studies show the ability of peptidyl transferase inhibitors to trigger a RSR by activating c-Jun N-terminal kinase 1 (JNK) (Iordanov, Pribnow et al. 1997), a stress-activated MAPK that signals the cellular response to stress, suggesting that trichothecene toxicity might not be a result of translational arrest (Iordanov, Pribnow et al.

1997). Studies in Jurkat T cells have shown that DON, NIV and T-2 have the ability to differentially up-regulate P38 and JNK, indicating that structural differences between trichothecenes influence the form of RSR they trigger, and that increased JNK/P38 activation is accompanied by increased activation of caspase-3 and apoptosis (Shifrin and Anderson 1999).

As mentioned before, trichothecenes have the ability to up-regulate COX2 and with this, lead to apoptosis. In murine macrophages, treatment with DON increased COX2 mRNA (Bunner and Morris) and protein levels, prostaglandin E2, and phosphorylation of extracellular signal-regulated kinases (ERK) 1/2, p38 MAPK and JNK 1/2 (Shifrin and Anderson 1999). The addition of p38 MAPK and ERK 1/2 inhibitor resulted in suppression of COX2 levels, suggesting a possible pathway for DON up-regulation of pro-inflammatory and apoptotic genes (Moon and Pestka 2002). Another possible player in COX2 up-regulation is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), a protein that controls transcription of DNA in cells that are under stress conditions, and is believed to regulate the immune response. Transfection of human and rat arterial smooth muscle cells with a dominant negative NF-kB reduced ERK 1/2 phosphorylation and blocked COX2 expression, suggesting that NF-kB is a potential RSR regulator (Yan, Subbaramaiah et al. 2000).

Other studies have focussed on the possibility that trichothecenes affect intercellular communications such as tight junctions. Treatment with DON has been shown to inhibit claudin-4, claudin-3 and ZO-1 protein levels in porcine intestinal cell lines (Pinton, Braicu et al. 2010, Diesing, Nossol et al. 2011). Genes involved in tight junctions, including C-terminal Src kinase (SRC), have been also studied. In

macrophages, MAPK activation after DON administration seems to be SRC-dependent, the use of protein phosphatase 1 (PP1) and protein phosphatase 1 (PP2) inhibitors significantly reduced phosphorylation of DON-induced P38 and c-jun, and completely abolished the increase of RSR related MAPK such as p38 (Zhou, Jia et al. 2005). PP1 reduced the expression of some transcription factors such as hematopoietic cell kinase (Hck), part of the Src family. Additional studies have shown an increase of Hck after treatment with DON, making the SRC-Hck a possible alternative pathway for RSR (Zhou, Jia et al. 2005).

The early stages of the RSR remain unclear. PKR is a 551 amino acid protein with two functional domains, an N-terminal double-stranded RNA (dsRNA) binding regulatory domain and a C-terminal kinase catalytic domain (Sunita, Schwartz et al. 2015). Its activity is mainly associated with the phosphorylation of eIF2a. Initially, it was identified and characterized as a translation inhibitor in an antiviral pathway, and nowadays it is known as a component of signal transduction pathways mediating cell growth, differentiation and early responses to stress (Donnelly, Gorman et al. 2013). Studies in the murine macrophage 264.7 cell-line demonstrated that DON increased PKR and eIF2a phosphorylation within 1 to 5 minutes (Zhou, Lau et al. 2003). Moreover, inhibition of PKR activity significantly reduced ERK 1/2, JNK and P38 phosphorylation, demonstrating that PKR lies upstream of these other MAPKs. The use of pharmaceutical inhibitors to determine the early stages of the RSR has revealed that inhibitors of protein kinase C (PKC), protein kinase A (PKA) and phospholipase C had no effect on ERK1/2 phosphorylation concluding that they do not interfere in early stages of the RSR (Zhou, Lau et al. 2003).

Chapter 2:
Hypothesis and objectives

Hypothesis and objectives

DON is known as a non-toxic fusarium toxin that is present in high concentrations in the field. Though its ability to trigger the RSR has been widely studied in different cell types, in cattle the effects of DON are completely unknown, partially because it is metabolized by bacteria in the rumen into its less toxic metabolite, DOM-1.

Our hypothesis is that both DON and DOM-1 can negatively impact bovine reproduction.

To test this hypothesis we established the following objectives:

1) To determine how and at what doses the major mycotoxin DON affects ovarian granulosa cell function and to elucidate its mechanism of action.

2) To determine how and at what doses the major mycotoxin DON and its metabolite affect ovarian theca cell function and to elucidate their its mechanism of action.

Chapter 3:
Articles

Article 1.

Effects of the mycotoxin, deoxynivalenol, on steroidogenesis and apoptosis in granulosa cells

Hilda M. Guerrero-Netro, Younès Chorfi and Christopher A. Price.

Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, J2S 7C6 QC Canada.

Correspondence should be addressed to CA Price: christopher.price@umontreal.ca

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Abstract

Mycotoxins can reduce fertility and development in livestock, notably in pigs and poultry, although the effect of most mycotoxins on reproductive function in cattle has not been established. One major mycotoxin, deoxynivalenol (DON), targets immune cells and activates the ribotoxic stress response involving MAPK activation, but also inhibits oocyte maturation in pigs. In the present study, we determined the effect of DON on bovine granulosa cell function using a serum-free culture system. Addition of DON inhibited oestradiol and progesterone secretion, and reduced levels of mRNA encoding estrogenic (CYP19A1) but not progestagenic (CYP11A1, STAR) proteins. Cell apoptosis was increased by DON, which also increased FASLG mRNA levels. The mechanism of action of DON was assessed by Western blotting and PCR experiments. Addition of DON rapidly and transiently increased phosphorylation of MAPK3/1, and resulted in a more prolonged phosphorylation of MAPK14 (p38) and MAPK8 (JNK). Activation of these pathways by DON resulted in time and dose-dependent increases in abundance of mRNA encoding the transcription factors FOS, FOSL1, EGR1 and EGR3. We conclude that DON is deleterious to granulosa cell function and acts through a ribotoxic stress response pathway.

Introduction

Fungal contamination of animal feed is a significant problem in many parts of the world (Marin et al. 2013). Contamination with Fusarium spp is common and results in significant accumulation of the mycotoxins zearalenone (ZEN) and deoxynivalenol (DON) among others (Rodrigues & Naehrer 2012). The actions of ZEN are well-known; it is estrogenic and affects the female reproductive system, particularly in pigs where symptoms include nymphomania, pseudopregnancy and ovarian atrophy (reviewed in (Cortinovis et al. 2013)). In cattle, ZEN intoxication is reported to result in reduced conception rates, possibly owing to toxic effects on the oocyte (Minervini et al. 2001).

Less is known about the effects of DON, a non-estrogenic compound, on the female reproductive system. In pigs, DON inhibited cumulus expansion and oocyte maturation in vitro (Alm et al. 2002, Malekinejad et al. 2007, Schoevers et al. 2010). The potential effects of DON on granulosa cells are unclear; DON has been reported to either increase or decrease progesterone secretion and to have a biphasic effect on oestradiol secretion from porcine granulosa cells in vitro (Ranzenigo et al. 2008, Medvedova et al. 2011). In cattle, there are preliminary data to suggest that DON increased levels of mRNA coding for the rate-limiting progestagenic enzyme cytochrome P450 cholesterol side-chain cleavage (CYP11A1), but had no effect on abundance of mRNA encoding the main estrogenic enzyme, cytochrome P450 aromatase (CYP19A1) in cultured granulosa cells (Pizzo et al. 2014); any effect of DON on abundance of mRNA encoding steroidogenic acute regulatory protein (STAR), the protein involved in the transport of cholesterol across the mitochondrial membrane, was not reported. To our knowledge, no other information is available 70 on the effects of DON on ovarian function in cattle.

The generally accepted mechanism of action of DON is through binding to ribosomes and initiation of the ribotoxic stress response (RSR). This involves activation of the p38 (MAPK14), ERK1/ 2 (MAPK3/ 1) and c-Jun N-terminal kinase (MAPK8) members of the mitogen-activated protein kinase (MAPK) family (Pestka 2008). As all these pathways are active in bovine granulosa cells (Evans & Martin 2000, Uzbekova et al. 2009, Abedini et al. 2015), we hypothesize that DON may activate one or more of these pathways to alter granulosa cell function. The objectives of the present study were to determine the effects of DON on granulosa cell steroidogenesis and apoptosis in a non-luteinizing serum-free culture system, and to determine whether DON acts through typical RSR intracellular signalling pathways, including early response genes.

Materials and methods

Cell culture

All materials were obtained from Life Technologies Inc. (Burlington, ON, Canada). Bovine granulosa cells were cultured in serum-free conditions that maintain oestradiol and progesterone secretion and responsiveness to FSH (Gutiérrez et al. 1997, Silva & Price 2000, Sahmi et al. 2004). Bovine ovaries were obtained from adult cows, independently of the stage of the oestrous cycle, at the slaughterhouse and transported to the laboratory at 30°C in phosphate-buffered saline (PBS) containing penicillin (100 IU) and streptomycin (100µg/ ml). Granulosa cells were harvested from follicles between 2-5mm diameter, and the cell suspension was filtered through a 150 mesh steel sieve (Sigma- Aldrich Canada, Oakville ON, Canada). Cell viability was assessed by Trypan blue dye exclusion. Cells were seeded into 24-well tissue plates (Sarstedt Inc., Newton,

NC, USA) at a density of 500,000 viable cells in 500µl DMEM/ F12 containing sodium bicarbonate (10mM), 25mM HEPES, sodium selenite (4ng/ ml), bovine serum albumin (BSA)(0.1%; Sigma-Aldrich), penicillin (100U/ ml) androstenedione (10^{-6}M) and bovine FSH (1ng/ ml starting on day 2, AFP5346D; National Hormone and Peptide Program, Torrance, CA, USA). Cultures were maintained at 37°C in 5% CO_2, 95% air for up to 6 days.

Experimental treatments

To determine the effects of DON on granulosa cell steroidogenesis, cells were treated from day 2 with 0, 1, 10 or 100ng/ ml DON (in methanol) or vehicle (methanol alone), and cells and media were recovered on day 6; these doses were based on concentrations of 2-14ng/ ml DON reported in serum of cattle fed a contaminated concentrate (Keese et al. 2008). Apoptosis was measured with an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich) after treating the cells with an effective dose of DON for 4 days. To assess the effect of DON on intracellular pathway activation, cells were treated on day 5 of culture with an effective dose of DON for 0, 5, 15, 30 and 120 min, and cells were recovered in RIPA buffer to measure the phosphorylation status of key protein kinases. The dose- and time-dependent effect of DON on abundance of mRNA of early-response genes was determined by treating cells on day 5 of culture with an effective dose of DON for 0, 1, 2, 4, 8 and 24h, and 116 by treating cells for 1 h with 0, 1, 10 or 100 ng/ ml DON. Cells were recovered for RNA extraction. All experiments were performed with three different pools of cells collected on different occasions.

Steroid assay

Oestradiol concentrations in conditioned medium were measured in duplicate as described (Jiang & Price 2012) with an antibody raised in rams (Sanford 1987). Intra and interassay coefficients of variation were 6% and 9%, respectively. Progesterone was measured in conditioned medium in duplicate as described (Bélanger et al. 1990, Price et al. 1995) with mean intra- and interassay coefficients of variation of 7.2% and 18%, respectively. Steroid concentrations in the culture medium were corrected for cell number by expressing the data per unit mass of total cell protein. The sensitivity of these assays was 10pg and 4pg per tube for oestradiol and progesterone, equivalent to 0.3 and 20ng/ μg protein, respectively.

Total RNA extraction and real -time PCR

After treatments medium was removed and total RNA was extracted using Trizol according to the manufacturer's instructions. Total RNA (0.5μg) was quantified by absorbance at 260nm and treated with 1U DNase (Invitrogen). RNA was reverse transcribed in the presence of 1mmol/ l oligo (dT) primer and 4U Omniscript RTase (Qiagen, Missauga, ON, Canada), 0.25mmol/ l dideoxynucleotide triphosphate (dNTP) mix and 19.33U RNase Inhibitor (GE Healthcare, Baie D´Urfé, QC, Canada) in a volume of 20μl at 37°C for 1h. The reaction was terminated by incubation at 93°C for 5min.

Real-time PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems, Streetsville ON, Canada) with Power SYBR Green PCR Master Mix. The bovine-specific primers have previously been published (Jiang et al. 2013). Common thermal cycling parameters (3min at 95°C, 40 cycles of 15s at 95°C, 30s at 59°C, and 30s

at 72°C) were used to amplify each transcript. Melting curve analyses were performed to verify product identity. Samples were run in duplicate and were expressed relative to histone H2AFZ as housekeeping gene. Data were normalized to a calibrator sample using the ΔΔCt method with correction for amplification efficiency (Pfaffl 2001).

Western blot

After challenge with DON, cells were washed with cold PBS and lysed in 100 μl/well cold RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, and protease inhibitor cocktail). The homogenate was centrifuged at 6000 g for 5 min at 4°C. The resulting supernatant was retained and stored at -20°C. Protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL, USA).

Samples were resolved on 12% SDS–polyacrylamide gels (10mg total protein/lane) and electrophoretically transferred onto nitrocellulose membrane in a Bio-Rad wet Blot Transfer Cell apparatus (transfer buffer: 39 mM glycine, 48 mM Trisbase, 1% SDS, 20% methanol, and pH 8.3). After transfer, the membranes were blocked in TTBS (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, and pH 7.5) for 1 h. Membranes were incubated overnight with the primary antibody (rabbit anti-rat MAPK3/1, #9102, 1:2000; rabbit anti-human phospho- MAPK3/1, #9101, 1:1000; rabbit anti-human MAPK14 #9211, 1:1000; rabbit anti-human phospho-MAPK14 #9215, 1:1000; rabbit anti-human MAPK8 #9251, 1:1000; and rabbit antihuman MAPK8 #9252, 1:1000; Cell Signaling Technology, Danvers, MA, USA) diluted in 5% BSA (total MAPK8) or TTBS (all other antibodies) at 4°C. The loading control was COX4I1

(#69359, 1:1000; Santa Cruz Biotechnology). After washing three times with TTBS, membranes were incubated for 2 h at room temperature with 1:10 000 anti-rabbit HRP-conjugated IgG (GE Healthcare Canada) diluted in TTBS. After five washes in TTBS, protein bands were revealed by ECL (Millipore, Billerica, MA, USA) using a gel imaging system (ChemiDoc XRS system, Bio-Rad). Semiquantitative analysis was performed using the Bio-Rad ChemiDoc XRS Software.

Statistical analysis

All statistical analyses were performed using the JMP Software (SAS Institute, Cary, NC, USA). Data were transformed to logarithms if they were not normally distributed (Shapiro–Wilk test). At instances where main effects were significant, the effect of time or treatment was tested using the Tukey–Kramer honest significant difference (HSD) test. The data are expressed as least square means ± SEM.

Results

DON suppressed steroid secretion and steroidogenic enzyme gene expression

We first assessed the effect of DON on steroidogenesis. Cultured bovine granulosa cells were challenged with 1, 10, and 100 ng/ml DON for 4 days. At the dose of 100 ng, DON significantly inhibited E2 and progesterone secretion (Fig. 1), and potently suppressed CYP19A1 mRNA levels but did not alter CYP11A1 and STAR mRNA levels (Fig. 2).

DON increases granulosa cell apoptosis

The ability of DON to inhibit E2 secretion prompted us to determine the effect of DON on granulosa cell health. Addition of an effective dose of DON (100 ng/ml) for 4 days increased the proportion of apoptotic cells by 15% (Fig. 3). In a subsequent experiment, DON was added for 24 h and increased levels of mRNA encoding the apoptosis-related genes FASLG and GADD45B (Fig. 3).

DON activates pathways related to RSR in granulosa cells

The main mechanism of action of DON is through RSR pathways; therefore, we assessed the activation of these pathways in granulosa cells. The addition of DON caused a rapid and transient increase in MAPK3/1 phosphorylation within 15 min and an increase in MAPK14 phosphorylation that was significant at 30 and 60 min (Fig. 4A and B). Furthermore, DON increased both MAPK8 (Fig. 4 C) and phospho-MAPK8 levels (Fig. 4 D), such that the ratio of phosphorylated to total MAPK8 did not change (not shown). Our next step was to determine whether the activation of these pathways by DON affected expression of specific target genes. Addition of DON increased EGR1, EGR3, FOS , and FOSL1 mRNA levels within 1–2 h, and levels declined to control values by 8 h for all genes. DON also increased PTGS2 mRNA levels, and this was not significant until 24 h of treatment (Fig. 5). We confirmed the effect on the early response genes with a dose–response study at 1 h of treatment, and mRNA levels of all four target genes were increased at the dose of DON that inhibited E2 secretion (Fig. 6).

Discussion

The results of this study clearly demonstrate that DON can have a significant negative impact on granulosa cell health and function, notably on E2 secretion and CYP19A1 mRNA levels, and that DON acts through typical RSR pathways involving activation of MAPK3/1, MAPK8, and MAPK14 kinases.

