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In vitro toxicological effects of *Fusarium* mycotoxins on bovine granulosa cells

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CHAPTER 1

Foreword

1. Foreword

1.1. Mycotoxins

Mycotoxins are secondary metabolites produced by molds (Hussein and Brasel, 2001; Binder et al., 2007). Their global occurrence is considered to be a major risk factor affecting human and animal health as it is estimated that 25% of the world's crop production is contaminated to some extent with mycotoxins (Pinton and Oswald, 2014). The most frequent toxigenic molds are *Fusarium*, *Aspergillus* and *Penicillium* species (Creppy, 2002). Mycotoxin contamination may occur in two ways: molds may grow as pathogens on plants in the field before harvesting or they may grow saprophytically on stored plants (Glenn, 2007). However the detection of molds does not imply necessarily the presence of mycotoxins since not all fungal growth leads to mycotoxin formation (Binder et al., 2007). Many factors are essential for mycotoxins production such as the presence of nutrients, temperature, water activity (a_w) and oxygen (Zaki et al., 2012).

Even if the occurrence of mycotoxins exhibits often a geographic pattern, the worldwide trade with food and feed commodities allows a wide distribution of contaminated material (Fink-Gremmels, 1999).

Mycotoxins have a wide spectrum of toxicological effects and the nature and the intensity of these effects are related to the level and duration of exposure (Fink-Gremmels, 1999). Since molds are able to produce different mycotoxins simultaneously, feed commodities are often contaminated with more than one mycotoxin and the co-presence of mycotoxins can result in additive, synergic or antagonistic effects (Tatay et al., 2014).

Although consumption of a mycotoxin-contaminated diet may induce acute effects, the chronic exposure to low doses of mycotoxins is of major concern (Fink-Gremmels and Malekinejad, 2007). The chronic effects may result in reduced weight gain, diminished productivity, reduced reproductive capacities and increased susceptibility to infections (Fink-Gremmels and Malekinejad, 2007; Pestka, 2007), which lead to economic losses (Huwig et al., 2001; Wu, 2004, 2006).

1.2. *Fusarium* mycotoxins

Fusarium species are able to produce a wide range of mycotoxins such as trichothecenes, deoxynivalenol (DON) and T-2 toxin (T-2), zearalenone (ZEA), and fumonisins (Placinta et al., 1999; Morgavi and Riley, 2007). *Fusarium* species are most prevalent in the northern hemisphere regions and they are commonly found on cereals grown in the temperate regions of Europe, America and Asia (Creppy, 2002; Larsen, 2004). The high moisture level promotes fungal growth in grains and cool temperatures can increase mycotoxins production. *Fusarium* species require a moisture content of 25% (Newman and Raymond, 2005) and toxicogenesis is closely influenced by temperature and a_w (Sweeney and Dobson, 1998).
Fusarium mycotoxins are able to induce both acute and chronic effects that are closely related to level and duration of exposure. Many syndromes in farm livestock that are linked with exposure to *Fusarium* mycotoxins have been observed (D'Mello, 1999).

1.2.1. Trichotecenes

Trichothecenes are a family of mycotoxins subdivided into four basic groups (A, B, C and D) according to their chemical structures. Type A and type B represent the most important members (Mostrom and Raisbeck, 2012).
The type A trichothecenes, which are more toxic compared to those in the type B category, include T-2, HT-2 toxin, neosolaniol and diacetoxyscirpetol (DAS) while type B include DON and its 3-acetyl and 15-acetyl derivates, nivalenol (NIV) and fusarenon X (Placinta et al., 1999). The tricyclic nucleus that contains an epoxide at C-