Reports on the effects of DON on steroidogenesis are contradictory. In pigs, DON increased E2 secretion and CYP19A1 mRNA levels at a concentration of 10 ng/ml and inhibited both at a concentration of 100 ng/ml (Ranzenigo et al. 2008), whereas, in bovine granulosa cells, DON (1000 ng/ml) increased CYP19A1 mRNA levels (Pizzo et al. 2014). In this study, no stimulatory effect of DON on E2 secretion or CYP19A1 mRNA levels was observed, although the decrease in E2 secretion and CYP19A1 mRNA levels with 100 ng/ml DON was consistent with the study by (Ranzenigo et al. 2008). Similarly, for progesterone, 100 ng/ml DON increased progesterone secretion in one study carried out on pigs (Medvedova et al. 2011), but inhibited secretion from granulosa cells in another study (Ranzenigo et al. 2008) and in this study with bovine cells. One difference between the previous and present studies is the use of serum in the culture medium in all previous studies; serum is known to alter granulosa cell steroidogenesis in vitro (Gutiérrez et al. 1997).

In pig granulosa cell cultures, DON at the doses used increased cell numbers and abundance of the proliferation marker PCNA without increasing apoptosis (Ranzenigo et al. 2008, Medvedova et al. 2011), whereas, in this study, DON increased the rate of apoptosis. This difference is again likely to be owing to the absence of serum in the current culture system, as this reduces the rate of proliferation of granulosa cells

(Gutiérrez et al. 1997). As an increase in the incidence of apoptotic granulosa cells (Irving-Rodgers et al. 2001) and a decrease in E2 secretion (McNatty et al. 1984) are characteristics of atretic follicles in vivo, the ability of DON to increase apoptosis and decrease E2 secretion suggests that it may be able to cause or promote follicle atresia.

The addition of DON increased the abundance of FASLG and GADD45B mRNAs, both of which have been linked to apoptosis. While FASLG is well known to induce apoptosis in a variety of cell types including granulosa cells (Porter et al. 2000), the role of GADD45B is much less clear. Granulosa cells of atretic bovine follicles contain less GADD45B mRNA than do those of healthy follicles (Mihm et al. 2008), and pro-apoptotic factors such as fibroblast growth factor 18 (FGF18) decrease GADD45B mRNA levels in granulosa cells in vitro (Portela et al. 2010), whereas mitogenic factors such as FGF2 increase GADD45B mRNA levels (Jiang et al. 2011). Thus, the increase in GADD45B mRNA levels with increased apoptosis is not consistent with the previous data, and supports the suggestion that the regulation of GADD45B mRNA abundance is context (ligand?) specific (Salvador et al. 2013). It has been suggested that GADD45B may enhance or mitigate FAS-mediated apoptosis, depending on the cell type (Zazzeroni et al. 2003, Cho et al. 2010), thus the increase in GADD45B mRNA levels occurring with increased FASLG mRNA abundance may be part of either the apoptotic mechanism or a DNA repair mechanism.

The intracellular pathways activated by DON in a variety of non-reproductive cell types include MAPK3/1, MAPK8, and MAPK14. In this study, we demonstrate for the first time that DON activates these MAPKs in granulosa cells. The time-course of DON-induced phosphorylation observed herein is similar to that observed in a number of cell

types, including murine macrophages (Moon & Pestka 2002, Pan et al. 2013), human intestine epithelial cells (Moon et al. 2007), and mouse skin (Mishra et al. 2014) among others. The activity of these pathways was demonstrated by the time- and dose-dependent increase in levels of mRNA encoding the transcription factors EGR1 and FOS; DON has previously been shown to increase Egr1 and Fos mRNAs in various cell lines (Moon et al. 2007, Nielsen et al. 2009) and mouse spleen (Kinser et al. 2004). In this study, we also identified FOSL1 and EGR3 as targets of DON activity, which are novel findings. EGR3 is a zinc finger-containing transcription factor that has been reported in breast cancer cells and in the mouse oocyte (Inoue et al. 2004, Shin et al. 2014). Although it is well known that EGR1 mRNA abundance in granulosa cells is increased by ligands including gonadotropins and growth factors (Espey et al. 2000, Russell et al. 2003, Sayasith et al. 2006, Jiang et al. 2013), we are unaware of any reports demonstrating the regulation of EGR3 mRNA abundance in granulosa cells. Interestingly, EGR3 but not EGR1 was shown to increase FASLG expression in T cells and fibroblasts (Mittelstadt & Ashwell 1998, Yoo & Lee 2004); therefore, the effect of DON on FASLG expression may be mediated in part through EGR3.

E2 is a major determinant of follicle development and decreases granulosa cell apoptosis in rodents in vivo (Billig et al. 1993). Studies with bovine granulosa cells have demonstrated that E2 can overcome the apoptotic effect of ligands such as FASLG and FGF18 (Quirk et al. 2006, Portela et al. 2015). Of the main RSR pathways, MAPK3/1 is known to alter CYP19A1 mRNA levels; inhibition of MAPK3/1 phosphorylation increased CYP19A1 mRNA levels in bovine and rodent granulosa cells (Moore et al. 2001, Silva et al. 2006) and reduced the ability of tumor necrosis factor alpha to increase

apoptosis (Morales et al. 2006). Therefore, one mechanism for the action of DON might be via MAPK3/1 inhibition of CYP19A1 expression and E2 secretion, which then predisposed cells to apoptosis.

In conclusion, this study demonstrates that, in vitro, the mycotoxin DON has a negative impact on granulosa cell steroidogenesis and survival, and that the mechanism of action probably involves activation of the RSR. The potential impact of natural intoxication with DON on fertility in cattle warrants investigation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

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Figures

Figure 1.

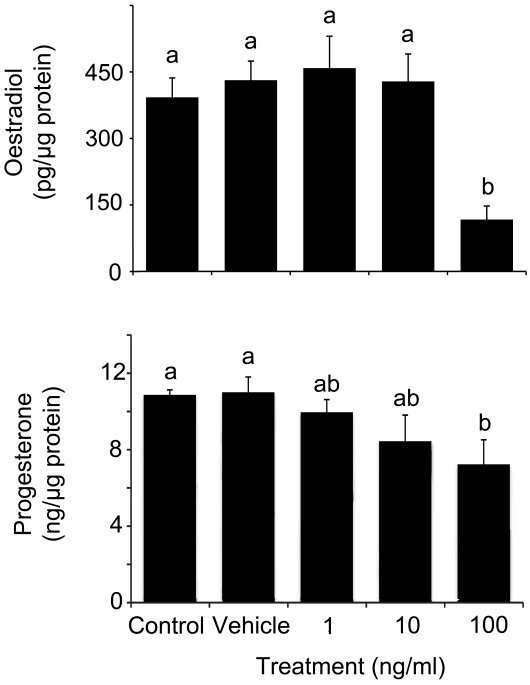

Fig. 1. Effect of DON on estradiol and progesterone secretion from bovine granulosa cells in a serum-free medium. Cells were cultured for 4 days with the doses of DON shown, and steroid in the medium measured by RIA. Concentrations were corrected for cell number (total cell protein) and represent the amount secreted during the last 2 days of culture. Data are expressed as means (± SEM.) of four independent cultures, and bars without common letters are significantly different ($P<0.05$, Tukey–Kramer HSD). Vehicle, methanol.

Figure 2.

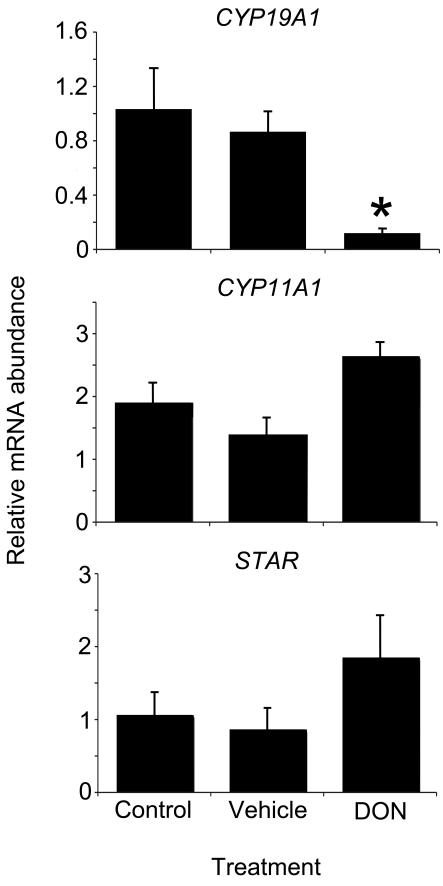
CYP19A1
1.6
1.2
0.8
0.4
0
*
CYP11A1
3
2
1
0
STAR
3
2
1
0
Relative mRNA abundance
Control
Vehicle
DON
Treatment

Fig. 2. Effect of DON on abundance of mRNA encoding steroidogenic proteins in bovine granulosa cells. Cells were cultured for 4 days with 100 ng/ml DON or methanol (vehicle), and RNA collected for real-time PCR. Data are expressed relative to a calibrator sample using the DDCt method with correction for amplification efficiency and are presented as means (± SEM.) of three independent cultures. Asterisk denotes treatment significantly different from control ($P<0.05$, Tukey–Kramer HSD).

Figure 3.

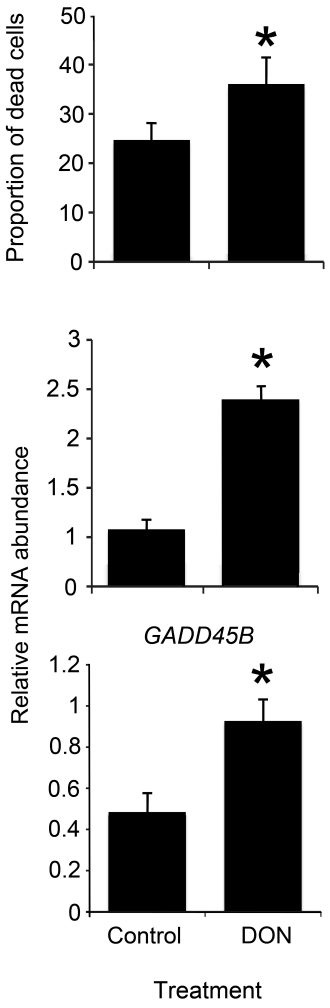
Proportion of dead cells
50
40
30
20
10
0
*
Relative mRNA abundance
3
2.5
2
1.5
1
0
*
GADD45B
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
DON
Treatment

Fig. 3. Addition of DON increased the proportion of dead cells and abundance of FASLG and GADD45B mRNA in bovine granulosa cells. Cells were cultured for 4 days with 100 ng/ml DON and recovered for either the measurement of apoptosis by flow cytometry (Annexin-V apoptosis kit) or for RNA measurement by real-time PCR. Data are expressed as means (± SEM.) of three independent cultures. Asterisk denotes treatment significantly different from control ($P<0.05$, Student's t-test).

Figure 4.

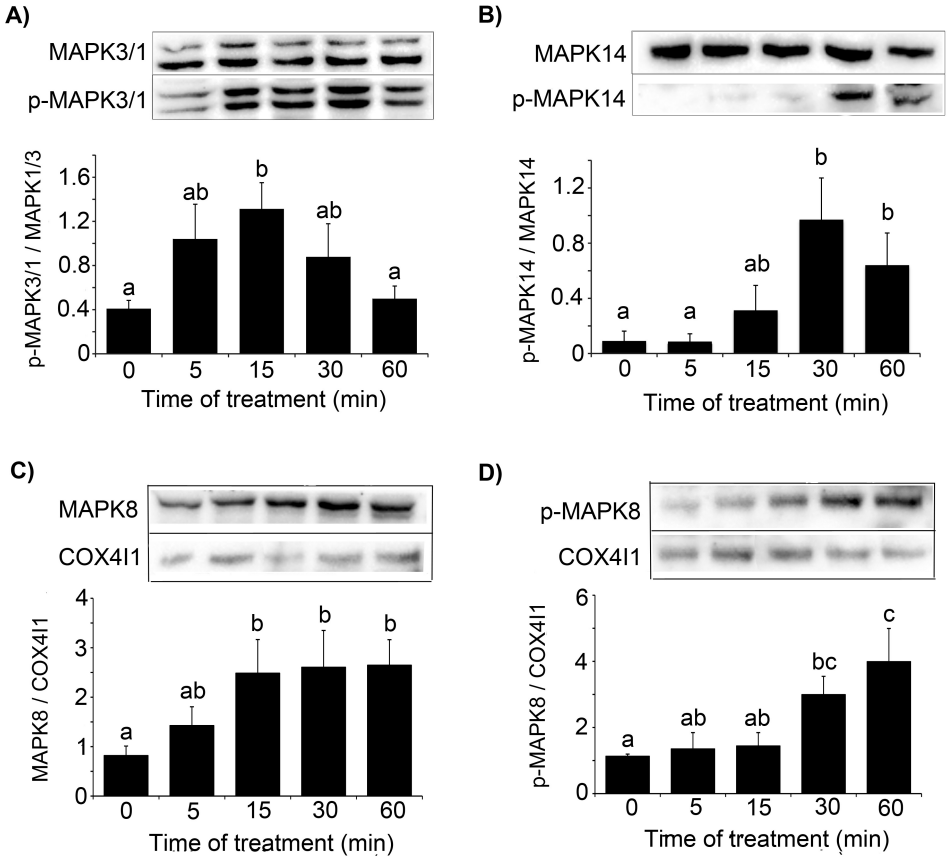
A)
MAPK3/1
p-MAPK3/1
p-MAPK3/1 / MAPK1/3
1.6
1.2
0.8
0.4
0
a
ab
b
ab
a
0
5
15
30
60
Time of treatment (min)
B)
MAPK14
p-MAPK14
p-MAPK14 / MAPK14
1.2
0.8
0.4
0
a
a
ab
b
b
0
5
15
30
60
Time of treatment (min)
C)
MAPK8
COX4I1
MAPK8 / COX4I1
4
3
2
1
0
a
ab
b
b
b
0
5
15
30
60
Time of treatment (min)
D)
p-MAPK8
COX4I1
p-MAPK8 / COX4I1
6
4
2
0
a
ab
ab
bc
c
0
5
15
30
60
Time of treatment (min)

Fig. 4. Intracellular pathways activated by DON in granulosa cells. Bovine granulosa cells were cultured in a serum-free medium and on day 5 were challenged with DON (100 ng/ml) for the times shown. Total cell protein was recovered for western blotting with antibodies against total and phosphorylated forms of (A) MAPK3/1, (B) MAPK14, and (C and D) MAPK8. Representative blots from one replicate are shown above the graphs, and samples were loaded in the same order as in the graphs. Data are represented as the ratio of phosphorylated: total protein for MAPK3/1 and MAPK14, and of each form of MAPK8: COX4I1 (housekeeping protein), and are means (± SEM) of three independent cultures; bars without common letters are significantly different ($P<0.05$, Tukey–Kramer HSD).

Figure 5.

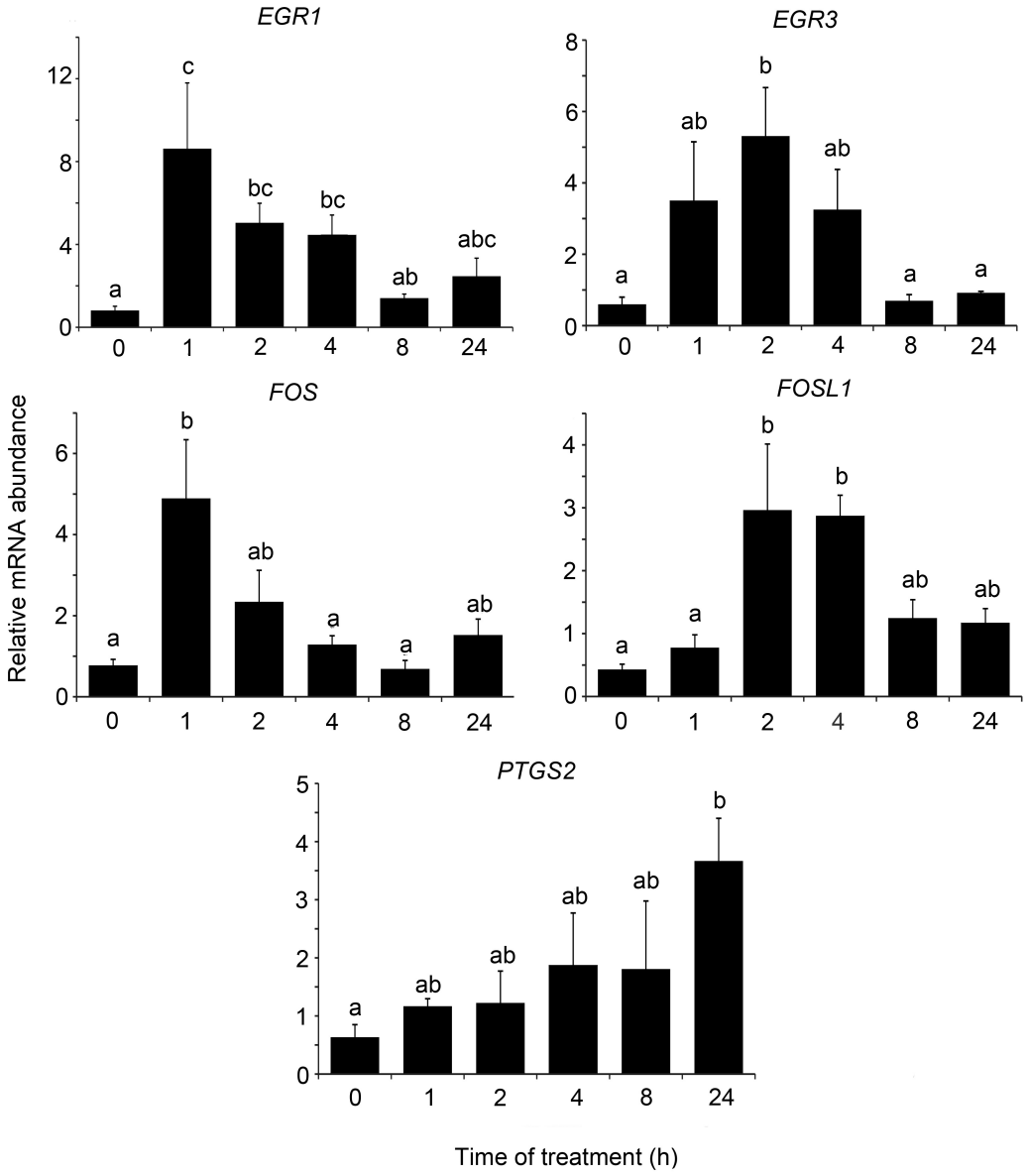
EGR1
EGR3
FOS
FOSL1
PTGS2
Relative mRNA abundance
Time of treatment (h)
0
1
2
4
8
24

Fig. 5. Acute effect of DON on early response genes in bovine granulosa cells. Cells were cultured in a serum-free medium and on day 5 were challenged with DON (100 ng/ml) for the times shown. Cells were recovered for RNA measurement by real-time PCR. Data are expressed relative to a calibrator sample using the DDCt method with correction for amplification efficiency, and are presented as means (± SEM) of three independent cultures. Bars without common letters are significantly different ($P<0.05$, Tukey–Kramer HSD).

Figure 6.

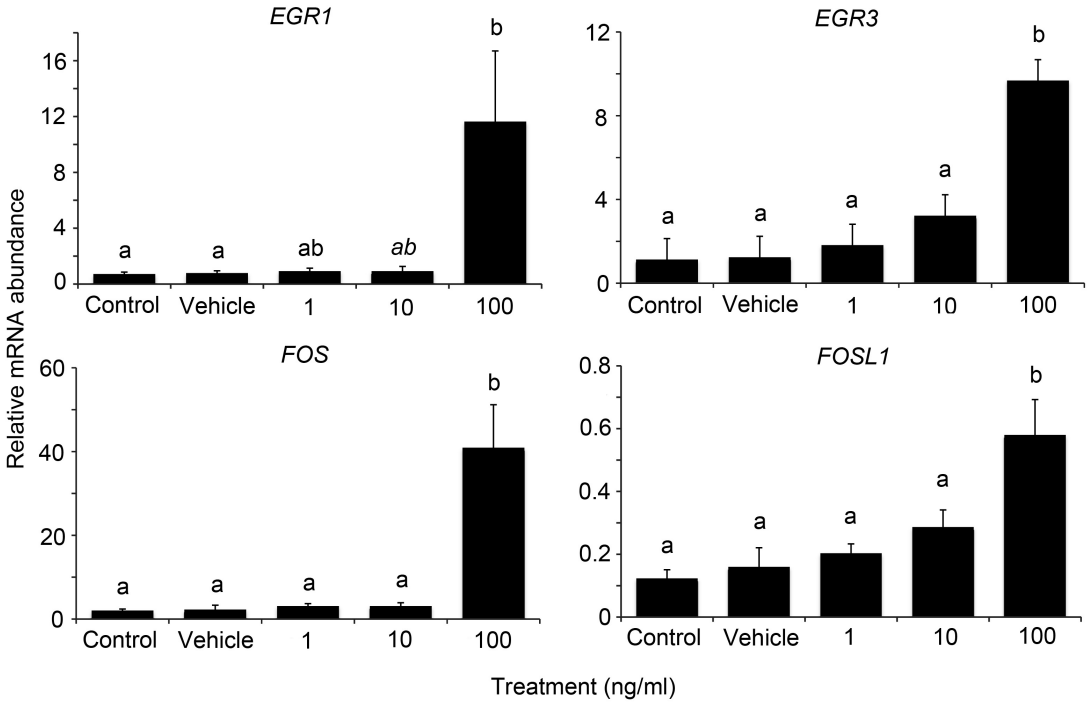
EGR1
EGR3
FOS
FOSL1
Relative mRNA abundance
Treatment (ng/ml)
Control
Vehicle
1
10
100
a
a
ab
ab
b
a
a
a
a
b
a
a
a
a
b
a
a
a
a
b

Fig. 6. Effect of DON on early response genes is dose dependent. Bovine granulosa cells were cultured in a serum-free medium and on day 5 were challenged withDONfor 1 h at the doses shown. Cells were recovered for RNA measurement by real-time PCR. Data are expressed relative to a calibrator sample using the DDCt method with correction for amplification efficiency and are presented as means (± SEM) of three independent cultures. Bars without common letters are significantly different ($P<0.05$, Tukey–Kramer HSD). Vehicle, methanol.

Article 2

The mycotoxin metabolite deepoxy-deoxynivalenol increases apoptosis in ovarian theca cells and induces follicle atresia.

Hilda M. Guerrero-Netro[1], Marcos H. Barreta[2], Valério M. Portela[2], Andre F. Goetten[2], Younès Chorfi[1] and Christopher A. Price[1]

[1] Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, J2S 7C6, QC Canada

[2] Laboratório de Fisiologia da Reprodução Animal, Universidade Federal de Santa Catarina, Curitibanos, SC Brazil.

Correspondence should be addressed to C A Price;

christopher.price@umontreal.ca

tel: 450-773-8521

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Abstract

The mycotoxin deoxynivalenol (DON) has been shown to inhibit ovarian granulosa cell function in cattle in vitro, and it is not known whether DON affects theca cell function. In ruminants, ingested DON is metabolized to deepoxy-DON (DOM-1) in the rumen and this is the predominant form in blood and follicular fluid. As DOM-1 is generally considered to be non-toxic, no studies on the effects of DOM-1 on the reproductive system have been reported. The objectives of this study were to determine the effects of DOM-1 and DON on bovine theca cells. Addition of DON decreased progesterone secretion at doses as low as 1 ng/ml but had no effect on testosterone secretion. Addition of DOM-1 inhibited progesterone and testosterone secretion at doses as low as 1 ng/ml. Treatment of cells with 1 ng/ml DOM-1 increased the proportion of atretic cells, whereas DON had no effect at this concentration. Addition of DON or DOM-1 (1 ng/ml) stimulated phosphorylation of PKR, MAPK3/1 and AKT. However, these compounds had divergent effects on MAPK14 (p38) phosphorylation : DON inhibited and DOM-1 stimulated pMAPK14 abundance. DON increased levels of mRNA encoding early-immediate genes *EGR1*, *EGR3* and *FOS*, whereas DOM-1 was without effect. The impact of DOM-1 on ovarian function in-vivo was tested by injecting DOM-1 directly into a growing follicle: DOM-1 treated follicles regressed whereas PBS-injected follicles continued to grow. These data demonstrate that DOM-1 has a major impact on theca cell function and follicle development in cattle.

Introduction

Contamination of animal feed with *Fusarium* fungi is a common occurrence in many parts of the world and leads to the accumulation of mycotoxins including zearalenone (ZEN) and deoxynivalenol (DON) (Rodrigues and Naehrer 2012, Marin, Ramos et al. 2013). ZEN is a highly estrogenic compound and ingestion has major repercussions for the reproductive system. Pigs are particularly sensitive to ZEN, and intoxication leads to nymphomania, pseudopregnancy and ovarian atrophy (reviewed in (Cortinovis, Pizzo et al. 2013)). ZEN has also been shown to reduce conception rates in cattle by decreasing the developmental competence of the oocyte (Minervini, Dell'Aquila et al. 2001).

DON is not estrogenic, but impacts cells by inducing ribotoxic stress response (RSR). In the current model of DON action, DON binds to ribosomal RNA and induces autophosphorylation and activation of double-stranded RNA-activated protein kinase (PKR; gene symbol EIF2AK2) (Zhou, He et al. 2014) and hematopoietic cell kinase (HCK) (Zhou, Jia et al. 2005). These events result in downstream activation of the p38 (MAPK14), ERK1/2 (MAPK3/1), and c-Jun N-terminal kinase (MAPK8) members of the mitogen-activated protein kinase (MAPK) family (Pestka 2008). DON also activates G protein-coupled receptors in the intestine (Zhou and Pestka 2015).

The effects of DON on the reproductive system have not been studied as extensively as those of ZEN. Addition of DON to porcine oocyte-cumulus complexes inhibited cumulus expansion and oocyte maturation (Alm, Greising et al. 2002, Malekinejad, Schoevers et al. 2007, Schoevers, Fink-Gremmels et al. 2010), and DON has been reported to either increase or decrease estradiol and progesterone secretion from

porcine granulosa cells *in vitro* (Ranzenigo, Caloni et al. 2008, Medvedova, Kolesarova et al. 2011, Cortinovis, Caloni et al. 2014). Ruminants are generally less affected than are pigs owing largely to the metabolism of DON to deepoxy-DON (DOM-1) in the rumen (Wu, Dohnal et al. 2010); although DOM-1 is less toxic than DON *in vitro* (Sundstøl Eriksen, Pettersson et al. 2004), it occurs in blood and in ovarian follicular fluid at greater concentrations than DON (Winkler, Kersten et al. 2014). It has recently been demonstrated that DON inhibits steroidogenesis in bovine granulosa cells and increases the rate of apoptosis *in vitro* (Guerrero-Netro, Chorfi et al. 2015, Pizzo, Caloni et al. 2016), suggesting that DON may affect follicle growth in cattle.

Although the two somatic cell layers of the follicle, the granulosa and the theca layers, are critical for follicle health, it is generally accepted that the granulosa cell layer is most affected by follicle atresia. Functional changes include a reduction in estradiol secretion (Ireland and Roche 1983, Price, Carrière et al. 1995) and expression of genes encoding cytochrome P450aromatase (gene symbol, CYP19A1), an enzyme critical for estradiol synthesis (Bao and Garverick 1998), as observed following treatment with DON (Guerrero-Netro, Chorfi et al. 2015). When antral follicles undergo atresia the theca layer remains relatively unaffected (Irving-Rodgers, van Wezel et al. 2001), nevertheless follicular estradiol secretion is entirely dependent on the production of androgens by the theca layer, suggesting that a toxin that alters theca function may have an impact on follicle development.

There are no studies to suggest whether DOM-1 can affect follicle function, and it is unknown whether any mycotoxin affects theca cells. The objectives of the present study were to determine the effects of DON and of DOM-1 on theca cell steroidogenesis

and apoptosis in a non-luteinizing serum-free culture system, and to determine the main pathways through which they act.

Materials and methods

Cell culture

All materials were obtained from Life Technologies Inc. (Thermo Fisher Scientific, Burlington, ON, Canada) unless otherwise stated. Bovine theca cells were cultured in serum-free conditions that maintain testosterone and progesterone secretion and responsiveness to LH (Glister, Richards et al. 2005). Bovine ovaries were obtained from adult cows, independently of the stage of the estrous cycle at the slaughterhouse and transported to the laboratory at 30 ºC in phosphate-buffered saline (PBS) containing penicillin (100 IU) and streptomycin (100 μg/mL). Follicles (4 – 6 mm diameter) were bisected within the ovarian stroma, gently scraped to remove granulosa cells, and the theca 'shells' were peeled from the stroma with forceps. Pooled theca layers were incubated with collagenase (type IV, 1 mg/mL; Sigma-Aldrich, Oakville, ON, Canada) and trypsin inhibitor (100 mg/mL; Sigma) in a water bath at 37 ºC for 45 min with agitation every 10 min. The resulting supernatant was filtered through a 150 mesh steel sieve (Sigma-Aldrich), centrifuged (800 g for 10 min) and the pellet resuspended in PBS before being subjected to an osmotic shock treatment to remove red blood cells. After washing, cells were resuspended in culture medium McCoy's 5A modified medium supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 1 μg/mL fungizone, 10 ng/mL bovine insulin, 2 mM L-glutamine, 10 mM HEPES, 5 μg/mL apotransferrin, 5 ng/Ml sodium selenite, and 0.1 % BSA (all purchased from Sigma-Aldrich) and LH. Cell

viability was assessed by Trypan blue dye exclusion, seeded into 24-well tissue plates (Sarstedt Inc., Newton, NC, USA) at a density of 250,000 viable cells in 1 mL, and cultured at 37 ºC in 5 % CO_2, 95 % air for up to 6 days with medium changes every 2 days.

Experimental treatments in vitro

Certified Biopure Standard grade DON and DOM-1 in acetonitrile were purchased from Romer Labs (Tulln, Austria), and were reconstituted in methanol for cell culture studies. To determine the effects of mycotoxins on theca cell steroidogenesis, cells were treated from day 2 with 0.005 - 100 ng/mL DON or DOM-1 (each in maximum volume of 1 mL methanol), or vehicle (1 mL methanol), and cells and media were recovered on day 6. Apoptosis was measured with an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich) after treating the cells with an effective dose of DON or of DOM-1 for 4 days. To assess the effect of DON and of DOM-1 on intracellular pathway activation, cells were treated on day 5 of culture with 1 ng/mL DON or DOM-1 for 0, 5, 15, 30 and 60 min, and cells were recovered in RIPA buffer to measure the phosphorylation status of key protein kinases. In some studies, cells were incubated for 1 h with pharmacological inhibitors of PKR (C16, Sigma-Aldrich) or HCK (PP2; Santa Cruz Biotechnology, Dallas, TX, USA) before adding DON or DOM-1. The effect of DON or of DOM-1 on abundance of mRNA of early-response genes was determined by treating cells on day 5 of culture with 1 ng/mL DON or DOM-1 for 0, 1, 2, 4 and 8 h. Cells were recovered for RNA extraction. All experiments were performed with three different pools of cells each collected on a different occasion.

Experimental treatments in vivo

All experimental procedures using cattle were reviewed and approved by the Universidade Federal de Santa Catarina Animal Care and Use Committee. Cycling crossbred heifers (*Bos taurus taurus x B t indicus)* with a body condition score of 3 or 4 on a scale from 1 (thin) to 5 (Adeyeye S.) were used in this study. The experiment was performed in spring.

Estrous cycles were synchronized by the placement of a progesterone releasing intravaginal device (1 g progesterone, DIB; Coopers, São Paulo, Brazil) and i.m. injections of 2 mg estradiol benzoate (Sincrodiol, Ourofino, Cravinhos, São Paulo, Brazil) and 500 mg sodium cloprostenol (Sincrocio, Ourofino) to induce regression of dominant follicles and a recruitment of a new follicular wave. Four days later, the progesterone devices were removed and ovaries were monitored daily for at least 3 days to ensure successful induction of a new follicle wave. Ovaries were examined by transrectal ultrasonography using an 8-MHz linear-array transducer (Mindray M5 Vet; Shenzhen Mindray Bio-medical Electronics Co. Ltd., Shenzhen, China) and all follicles larger than 5 mm were recorded on three virtual slices of the ovary, allowing a three-dimensional localization and monitoring of individual follicles during the follicular wave. Only cows without a corpus luteum in the ultrasound image were included in the study.

When the largest follicle of the growing cohort reaches a diameter larger than 7 mm it is reliably identifiable as the sole future dominant follicle (Ferreira, Gasperin et al. 2011). Following identification, it was injected with PBS (n=3) or DOM-1 diluted in PBS (n=4) to a final intrafollicular concentration of 100 ng/ml. Intrafollicular injections were performed with a double needle system and guided by ultrasound as described in detail

(Ferreira, Oliveira et al. 2007); under epidural anesthesia, the needle was guided through the vaginal wall, and the ovary manipulated transrectally to allow the needle to penetrate the follicle through the ovarian stroma at the base of the follicle. Two hours after the injections, follicles were examined by ultrasonography to ensure that no follicle damage occurred as a result of the injection; a reduction in diameter larger than 1 mm within 2 h of injection is evidence of follicle leakage. Animals were monitored daily for 3 consecutive days by ultrasonography to evaluate the effect of DOM-1 on follicle growth.

Steroid assay

Progesterone was measured in conditioned medium in duplicate as described (Bélanger, Couture et al. 1990, Price, Carrière et al. 1995) with mean intra- and interassay coefficients of variation of 2.2 % and 3.2 %, respectively. Testosterone levels were measured in conditioned medium in duplicate by ELISA using an anti-testosterone monoclonal antibody (MO21812, Fitzgerald Industries, Acton MA, USA), mean intra- and interassay coefficients of variation of 2.3 % and 8.7 %. Steroid concentrations in the culture medium were corrected for cell number by expressing the data per unit mass of total cell protein. The sensitivity of these assays was 4 and 1.5 pg/mL for progesterone and testosterone, equivalent to 20 and 24 ng/µg protein, respectively.

Total RNA extraction and real-time PCR

After treatments medium was removed and total RNA was extracted using Trizol according to the manufacturer's instructions. Total RNA (0.5 μg) was quantified by absorbance at 260 nm and treated with 1 U DNase (Invitrogen; Thermo Fisher Scientific).

RNA was reverse transcribed in the presence of 1 mmol/L oligo (dT) primer and 4 U Omniscript RTase (Qiagen, Toronto, ON, Canada), 0.25 mmol/L dideoxynucleotide triphosphate (dNTP) mix and 19.33 U RNase Inhibitor (GE Healthcare, Baie D´Urfé, QC, Canada) in a volume of 20 μL at 37 °C for 1 h. The reaction was terminated by incubation at 93 °C for 5 min.

Real-time PCR was performed on a 7300 Real-Time PCR system (Applied Biosystems, Mississauga, ON, Canada) with Power SYBR Green PCR Master Mix. The bovine-specific primers have previously been published (Jiang, Guerrero-Netro et al. 2013) except *EGR3* (forward 5'- AGCGCGCTCAACCTCTTTT-3'; reverse 5'-GGTCAGACCGATGTCCATC-3'). Common thermal cycling parameters (3 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 59 °C, and 30 s at 72 °C) were used to amplify each transcript. Melting curve analyses were performed to verify product identity and the novel *EGR3* amplicon was sequenced to verify authenticity. Samples were run in duplicate and were expressed relative to histone H2AFZ as housekeeping gene. Data were normalized to a calibrator sample using the ΔΔCt method with correction for amplification efficiency (Pfaffl 2001).

Western Blot

After challenge with DON/DOM-1, cells were washed with cold PBS and lysed in 100µL/well cold RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS, 1 mM sodium orthovanadate, and protease inhibitor cocktail). The homogenate was centrifuged at 6000*g* for 5 min at 4°C. The resulting supernatant was retained and stored at –20°C. Protein concentrations were determined

with the Pierce BCA protein assay (Thermo Fisher Scientific).

Samples were resolved on 12 % SDS-polyacrylamide gels (10μg total protein per lane) and electrophoretically transferred onto nitrocellulose membrane in a Bio-Rad wet Blot Transfer Cell apparatus (transfer buffer: 39 mM glycine, 48 mM Tris-base, 1 % SDS, 20 % methanol, pH 8.3). After transfer, the membranes were blocked in TTBS (10 mM Tris–HCl, 150 mM NaCl, 0.1 % Tween-20, pH 7.5) for 1 h. Membranes were incubated overnight with primary antibodies against phospho-PKR (#ab47377, Abcam, Cambridge MA, USA, 1:1000), MAPK3/1 (#9102, 1:2000, Cell Signaling Technology, Danvers, MA, USA), phospho-MAPK3/1 (#9101, 1:1000, Cell Signaling Technology), MAPK14 (#9211, 1:1000, Cell Signaling Technology), phospho-MAPK14 (#9215, 1:1000, Cell Signaling Technology), phospho-MAPK8 (# 9251, 1:1000, Cell Signaling Technology), MAPK8 (# 9252, 1:1000; Cell Signaling Technology) and phospho-AKT (#sc-101629, 1:200, Santa Cruz Biotechnology) diluted in 5 % bovine serum albumin (total MAPK8) or TTBS (all other antibodies) at 4 °C. The loading control was COX4I1 (#69359, 1:1000; Santa Cruz Biotechnology). After washing three times with TTBS, membranes were incubated for 2 h at room temperature with 1:10,000 anti-rabbit HRP-conjugated IgG (GE Healthcare) diluted in TTBS. After five washes in TTBS, protein bands were revealed by chemiluminescence (Millipore ECL; Fisher Scientific, Ottawa, ON, Canada) with a gel imaging system (ChemiDoc XRS system, Bio-Rad, Mississauga, ON, Canada). Semiquantitative analysis was performed with Bio-Rad ChemiDoc XRS software.

Statistical analysis

All statistical analyses were performed with JMP software (SAS institute, Cary, NC, USA). Data were transformed to logarithms if they were not normally distributed (Shapiro–Wilk test). Where main effects were significant, the effect of time or treatment was tested with the Tukey–Kramer HSD test. Follicle growth data were analysed by repeated measures ANOVA. The data are presented as least square means ± SEM.

Results

Effect on steroidogenesis

Theca cells cultured in the current conditions responded to LH with a significant increase in testosterone and progesterone secretion, and the addition of vehicle (methanol) did not alter this response (Fig 1). An initial dose-response study with 1, 10 and 100 ng/mL DON or DOM-1 revealed that all doses completely suppressed progesterone secretion (not shown); as a consequence, a further dose-response was performed with concentrations from 0.005 to 1 ng/mL. Addition of DON to theca cells significantly inhibited LH-stimulated progesterone secretion at 0.5 and 1 ng/mL, but had no effect on testosterone secretion. Treatment of cells with DOM-1 resulted in significant inhibition of progesterone and testosterone secretion at doses of 0.5 ng/mL and higher (Fig 1).

At the dose of 1 ng/mL, DON decreased abundance of *STAR* and *HSD3B1* mRNA levels and DOM-1 decreased *CYP11A1*, *STAR* and *HSD3B1* mRNA abundance (Fig 2). Neither treatment significantly altered *CYP17A1* mRNA levels. Methanol alone did not alter abundance of mRNA of any gene measured.

Effect on apoptosis

Exposure of theca cells to DOM-1 increased the proportion of annexin positive apoptotic cells, and also increased abundance of mRNA encoding GADD45B, FASLG and BID, proteins associated with apoptosis. Addition of DON did not increase the proportion of apoptotic cells or *GADD45B* or *FASL* mRNA levels, but did increase levels of *BID* mRNA (Fig 3).

Signaling pathways activated by DON and DOM-1

The intracellular signaling pathways activated by DON and by DOM-1 in theca cells were investigated by Western blotting. Addition of either DON or DOM-1 increased pPKR levels within 15 or 5 min, respectively, and phosphorylation remained elevated for at least an hour (Fig 4A). Addition of DON caused a rapid (within 5 min) and transient (return to baseline by 30 min) increase in MAPK3/1 phosphorylation, whereas DOM-1 resulted in a weaker but more sustained phosphorylation of MAPK3/1 (Fig 4B). DON and DOM-1 had opposite effects on activation of MAPK14; DON decreased pMAPK14 levels whereas DOM-1 increased pMAPK14 levels (Fig 4C).

Addition of either DON or DOM-1 increased phosphorylation of AKT, although the timing differed between treatments; DON increased pAKT at 15 min whereas DOM-1 increased pAKT at 5 min only (Fig 4D). Both mycotoxins transiently stimulated the phosphorylation of MAPK8 at 30 min post-treatment (Fig 4E).

Addition of DON resulted in a rapid and transient increase in *EGR1, EGR3,* and *FOS* mRNA levels, and a decrease in *FOSL1* mRNA abundance (Fig 5). Treatment with DOM-1 had no effect on these immediate-early genes.

The importance of the RSR for the downstream effects of DON and DOM-1 was tested with the addition of PKR (C16) or HCK (PP2) inhibitors. Preliminary experiments demonstrated effective inhibition of PKR phosphorylation by 1 mg/mL inhibitor (Fig 6), and this dose abolished the stimulatory effect of DON or DOM-1 on MAPK3/1 (Fig 6). Inhibition of PKR activity increased pMAPK14 irrespective of mycotoxin added (Fig 6).

Addition of PP2 abolished the ability of DON or DOM-1 to stimulate MAPK3/1 phosphorylation, and suppressed MAPK14 phosphorylation to levels significantly lower than controls (Fig 7). Inhibition of HCK has no effect on DON or DOM-1 stimulated PKR phosphorylation.

Effect of DOM-1 on follicle growth in-vivo

As DOM-1 increased the proportion of atretic cells and activated MAPK14, we sought to determine of DOM-1 alters follicle growth *in vivo* by injected DOM-1 directly into a growing follicle. The growing follicle that was injected with PBS continued its growth trajectory, and was significantly larger at 48 and 72 h after injection compared with at the time of injection. Injection of DOM-1 directly into a growing follicle caused cessation of follicle growth, and the follicles were significantly smaller than the PBS-injected follicles at 48 and 72 h after injection (Fig 8).

Discussion

This study reveals several novel and striking findings on the impact of DON and DOM-1 on theca cell function and follicle growth in cattle. These include the unexpected sensitivity of theca cells to DON and DOM-1, the proapoptotic effects of DOM-1 on follicle growth, and the likely mechanism of action of DOM-1 through the RSR.

The deleterious effects of DON on numerous cell types are well known. In the ovary, DON has been shown to inhibit granulosa cell function (steroidogenesis) and health in cattle at relatively high doses (30 – 100 ng/mL) (Guerrero-Netro, Chorfi et al. 2015, Pizzo, Caloni et al. 2016). Theca cells are not generally considered to be affected by follicle atresia (Irving-Rodgers, van Wezel et al. 2001), therefore it was surprising to observe that theca cells are highly sensitive to DON, with an inhibition of progesterone secretion occuring at a dose 100-fold lower than that required in granulosa cells (Guerrero-Netro, Chorfi et al. 2015). This was logically accompanied by decreases in levels of mRNA encoding main enzymes involved in progesterone production, STAR and HSD3B.

More surprising however, was the dramatic reduction of progesterone and testosterone secretion and increase in apoptosis caused by DOM-1. This is in contrast to the considerably lower cytotoxicity of DOM-1 compared with DON in mouse fibroblasts, porcine peripheral blood mononuclear cells or intestinal epithelial cell lines (Sundstøl Eriksen, Pettersson et al. 2004, Dänicke, Hegewald et al. 2010
). The decrease in steroid secretion was accompanied by a decrease in the abundance of mRNA encoding not only STAR and HSD3B as observed for DON, but also CYP11A1. These proteins act sequentially to import cholesterol into the mitochondrion (STAR),

convert cholesterol into pregnenolone (CYP11A1) and pregnenolone into progesterone (HSD3B1) (see (Conley and Bird 1997) for a detailed description of the steroidogenic pathway). Pregnenolone is a precursor for testosterone, and it is likely that the additional reduction of pregnenolone synthesis in DOM-1 treated cells caused by the reduction of CYP11A1 is responsable for the reduction of testosterone secretion at low doses of DOM-1. We are unaware of any reports demonstrating an inhibitory effect of DOM-1 on endocrine function in any cell type.

The increase in the proportion of apoptotic cells caused by DOM-1 was accompanied with an increase in abundance of *GADD45B* and *FASLG* mRNA levels. FASLG is a major 'death ligand' that binds to the receptor FAS; the involvement of FASLG in inducing apoptosis in granulosa cells is well-known (Quirk, Cowan et al. 1995), and DON potentiates FAS-induced apoptosis in leucocytes (Uzarski, Islam et al. 2003). The role of GADD45B is unclear as it has been shown to either mediate FAS-induced apoptosis in hepatocytes (Cho, Park et al. 2010) or to repress FAS-induced apoptosis in B lymphocytes (Zazzeroni, Papa et al. 2003). Nevertheless, these data collectively demonstrate that DOM-1 but not DON increases apoptosis in theca cells.

In a study of cattle fed with a diet containing 5 mg/kg DON, which is the European limit for animal feed (EuropeanCommission 2006), the concentrations of DOM-1 in follicular fluid ranged from 17 – 88 ng/mL (Winkler, Kersten et al. 2014), which are higher than those required to increase theca cell death *in vitro* (present study). We therefore tested whether DOM-1 alters follicle development *in vivo* by injecting DOM-1 to a final concentration of 100 ng/mL, although allowing for procedural losses and diffusion from the follicle into blood and surrounding tissue, the concentrations

achieved were likely less than 100 ng/mL. The clear cessation of follicle growth demonstrates the adverse effects of DOM-1 on follicle function, either acting on theca, granulosa cells or both.

As DOM-1 is generally considered to be non-toxic, little attention has been paid to the mechanism of action of this molecule. In the present study, both DON and DOM-1 activated PKR, MAPK3/1 and AKT, which is fully expected for DON (Zhou, Islam et al. 2005, Pestka 2008, Zhou, He et al. 2014) but not previously described for DOM-1. However, the effects of DON and DOM-1 on MAPK14 phosphorylation were not expected. It is generally accepted that DON increases pMAPK14 levels but doses used are typically above 100 ng/mL (Zhou, Lau et al. 2003), although studies with doses between 25 - 50 ng/mL DON show weak effects on MAPK14 activation (Islam, Gray et al. 2006, Mishra, Tripathi et al. 2014). Thus the inhibitory effect of DON on MAPK14 phosphorylation observed in the present study may be an effect of very low concentrations of DON. In contrast, this same dose of DOM-1 increased MAPK14 phosphorylation in the present study, which has not been previously reported. Recently Pierron and colleagues showed that DOM-1 had no effect on MAPK14 phosphorylation in porcine jejunal explants and the Caco-2 human intestinal cell-line (Pierron, Mimoun et al. 2016); the discrepancies between our study and that of Pierron et al may be caused by the different doses of DOM-1 used (1 ng/mL compared with 2800 ng/mL), the different cell types and/or different species used.

Activation of these pathways by DON resulted in increased abundance of mRNA encoding the early-immediate genes *EGR1, EGR3* and *FOS*, which is consistent with previous studies with ovarian granulosa, intestinal epithelial and hepatoma cells (Moon,

Yang et al. 2007, Nielsen, Lippke et al. 2009, Guerrero-Netro, Chorfi et al. 2015). However, as for all the other effects noted here, theca cells are more sensitive than granulosa cells to DON; in granulosa cells, DON stimulation of *EGR1, EGR3* or *FOS* mRNA levels was observed at 100 ng/mL but not at 1 ng/mL (Guerrero-Netro, Chorfi et al. 2015). In contrast, DOM-1 failed to alter levels of mRNA transcripts of these genes, and this is most likely owing to differences in the pattern of MAPK3/1 activation by DON (strong and transient) and DOM-1 (weaker and sustained); several studies suggest that transient vs sustained MAPK3/1 activation leads to distinct cell responses (Murphy, MacKeigan et al. 2004, Glotin, Calipel et al. 2006, Shaul and Seger 2007).

The activation of PKR and HCK was necessary for the activation of downstream pathways by DOM-1 as it is for DON, as treatment with a PKR inhibitor or a HCK inhibitor blocked the ability of DOM-1 to phosphorylate MAPK3/1, and blocking HCK suppressed MAPK14 phosphorylation as previous reported (Bae, Gray et al. 2010). Treating cells with the imidazole-oxindole PKR inhibitor C16 increased pMAPK14 levels irrespective of treatment, which suggests a non-specific action of this inhibitor, and caution is therefore advised for its use to dissect signaling pathways. This implies that DOM-1 associates with the ribosome to activate PKR and HCK, and molecular modeling suggests that DOM-1 can indeed bind to the 60S ribosome, albeit with a lower number of hydrogen bonds compared to the binding of DON (Pierron, Mimoun et al. 2016).

In conclusion, this study identifies a biological action of DOM-1 in ovarian theca cells. DOM-1 activates PKR and HCK through unknown mechanisms, which leads to a low but sustained increase in MAPK3/1 phosphorylation and activation of MAPK14. This stimulates apoptosis and leads to follicle atresia. It is thus unwise to dismiss DOM-1,

even at levels as low as 1 ng/mL, as an inactive metabolite of DON with no deleterious effects on organ systems.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this work.

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Figures

Figure 1.

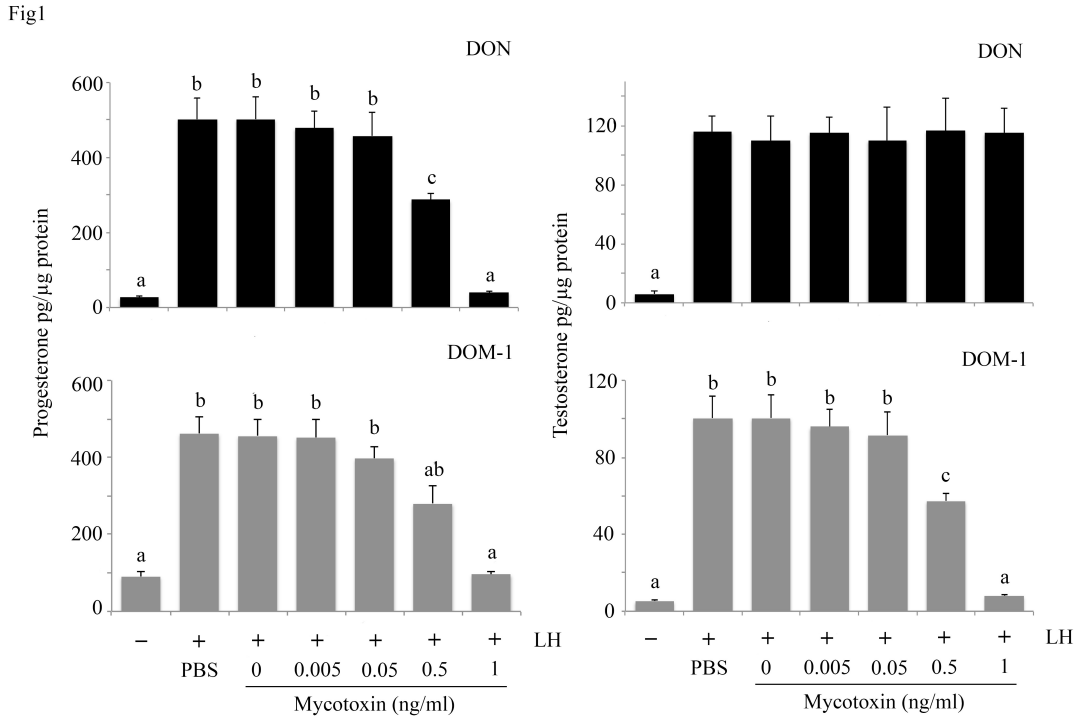

Fig. 1. Progesterone and testosterone secretion from bovine theca cells after treatment with DON or DOM-1 *in vitro*. Cells were cultured in serum-free medium for 6 days with medium changes on days 2 and 4. From day 2, cells were cultured without LH, with LH (0.8 ng/mL), or with LH and DON or DOM-1 in methanol. An additional control group received LH and PBS. Mass of steroid in medium on day 6 is expressed relative to cell number (total cell protein). Data are presented as means ± SEM; bars without common letters are significantly different ($p<0.05$)

Figure 2.

Fig2

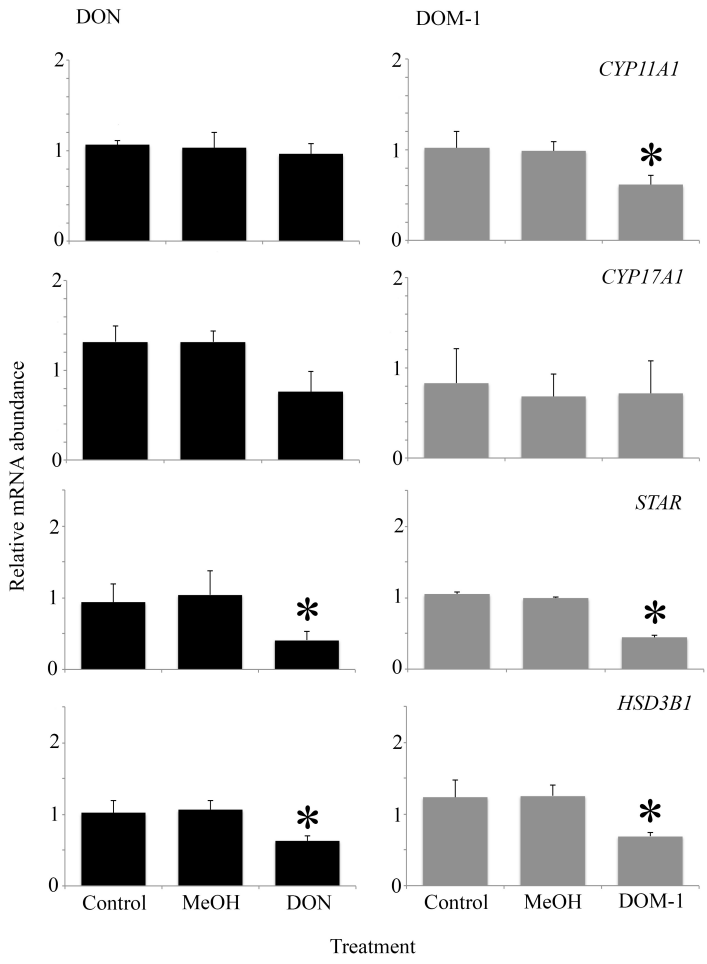

Fig. 2. Effect of mycotoxins on the abundance of mRNA encoding key steroidogenic proteins in bovine theca cells. Cells were cultured as described in Fig 1 in the presence of LH alone (Control), LH plus methanol (MeOH) or LH plus DON or DOM-1 (1 ng/mL). Total RNA was recovered on day 6 for analysis by real-time PCR. Data are presented as means ± SEM; asterisks indicate means that are significantly different from control ($p<0.05$)

Figure 3.

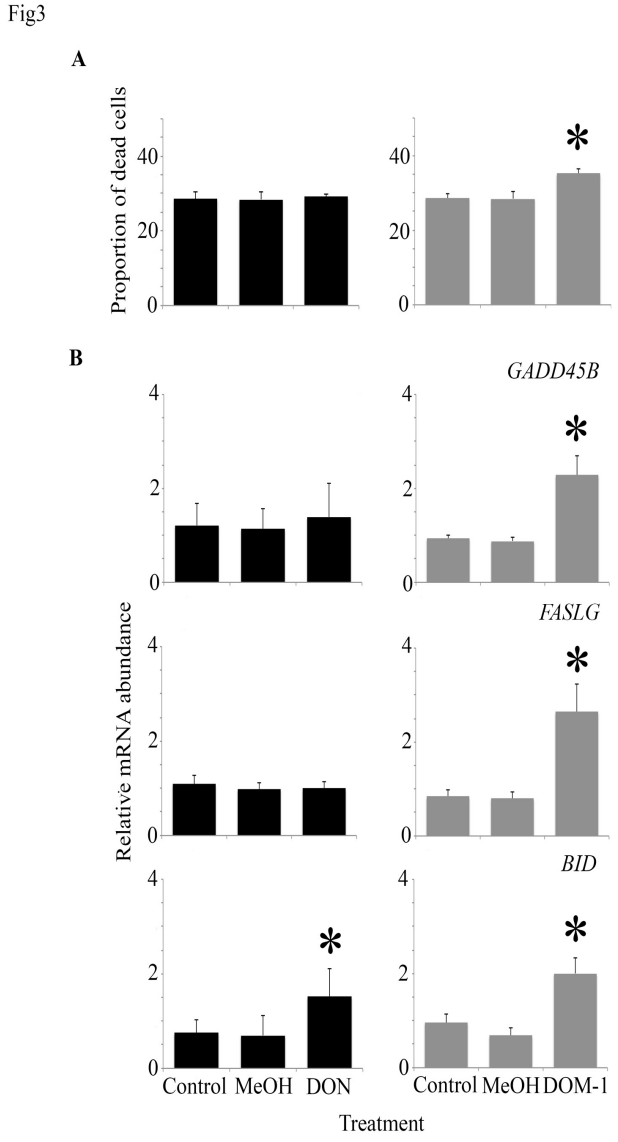
Fig3
A
Proportion of dead cells
40
20
0
*
B
GADD45B
FASLG
BID
Relative mRNA abundance
4
2
0
Control MeOH DON
Control MeOH DOM-1
Treatment

Fig. 3. Apoptosis in theca cells treated with DON or DOM-1. Cells were cultured as described in Fig 2, and apoptosis was assessed with the Annexin apoptosis kit (A), and abundance of mRNA encoding pro-apoptotic proteins (B). Data are presented as means ± SEM; asterisks indicate means that are significantly different from control ($p<0.05$).

Figure 4.

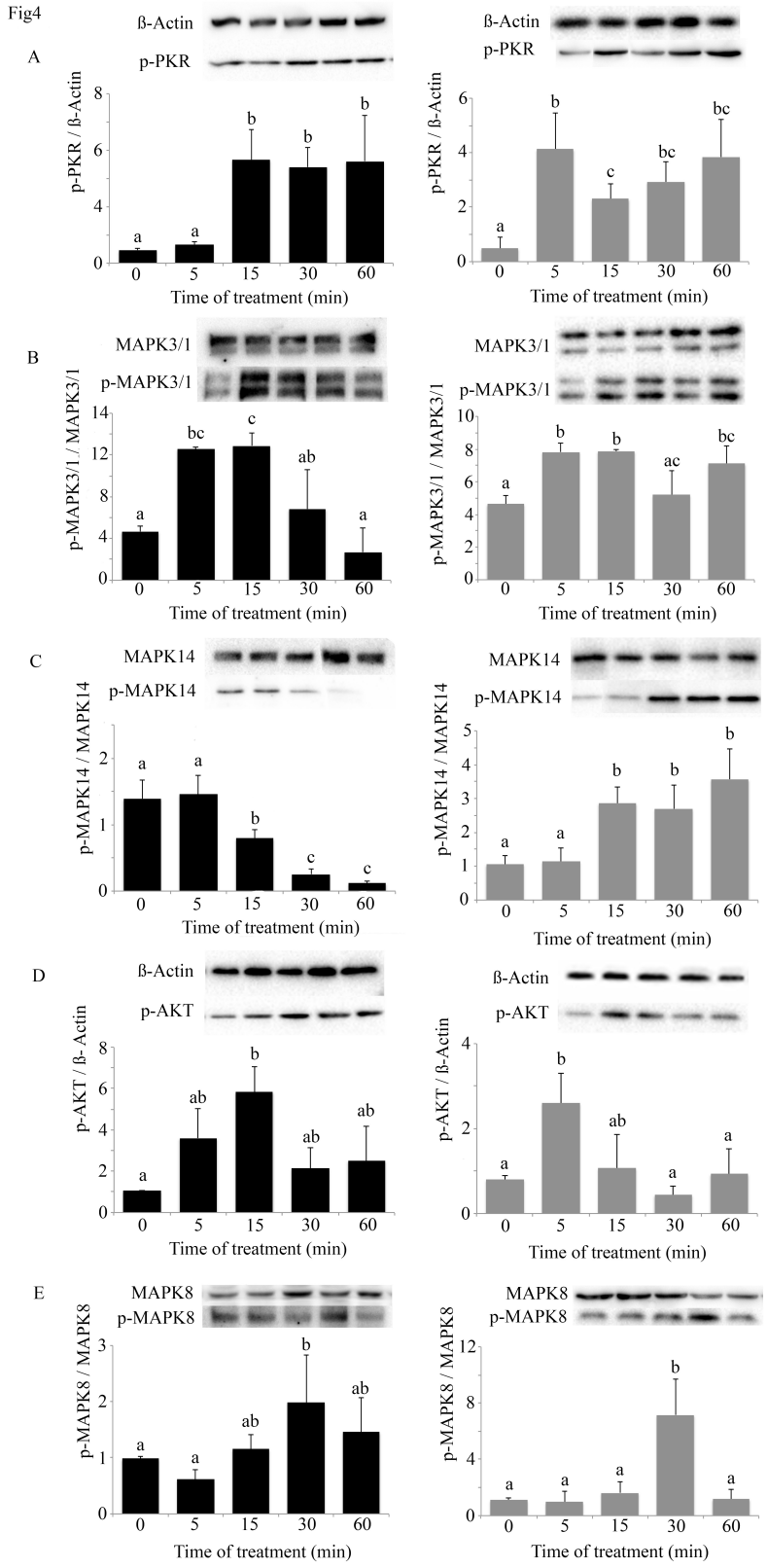
Fig4
A
ß-Actin
p-PKR
p-PKR / ß-Actin
Time of treatment (min)
B
MAPK3/1
p-MAPK3/1
p-MAPK3/1 / MAPK3/1
C
MAPK14
p-MAPK14
p-MAPK14 / MAPK14
D
p-AKT
p-AKT / ß-Actin
E
MAPK8
p-MAPK8
p-MAPK8 / MAPK8
0
5
15
30
60

Fig. 4. Intracellular pathways activated by DON and DOM-1 in bovine theca cells. Cells were cultured in serum-free medium and on day 5, DON or DOM-1 were added (1 ng/mL). Total cell protein was recovered at the times given and abundance of phosphorylated PKR (panel A), MAPK3/1 (B), MAPK14 (C), AKT (D) and MAPK8 (E) was measured by Western blotting. Phosphorylated protein was expressed relative to total protein, except for PKR and AKT which were expressed relative to ß-actin. Data are presented as means ± SEM; bars without common letters are significantly different ($p<0.05$).

Figure 5.

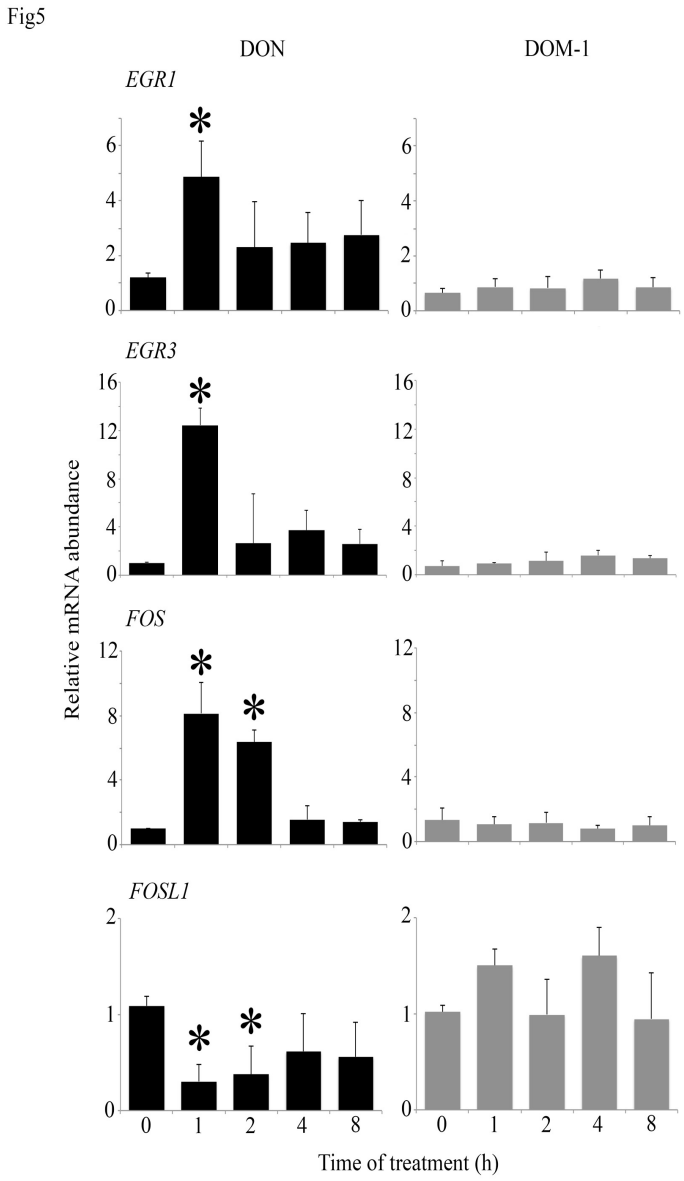
Fig5
DON
DOM-1
EGR1
EGR3
FOS
FOSL1
Relative mRNA abundance
Time of treatment (h)
0
1
2
4
8

Fig. 5. Effect of DON or DOM-1 on early-immediate gene mRNA levels in theca cells. Cells were cultured in serum-free medium and on day 5, DON or DOM-1 were added (1 ng/mL) for the times given. Total RNA was recovered for analysis by real-time PCR. Data are presented as means ± SEM; asterisks indicate means that are significantly different from control ($p<0.05$).

Figure 6.

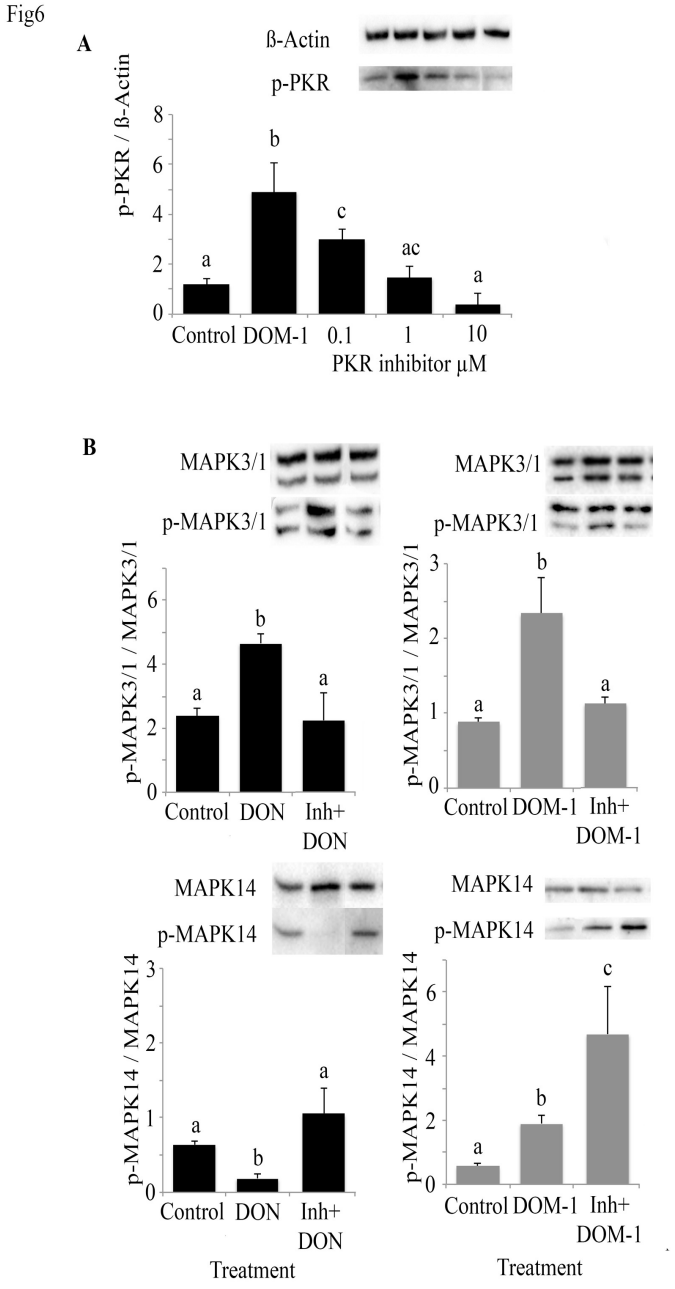
Fig6
A
ß-Actin
p-PKR
p-PKR / ß-Actin
8
6
4
2
0
a
b
c
ac
a
Control DOM-1 0.1 1 10
PKR inhibitor µM
B
MAPK3/1
p-MAPK3/1
p-MAPK3/1 / MAPK3/1
6
4
2
0
a
b
a
Control DON Inh+ DON
MAPK3/1
p-MAPK3/1
p-MAPK3/1 / MAPK3/1
3
2
1
0
a
b
a
Control DOM-1 Inh+ DOM-1
MAPK14
p-MAPK14
p-MAPK14 / MAPK14
3
2
1
0
a
b
a
Control DON Inh+ DON
Treatment
MAPK14
p-MAPK14
p-MAPK14 / MAPK14
6
4
2
0
a
b
c
Control DOM-1 Inh+ DOM-1
Treatment

Fig. 6. Role of PKR in DON and DOM-1 action. (A) Cells were cultured in serum-free medium and on day 5, DOM-1 (1 ng/mL) was added with or without pretreatment with 0, 0.1, 1 or 10 mM inhibitor for 1 h. (B) Cells were pretreated with 1 mM inhibitor for 1 h before adding DON or DOM-1 for 15 min. Total cell proteins were recovered for Western blot analysis for abundance of PKR, MAPK3/1 and MAPK14 phosphorylation. Data are presented as means ± SEM; bars without common letters are significantly different ($p<0.05$).

Figure 7.

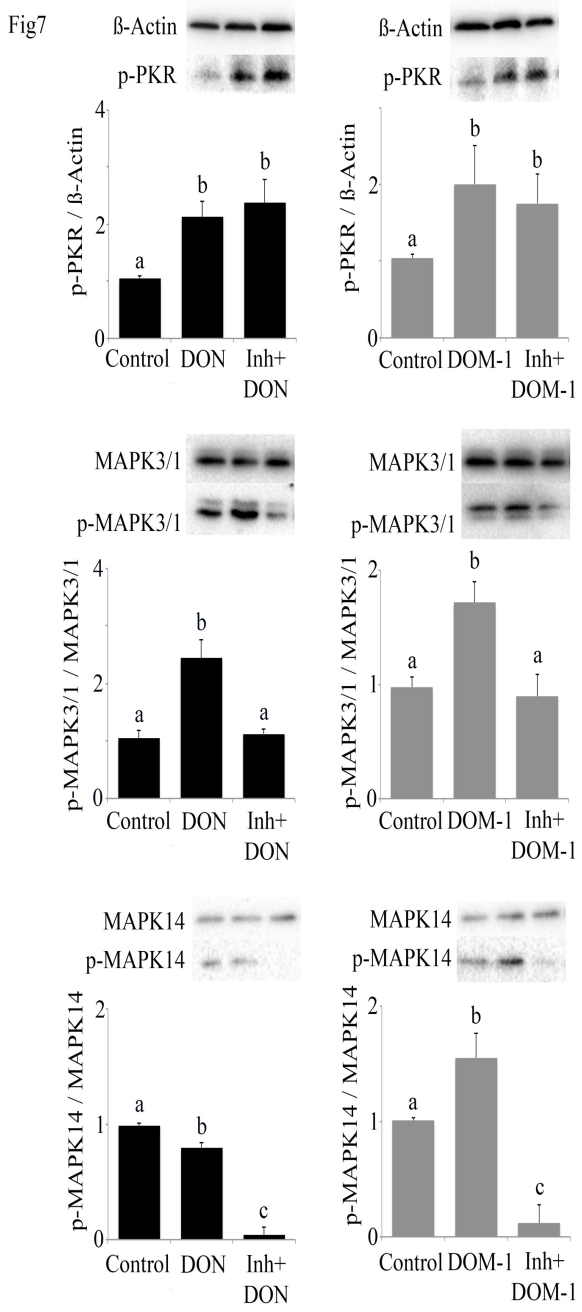
Fig7
ß-Actin
p-PKR
p-PKR / ß-Actin
Control
DON
Inh+ DON
DOM-1
Inh+ DOM-1
MAPK3/1
p-MAPK3/1
p-MAPK3/1 / MAPK3/1
MAPK14
p-MAPK14
p-MAPK14 / MAPK14

Fig. 7. Role of HCK in DON and DOM-1 action. Cells were pretreated with 1 mM inhibitor for 1 h before adding DON or DOM-1 for 15 min. Total cell proteins were recovered for Western blot analysis for abundance of PKR, MAPK3/1 and MAPK14 phosphorylation. Data are presented as means ± SEM; bars without common letters are significantly different ($p<0.05$)

Figure 8.

Fig8

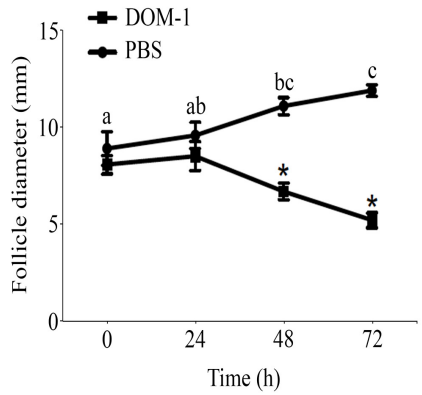

Fig. 8. Injection of DOM-1 directly into a growing follicle caused cessation of growth. DOM-1 (100 ng/ml) or PBS was injected into the dominant follicle and diameters were measured by ultrasound imaging. Data are presented as means ± SEM; a,b,c, for PBS injected follicles, means with different letters are significantly different; asterisks indicate means that are significantly different from control ($p<0.05$)

Article 3 (in preparation)

The mycotoxin deoxynivalenol (DON) and its main metabolite de-epoxy (DOM-1) increase autophagy in bovine reproductive cells.

Hilda M. Guerrero-Netro, Younès Chorfi and Christopher A. Price

Centre de recherche en reproduction animale, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, J2S 7C6, QC Canada

Keywords: fertility, autophagy, ovary, follicle, granulosa cells, theca cells, deoxynivalenol, de-epoxy deoxynivalenol

Abstract

Autophagy can be simply defined as cellular "self-eating", during which the cell forms a phagophore or isolation membrane that has the function of enclosing cytoplasmatic organelles by forming the autophagosome, which ensures the degradation of damaged material. Autophagy-related proteins (Atg) are restricted to the pre-autophagosomal structure and are involved in its formation, but from them, only LC3 (Atg8) is present in the autophagosomes making it the best marker for autophagy. Deoxynivalenol (DON) and its metabolite de-epoxy deoxynivalenol (DOM-1) increase apoptotic cell death in granulosa and theca cells, respectively. The objective of this study was to determine if DON and DOM-1 activate the autophagy pathway in bovine granulosa and theca cells. Addition of DON or DOM-1 increased mRNA levels of LC3 and Beclin 1 at 100 ng/ml and 1ng/ml, in cultured granulosa and theca cells. Additional exposure to DON resulted in a gradual increase for LC3-I and LC3-II protein levels reaching a maximum peak after 12 hours of treatment. Granulosa and theca cells fixed after 12 hours exposure to DON or DOM-1 respectively, showed an increased in immunofluorescence for LC3 compared to controls. The addition of ULK1 inhibitor to DON-treated granulosa cells resulted in a dramatic increase of apoptotic cells when compared to DON alone. These data demonstrate that DON and DOM-1 can trigger autophagy in granulosa and theca cells.

Introduction

Contamination of animal feed with *Fusarium* fungi is a common occurrence in many parts of the world and leads to the accumulation of mycotoxins including zearalenone (ZEN) and deoxynivalenol (DON) (Rodrigues and Naehrer 2012, Marin, Ramos et al. 2013). DON impacts cells by binding to ribosomal RNA and inducing autophosphorylation and activation of double-stranded RNA-activated protein kinase (PKR; gene symbol *EIF2AK2*) (Zhou, He et al. 2014) and hematopoietic cell kinase (HCK) (Zhou, Jia et al. 2005). These events result in downstream activation of the p38 (MAPK14), ERK1/2 (MAPK3/1), and c-Jun N-terminal kinase (MAPK8) members of the mitogen-activated protein kinase (MAPK) family (Pestka 2008) in a process known as the ribotoxic stress response (RSR).

Several studies have demonstrated that DON negatively impacts the reproductive system, particularly the ovarian follicle. In pigs, DON inhibited cumulus expansion and oocyte maturation *in vitro* (Alm et al. 2002, Malekinejad et al. 2007, Schoevers et al. 2010), and altered steroid secretion from granulosa cells (Ranzenigo et al. 2008, Medvedova et al. 2011). In cattle, DON inhibited steroidogenesis in granulosa cells *in vitro* and increased the apoptosis rate through the RSR (Guerrero-Netro, Chorfi et al. 2015, Pizzo, Caloni et al. 2016), and the major metabolite of DON, deepoxy-DON (DOM-1), increased apoptosis in theca cells (Article 2).

Programmed cell death can occur through the activation of caspases and DNA fragmentation (apoptosis) or intracellular degradation of organelles in lysosomes (autophagy)(Mizushima 2007). A body of evidence shows that granulosa cells undergo autography in response to cell stressors and toxins including oxidized low-density lipoproteins (Gannon, 2013 #20), cigarette smoke and atresia induced by gonadotropin

withdrawal (Gannon, 2012 #19). Cell stress activates the autophagy-related (Atg) protein ULK1 (Atg1), which then phosphorylates and activates beclin-1 (BECN1) to promote formation of the autophagosome and autophagy (Russell, Tian et al. 2013). An essential step in the maturation of the autophagosome is the conversion of the cytosolic form of microtubule associated protein 1 light chain 3 alpha (MAP1LC3A), known as LC3-I, to the membrane-bound form known as LC3-II (Tanida, Ueno et al. 2008, Mizushima, Yoshimori et al. 2010).

There are no studies to suggest whether DON or DOM-1 can trigger autophagy in ovarian cells. The objective of this study was to determine if DON or DOM-1activates the autophagy pathway in bovine granulosa and theca cells, respectively.

Materials and methods

Granulosa cell culture

All materials were obtained from Life Technologies Inc. (Burlington, ON, Canada) unless otherwise stated. Bovine ovaries were obtained from adult cows, independently of the stage of the oestrous cycle, at the slaughterhouse and transported to the laboratory at 30°C in phosphate-buffered saline (PBS) containing penicillin (100 IU) and streptomycin (100μg/ml). Bovine granulosa cells were cultured in serum-free conditions that maintain oestradiol and progesterone secretion and responsiveness to FSH (Gutiérrez et al. 1997, Silva & Price 2000, Sahmi et al. 2004). Granulosa cells were harvested from follicles measuring between 2 and 5mm in diameter, and the cell suspension was filtered through a 150 mesh steel sieve (Sigma- Aldrich Canada, Oakville ON, Canada). Cell viability was assessed by Trypan blue dye exclusion. Cells were seeded into 24-well tissue culture plates (Sarstedt Inc., Newton, NC, USA) at a density of

500,000 viable cells in 500µl DMEM/ F12 containing sodium bicarbonate (10mM), 25mM HEPES, sodium selenite (4ng/ml), bovine serum albumin (BSA)(0.1%; Sigma-Aldrich), penicillin (100U/ml) androstenedione (10^{-6}M) and bovine FSH (1ng/ml starting on day 2, AFP5346D; National Hormone and Peptide Program, Torrance, CA, USA). Cultures were maintained at 37°C in 5% CO_2, 95% air for up to 6 days.

Theca cell culture

Bovine theca cells were cultured in serum-free conditions that maintain testosterone and progesterone secretion and responsiveness to LH (Glister, Richards et al. 2005). Follicles (4–6 mm in diameter) were bisected within the ovarian stroma, gently scraped to remove granulosa cells, and the theca 'shells' were peeled from the stroma with forceps. Pooled theca layers were incubated with collagenase (type IV, 1 mg/mL; Sigma-Aldrich, Oakville, ON, Canada) and trypsin inhibitor (100 mg/mL; Sigma-Aldrich) in a water bath at 37 ºC for 45 min with agitation every 10 min. The resulting supernatant was filtered through a 150 mesh steel sieve (Sigma-Aldrich), centrifuged (800xg for 10 min) and the pellet resuspended in PBS before being subjected to an osmotic shock treatment to remove red blood cells. After washing, cells were resuspended in McCoy's 5A modified medium supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 1 μg/mL fungizone, 10 ng/mL bovine insulin, 2 mM L-glutamine, 10 mM HEPES, 5 μg/mL apotransferrin, 5 ng/mL sodium selenite, and 0.1 % BSA (all purchased from Sigma-Aldrich) and LH (National Hormone and Peptide Program, Torrance, CA, USA). Cell viability was assessed by Trypan blue dye exclusion, and cells were seeded into 24-well tissue culture plates (Sarstedt Inc., Newton, NC, USA)

at a density of 250,000 viable cells in 1 mL, and cultured at 37 ºC in 5 % CO_2, 95 % air for up to 6 days with medium changes every 2 days.

Experimental treatments

To determine the role of DON and DOM-1 in ovarian cells, cells were treated from day 2 with different doses of DON (0 and 100 ng/ml in GC, and 0,1 and 10 ng/ml in TC) or DOM-1 (0 and 1 ng/ml in TC) and cells were recovered on day 6 for mRNA extraction. The time-dependent effects of DON on abundance of LC3 protein was determined by treating cells on day 5 of culture with an effective dose of DON for 0, 4, 8, 12, 24 and 32h, and cells were recovered in RIPA buffer. Additional cells were treated for 12h with DON (GC) or DOM-1 (TC) and fixed to measure LC3 fluorescence. Apoptosis was measured with an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich) after treating the cells with an effective dose of DON for 12 hours with or without ULK1 inhibitor (Bafilomycin A1, Sigma-Aldrich)

Total RNA extraction and real-time PCR

Total RNA was extracted using Trizol according to the manufacturer's instructions. Total RNA (0.5µg) was quantified by absorbance at 260nm and treated with 1U DNase (Invitrogen). RNA was reverse transcribed in the presence of 1mmol/ l oligo (dT) primer and 4U Omniscript RTase (Qiagen, Mississauga, ON, Canada), 0.25mmol/l dideoxynucleotide triphosphate (dNTP) mix and 19.33U RNase Inhibitor (GE Healthcare, Baie D´Urfé, QC, Canada) in a volume of 20µl at 37°C for 1h. The reaction was terminated by incubation at 93°C for 5min.

Real-time PCR was performed on a CFX96 Real-Time PCR system (BioRad) with Sso Advanced Universal SYBR Green. The bovine-specific primers have previously been detailed (Jiang et al. 2013). Common thermal cycling parameters (3min at 95°C, 40 cycles of 15s at 95°C, 30s at 59°C, and 30s at 72°C) were used to amplify each transcript. Melting curve analyses were performed to verify product identity. Samples were run in duplicate and were expressed relative to histone H2AFZ as a housekeeping gene. Data were normalized to a calibrator sample using the ΔΔCt method with correction for amplification efficiency (Pfaffl 2001).

Western blot

After challenge with DON, cells were washed with cold PBS and lysed in 100 ml/well cold RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, and protease inhibitor cocktail). The homogenate was centrifuged at 6000 g for 5 min at 4ºC. The resulting supernatant was retained and stored at -20 ºC. Protein concentrations were determined by BCA Protein Assay (Pierce, Rockford, IL, USA).

Samples were resolved on 12% SDS–polyacrylamide gels (15 μg total protein/lane) and electrophoretically transferred onto a nitrocellulose membrane in a Bio-Rad wet Blot Transfer Cell apparatus (transfer buffer: 39 mM glycine, 48 mM Trisbase, 1% SDS, 20% methanol, and pH 8.3). After transfer, the membranes were blocked in TTBS (10 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, and pH 7.5) for 1 h. Membranes were incubated overnight with the primary antibody against LC3 (1:1000, Abcam, ab128025) at 4ºC. After washing three times with TTBS, the membranes were

incubated for 2 h at room temperature with 1:10 000 anti-rabbit HRP-conjugated IgG (GE Healthcare Canada) diluted in TTBS. After five washes in TTBS, protein bands were revealed by ECL (Millipore, Billerica, MA, USA) using a gel imaging system (ChemiDoc XRS system, Bio-Rad). Quantitative analysis was performed using the Bio-Rad ChemiDoc XRS Software.

Immunofluorescence

Cultured cells were fixed in 4% paraformaldehyde for 20 min, washed sequentially in 2% Triton-X and 0.05% Tween, blocked in 5% BSA, and incubated with the LC3 antibody (1:150). After the primary antibody, the cells were washed in PBS and then incubated with Cy3-conjugated second antibody (Jackson ImmunoResearch) and counterstained with 4',6-diamidino-2-phenylidole (DAPI). Cells were examined under a Zeiss epifluorescence microsope, digital images were captured and mean fluorescence intensities in each field for Cy3 and DAPI were quantified with ImageJ software (NIH). Results are expressed relative to DAPI.

Statistical analysis

All statistical analyses were performed using the JMP Software (SAS Institute, Cary, NC, USA). Data were transformed to logarithms if they were not normally distributed (Shapiro–Wilk test). Where main effects were significant in ANOVA, the effect of time or treatment was tested using the Tukey–Kramer honestly significant difference (HSD) test. The data are expressed as least square means ± SEM.

Results

DON and DOM-1 induce autophagy pathway

Treatment with DON significantly increased *LC3* and *BECN1* mRNA levels in granulosa cells (Fig 1A), and DOM-1 increased *LC3* and *BECN1* mRNA levels in theca cells (Fig 1B). In theca cells, DON did not alter *LC3* mRNA levels in theca cells (Fig 2). Inmmunofluorescence demonstrated significantly increased LC3 protein levels in granulosa cells after addition of DON (Fig 3) and in theca cells after the addition of DOM-1 (Fig 4). Western blot analysis of granulosa cells treated with DON demonstrated significant upregulation of LC3-I and LC3-II protein levels (Fig 5).

Autophagy pathway

To determine the relationship between autophagy and apoptosis in granulosa and theca cells, we inhibited autophagy by the addition of a ULK1 inhibitor. Cotreatment of granulosa cells with DON plus ULK-1 inhibitor significantly increased the percentage of apoptotic cells compared with DON alone (Fig 6). On the other hand addition of ULK-1 inhibitor plus DOM-1 to theca cells had no effect on the percentage of apoptosis (Fig. 7).

Discussion

It is well known that DON induces cell death in a variety of cells (Pestka 2008) and it has recently been shown to induce autophagy in porcine oocytes. In the present study we demonstrate that DON causes autophagy in granulosa cells, and also that the DON metabolite DOM-1 causes autophagy in theca cells.

Autophagy can be triggered during cellular stress with the purpose of selectively removing damaged organelles and therefore prevent cell death, or alternatively it may be a mechanism of cell death (Mizushima, Yoshimori et al. 2010). As DON has been shown to increase the rate of apoptosis in granulosa cells and DOM-1 increased apoptosis in theca cells, we investigated whether these compounds also increased autophagy. Both DON in granulosa cells and DOM-1 in theca cells increased levels of *BECN1* and *LC3* mRNA and protein. Beclin-1 has a critical role during autophagy and apoptosis as it can interact with BID and induce apoptosis (Kang, Zeh et al. 2011) or interact with ULK-1 and induce autophagy (Russell, Tian et al. 2013). The membrane-bound form of LC3 is a key marker of autophagy as it is the only Atg present in the autophagosomal membranes (Mizushima, Yoshimori et al. 2010), and treatment of DON increased both the cytosolic and membrane-bound forms of LC3. It is noteworthy that DON does not increase apoptosis in theca cells (Article 2) and did not alter autophagy in the present study. To our knowledge these are the first data to suggest that DOM1 activates autophagy in any cell type.

Inhibition of ULK-1 activity in granulosa cells treated with DON resulted in a significant increase in cell death and apoptosis. This is consistent with the increase in apoptosis observed after inhibition of ULK1 endometrial stromal cells (Stephens C 2016). This implies that the autophagic response to DON is a defense mechanism to prevent cell death, and in the absence of this ability, more cells enter apoptosis in response to DON administration.

In conclusion, the mycotoxins DON and DOM1 activate the ribotoxic stress response and initiate both apoptotic and autophagic pathways. The autophagic pathway is likely a defense mechanism to mitigate the apoptotic effects of mycotoxins.

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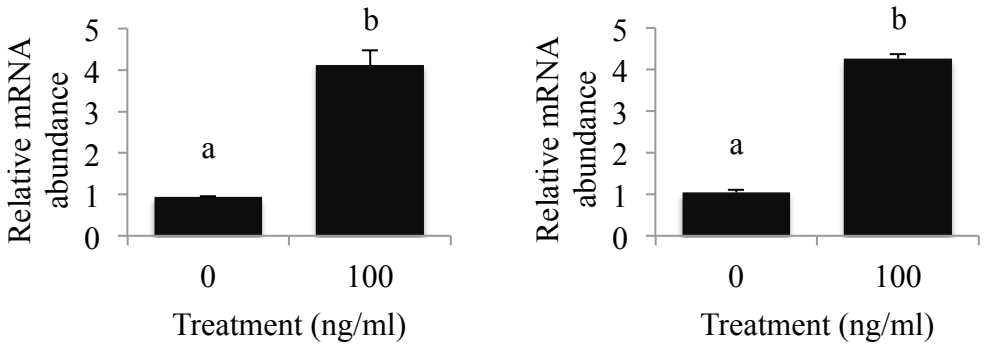
Figures

Figure 1.

1A

LC3 *BECN1*



1B

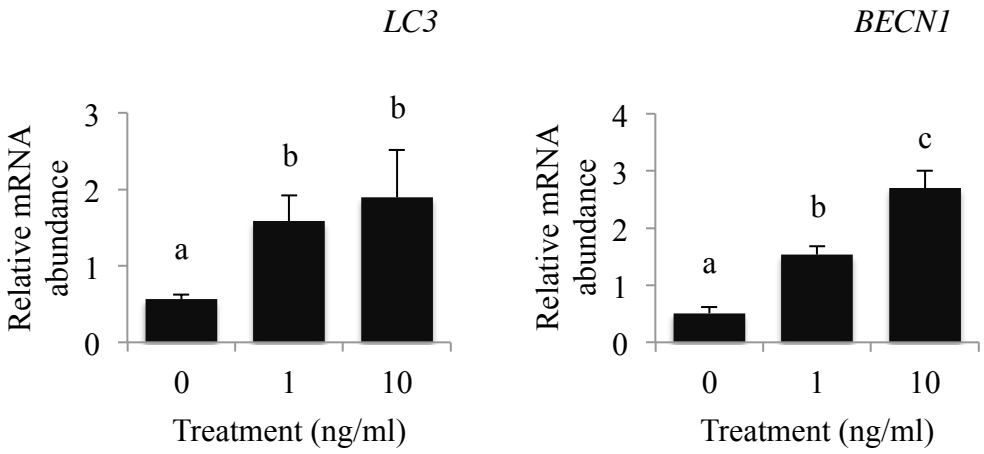


Fig. 1. Role of DON (GC) and DOM-1 (TC) on abundance of *LC3* and *BECN1* mRNA. (A) Granuloca cells were cultured in serum-free medium in the presence of DON (100ng/ml) for 4 days. (B) Theca cultured in serum-free medium in the presence of DOM-1 (1 and 10 ng/ml) for 4 days. Total cell mRNA was recovered for real time PCR

analysis to meassure the abundance of *LC3* and *BECN1*. Data are presented as means ± SEM; bars without common letters are significantly different ($p<0.05$).

Figure 2.

LC3

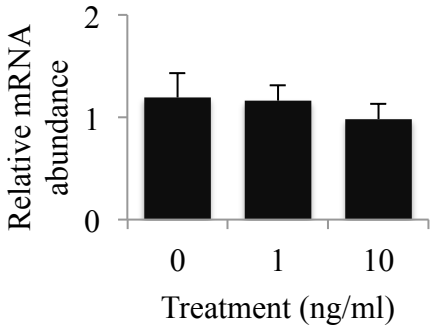
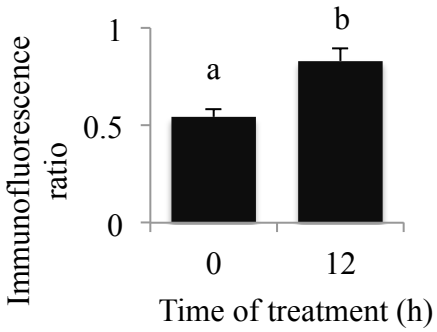


Fig. 2. Role of DON in theca cell autophagy. Theca cells were cultured in serum-free medium in the presence of DON (1ng/ml) for 4 days. Total cell mRNA was recovered for

real time PCR analysis to measure the abundance of *LC3* mRNA. Data are presented as means ± SEM.

Figure 3.



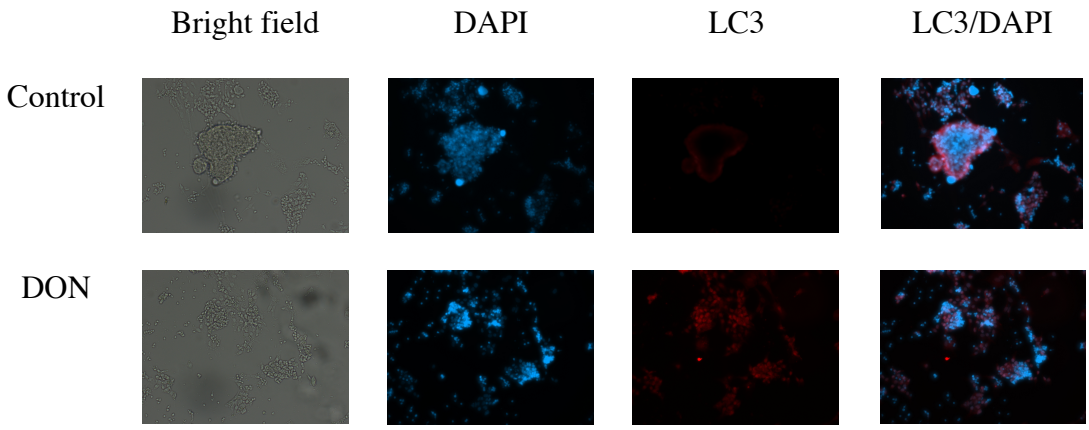


Fig. 3. DON increases LC3 proteins levels in GC. Granulosa cells were cultured in serum-free medium in the presence of DON (100 ng/ml) for 12 hours. Cells were

recovered and fixed for immunofluorescence. Total fluorescence was measured for DAPI and LC3 using Image J software. Data are presented as means ± SEM; bars without common letters are significantly different ($p<0.05$).

Figure 4.

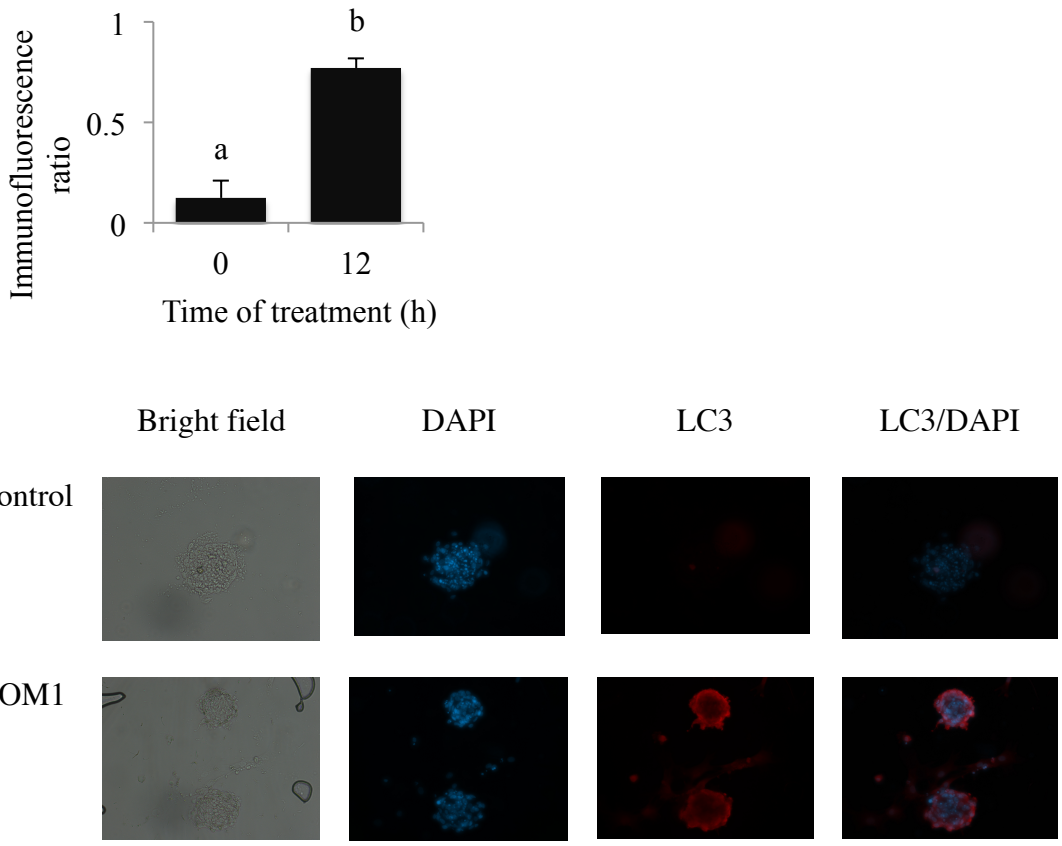


Fig. 4. DOM-1 increased LC3 protein abundance in TC. Theca cells were cultured in serum-free medium in the presence of DOM-1 (1 ng/ml) for 12 hours. Cells were

recovered and fixed for immunofluorescence. Total fluorescence was measured for DAPI and LC3, using Image J software. Data are presented as means ± SEM; bars without common letters are significantly different ($p<0.05$).

Figure 5.

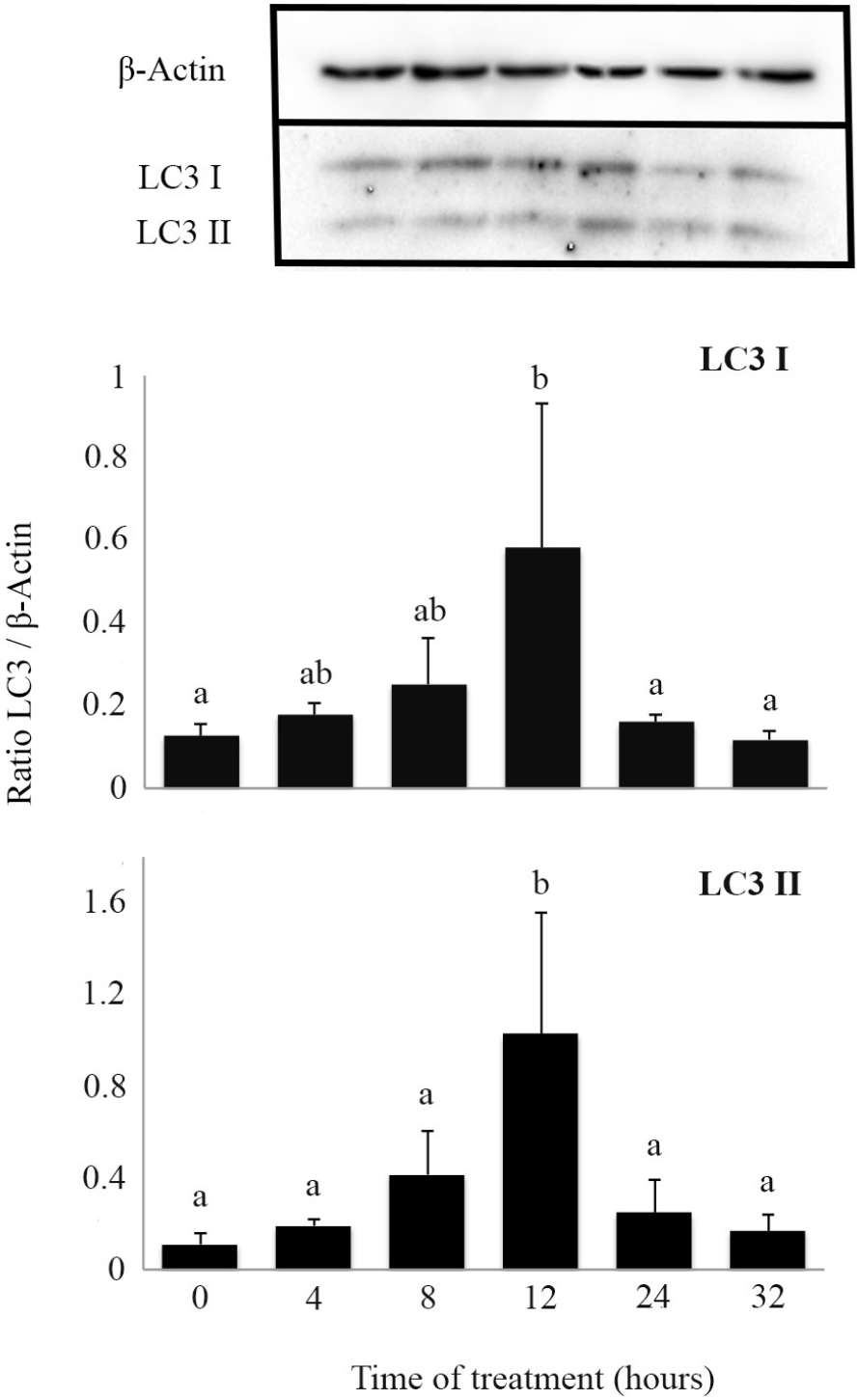
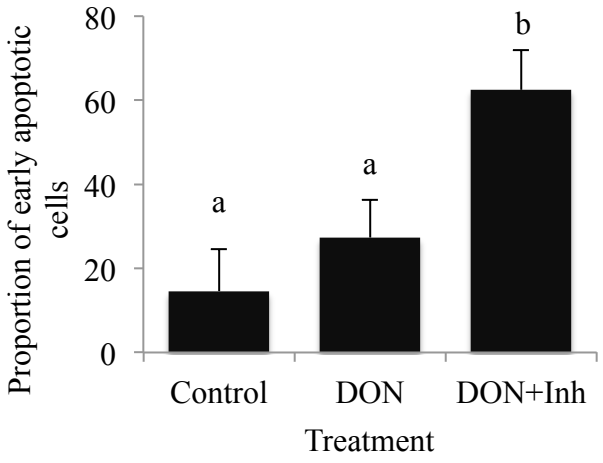

β-Actin
LC3 I
LC3 II
LC3 I
Ratio LC3 / β-Actin
1
0.8
0.6
0.4
0.2
0
a
ab
ab
b
a
a
LC3 II
1.6
1.2
0.8
0.4
0
a
a
a
b
a
a
0
4
8
12
24
32
Time of treatment (hours)

Fig. 5. DON increased abundance of LC3-I and LC3-II protein in GC. Granulosa cells were cultured in serum-free medium in the presence of DON (100 ng/ml) for 0, 4, 8, 12, 24 and 32 hours. Cells were recovered for protein extraction and measurement by Western blot. Data are presented as means ± SEM; bars without common letters are significantly different ($p<0.05$).

Figure 6.

6A



6B

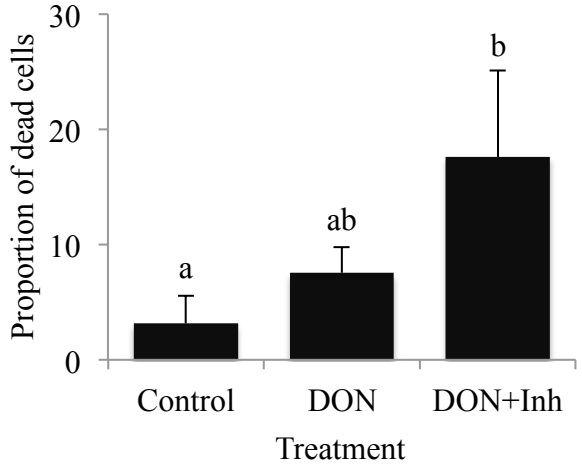
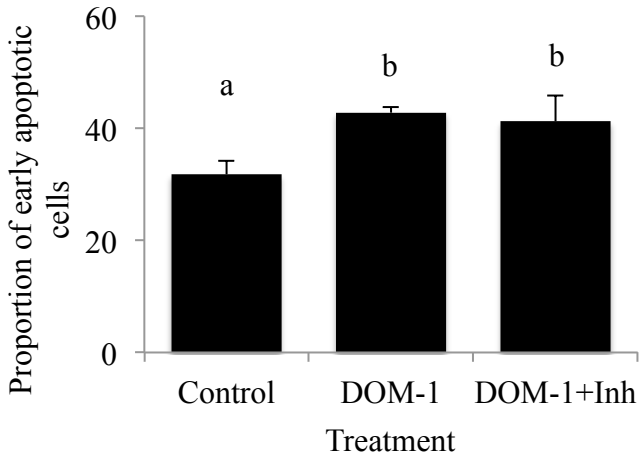


Fig. 6. Inhibition of autophagy increases apoptosis in DON-treated granulosa cells. Cells were cultured in serum-free medium in the presence of DON (100ng/ml) or DON plus ULK-1 inhibitor. Early apoptotic cells (A) and apoptotic cells (B) were assessed with the Annexin apoptosis kit. Data are presented as means ± SEM; bars without common letters are significantly different ($p<0.05$).

Figure 7.

7A



7B

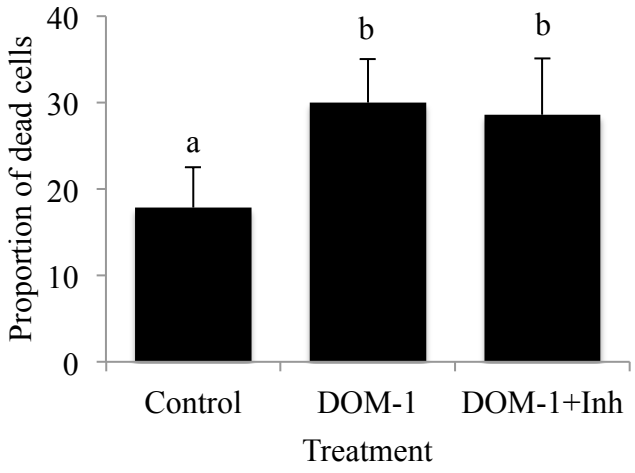


Fig. 7. Inhibition of autophagy does not alter apoptosis in DOM-1 treated theca cells. Theca cells were cultured in serum-free medium in the presence of DOM-1 (1ng/ml) or DOM-1 plus ULK-1 inhibitor. Early apoptotic cells (A) and apoptotic cells (B) were assessed with the Annexin apoptosis kit. Data are presented as means ± SEM; bars without common letters are significantly different ($p<0.05$).

Chapter 4:
General discussion

General discussion

This study provides the first investigation of the effects of the mycotoxin DON and its non-toxic metabolite DOM-1 in bovine reproduction. We identify pathways activated by DON in GC, important differences in biological effects of DON and DOM-1 in theca cells, and an unexpected sensitivity of TC to DOM-1. Mycotoxin contamination is a major problem that ranges from minor husbandry issues to the death of a herd, and although it has been widely studied, the mechanisms of action still require further investigation.

DON is also known as vomitoxin due to its strong emetic effects after ingestion and it can also produce changes in long-term feeding behavior; these effects are a result of DON action on the serotonergic activity in the central nervous system (Fitzpatrick, Boyd et al. 1988). One of the main characteristics of DON is that it is capable of crossing the blood-brain barrier through gaps between the endothelial cells (Behrens, Huwel et al. 2015). Although it is not yet know how fast DON can reach and affect the brain, this ability to cross into different organs is of main relevance in female reproduction. DON can easily go from the bloodstream into the ovarian follicle by a passive diffusion mechanism. Once inside the follicle DON cannot saturate the follicular fluid as it acts as a polar molecule. On the other hand, DOM-1 has decreased polarity compared to DON, therefore it might have a higher ability to diffuse and accumulate within the ovarian follicle (Maresca 2013).

In general, ruminants are considered less susceptible to the effects of mycotoxins than monograstric species. In cattle, only a few cases of intoxication by DON have been reported and are always attributed to a combined action of different mycotoxins (Danicke, Matthaus et al. 2005), therefore, there is not a lot of information available on the effects

of DON in cattle. A second reason and probably the most important one, is that DON is metabolized by bacteria in the rumen to DOM-1, tto he apparently non-toxic metabolite of DON. Previous publications demonstrated that in cows fed with 2.62 mg/kg (2.6 ppm) of DON, plasma levels reach around 1.35 ng/ml DON while DOM-1 levels are much higher, at around 20.7 ng/ml; similarly, a diet with a dose of 4.9 mg/kg DON resulted in 3.5 ng/ml of DON and 54 ng/ml of DOM-1 in blood (Winkler, Kersten et al. 2014). Although concentrations may vary during the day, after oral intake DON can follow three different processes: 1) it can be metabolized by bacteria, and perhaps preferentially by protozoa in the rumen, into DOM-1 (around 93-98%), 2) it can be excreted in faeces by xenobiotic metabolism (phase I) and, 3) in lower doses (around 1%), it can cross the intestinal barrier along with DOM-1 and reach the blood stream where DON can reach a peak 30 min after ingestion (Winkler, Kersten et al. 2015). Blood-borne DON and DOM-1 are conjugated with glucuronic acid and excreted in urine by xenobiotic metabolism (phase II), which takes 4-24 hours, therefore DOM-1 is always in higher concentrations and it takes longer to be conjugated and eliminated in urine (Danicke, Matthaus et al. 2005).

Levels of DON and DOM-1 have also been measured in the ovarian follicle. DOM-1 has the tendency to accumulate as it enters the follicle and it can reach levels of 34.4-88.8 ng/ml while DON only reaches concentrations around 2 ng/ml (Winkler, Kersten et al. 2015). In conclusion ruminants can transform almost all DON from oral intake into DOM-1, which can remain in the organism for long periods and accumulate within the ovarian follicle. Taking into account these previous publications, we designed an in vivo experiment where large follicles were injected with 100 ng/ml of DOM-1.

Accounting for losses during the injection and through diffusion into other cells and the blood stream, we expect that the intrafollicular levels of DOM-1 were similar to those achieved by a diet naturally contaminated with DON, as previously discussed. Under this treatment, the diameter of large follicles was reduced compared to that of controls, this regression being the first sign of a negative effect of DOM-1. Whereas DON induces apoptosis in leukocytes, DOM-1 failed to increase the rate of apoptotsis, and was therefore classified as a non-toxic metabolite (Pestka 2008). This, however does not discard the possibility in having a biological effect in cells as shown in our *in vivo* study, although further experiments are necessary to determine the mechanism of action of DOM-1.

Mycotoxins are known for having a "Janus face" as they can have biphasic effects according to the dose; as an example, they can increase or reduce secretion of hormones such as E2 depending on the dose used (Pizzo, Caloni et al. 2015). Within the ovary, E2, P4 and testosterone are usually referred to as health markers. In TC, P4 and testosterone secretion is essential for development and the same applies for E2 and P4 in GC. In published studies, DON at lower doses (10 ng/ml) could increase secretion of E2 in pig granulosa cells while higher doses (100 and 1000 ng/ml) decreased it (Ranzenigo, Caloni et al. 2008). In our studies, DON has the ability to decrease hormone secretion in both GC and TC; in GC a dose of 100 ng/ml of DON was potent enough to reduce E2 and P4 secretion, while lower doses (1 and 10 ng/ml) caused no changes in E2 secretion but slightly reduced P4 levels. In our TC culture, DON (1 ng/ml) could only reduce the secretion of P4, whereas DOM-1 (1 ng/ml) decreased both P4 and testosterone. This loss of differentiated function is consistent with our in vivo experiment where DOM-1 had a

negative impact on follicular growth. TC play an important role during steroid secretion as they produce androgens that diffuse to GC to be converted into E2, and therefore impact oocyte quality. As the vascular part of the follicle, TC represents the first defense barrier against the effects of DON and DOM-1 coming from the blood stream, but experiments performed in TC to assess the effects of other mycotoxins like ZEA in more sensitive species such as pigs are usually performed in co-culture with GC (Jakimiuk, Gajecka et al. 2010); consequently, there is no published information about the effects of DON or DOM-1 in TC.

The negative impact of DOM-1 and DON on steroid secretion by TC is directed at several loci, as DOM-1 down-regulated mRNA encoding CYP11A1, StAR and HSD3B1 and DON reduced both StAR and HSD3B1; in GC however, DON only reduced CYP9A1 levels. A recent study explored the effects of DON in cultured bovine GC in the presence of serum, in which 3.3 μM (1000 ng/ml) reduced levels of both E2 and P4, but failed to decreased GC cell number (Ranzenigo, Caloni et al. 2008), while lower doses such as 0.3 (100 ng/ml) and 0.1 μM (33.3 ng/ml) had no effect on steroidogenesis. A subsequent paper reported that DON increased mRNA levels encoding for CYP11A1 but had no effect on mRNA levels of CYP19A1 (Pizzo, Caloni et al. 2016). These differences in cell sensitivity to DON can be attributed to two main differences in the cell culture: the presence of fetal bovine serum may affect the natural response of GC to DON, and the use of FSH in a higher concentration (30 ng/ml) may also produce a protective effect on the cells. Unfortunately, there is no more information available on the effects of DON or DOM-1 on steroidogenesis in bovine GC and TC, therefore the effects are usually compared with those documented in pigs, a more sensitive species to the effects

of mycotoxins.

The main mechanism of action of DON is through the RSR that is activated when DON binds to the 60S sub-unit of the ribosome. The RSR results in the activation of MAPK signaling, usually involved in many physiological processes such as cell growth and differentiation, but also in apoptosis. Upstream of MAPK, one of the first proteins to be activated during RSR is PKR. In the present study DON and DOM-1 increased phosphorylation of PKR, MAPK3/1, MAPK8 and AKT, as has been previously reported for DON (Pestka 2008), but was unexpected for DOM-1. Interestingly, there was a significant difference between DON and DOM-1 in TC, as DOM-1 increased phosphorylation of MAPK14 while DON decreased it, contrary to previous experiments were DON could phosphorylate MAPK14 by phosphorylation of eIF2α by PKR. We believe that this difference is caused by the use of a high dose of DON (100 ng/ml) (Zhou, Lau et al. 2003), as GC from our experiments treated with 100 ng/ml were also capable of inducing phosphorylation of MAPK14.

To study this difference in MAPK14 activation a PKR blocker was added to the TC, which blocked phosphorylation of MAPK3/1 but failed to block MAPK14. The PKR pathway is known as an integrator for ligand-activated stress-activated protein kinase pathways that lead to stimulation of MAPK14 (Williams 2001). We attribute the inability of the PKR inhbitor to decrease MAPK14 activation to an additional RSR protein known as Hck. Hck is rapidly activated after DON exposure and can be inhibited by tyrosine kinase inhibitors PP1 and PP2 (Zhou, Jia et al. 2005). We, therefore, measured the role of Hck in TC by blocking its function with PP2. Addition of DON or DOM-1 to PP2-treated cells did not affect levels of PKR but decreased phosphorylation of both MAPK1/3 and

MAPK14, which is consistent with previous publications in which PP2 abolished phosphorylation of MAPK such as MAPK 1/3, MAPK14 and MAPK8 (J.J. Pestka, 2003 #164).

Activation of MAPK1/3 pathway by DON resulted in significant changes in mRNA levels for EGR1, EGR3 and FOS in both GC (100 ng/ml) and TC (1 ng/ml). Strikingly, even though TC seem to be sensitive to DOM-1, it did not alter levels of mRNA of these early response genes. One possible reason for this is the pattern of phosphorylation of MAPK1/3; the determination of its downstream signals seem to be related to the duration and intensity of its phosphorylation (Shaul and Seger 2007) and, in our experiments, DON induced a strong (3 fold change) and short phosphorylation (decreased by 30 min) while DOM-1 induced a weaker (2 fold change) and more stable phosphorylation (up to 60 min).

The activation of the RSR pathway by DON should induce apoptosis and this was observed in GC, but for the first time we demonstrated that DOM-1 increased the percentage of apoptotic cells by 8%, while DON produced no changes in apoptosis in TC. The inefficiency of DON to induce apoptosis has been previously reported in murine macrophage cell lines where low doses of DON (5 and 10 ng/ml) did not alter the percentage of apoptotic cells when compared to other trichothecene toxins such as satratoxin that can induce apoptosis with very low doses (Yang, Jarvis et al. 2000). In order for DON to induce apoptosis, a higher dose (1000 ng/ml) is required in other cell types (Pestka 2008). In our GC experiments, DON at a concentration of 100 ng/ml was enough to increase the percentage of apoptotic cells by 15%, suggesting that the doses of DON are key for the induction of apoptosis and that lower doses may induce some

different mechanism within the cells.

Along with this increase in apoptosis, DOM-1 was able to induce changes in mRNA of genes related to the apoptotic pathway, including FASLG, a ligand that binds to FAS receptor and is involved in apoptosis in human GC (Quirk, Cowan et al. 1995). DON also increased FASLG mRNA levels in GC while in TC it had no effects, which may explain the lack of increase in percentage of apoptotic cells. Interestingly both DON in GC and DOM-1 in TC increased mRNA levels of BID, a pro-apoptotic member of the Bcl-2 family that can be cleaved by caspases or bind to anti-apoptotic Bcl-2 (Billen, Shamas-Din et al. 2008). Bcl-2 is gaining attention as its homolog, Beclin1 (Atg6), is an essential initiator of autophagy, having a key role in determining whether cells go on to apoptosis or autophagy (Tsujimoto 1998). This cross-talk between apoptosis and autophagy occurs under conditions of cellular stress (including the RSR) that lead to an up-regulation of caspase 8, which, in its active form, promotes Beclin1 cleavage by BID and induces apoptosis (Kang, Zeh et al. 2011). However, if Beclin1 is cleaved directly by caspase 8 this will lead to cleavage of Atg4 by caspase 3 that will result in autophagy, inhibiting Beclin1 and BID interaction and therefore blocking apoptosis (Kang, Zeh et al. 2011). As the addition of DON in GC and DOM-1 in TC led to only a slight increase in apoptosis, we hypothesized a possible role of autophagy. Both DON (100 ng/ml in GC) and DOM-1 (1 ng/ml) were able to up-regulate levels of Beclin1, and increased levels of mRNA encoding LC3, and DON increased protein levels for LC3-I (cytosolic form) and LC3-II (autophagosomal membrane-bound form). To further assess the autophagy pathway, pharmacological inhibition of ULK1 (Atg1) was employed, which resulted in an increase in apoptosis in GC cells. In some cell types, autophagy is a protective

reaction to cell damage, by destroying damaged organelles in autophagosomes (Mizushima 2007). We propose that this mechanism is activated also in both TC and GC in response to DON and DOM-1.

While in other species we know that DON can affect brain activity and disregulate immunologic response (Behrens, Huwel et al. 2015), for DOM-1 we know nothing. In the reproductive field, it would be interestering to observe this new "toxic" effect in other cells types such as oocytes, sperm or embrios, as it may not cause rapid effects that kill the cell, but exert long term negative effects that can be observed much later, such as a disrumption of new follicular waves, abnormalities in embryo development or even low fertility in the offspring of exposed animals. Therefore there is still a lot of research that needs to be done in the mechanism of action of DON, DOM-1 or both.

Final conclusions

In conclusion, these studies have shown for the first time the negative impact of the major mycotoxin DON and its main metabolite DOM-1 on bovine reproduction, demonstrating that the non-toxic metabolite DOM-1 increases levels of cellular stress in TC, and can inhibit follicle growth in vivo. There are an increasing number of feed additives on the market that are modifed bacteria with high de-epoxydase activity and which convert DON to DOM-1 in feed. These data suggest that DON is a potential problem for ruminants, and calls into the question the use of such products until the effects of DOM-1 in other species such as pigs and birds has been evaluated.

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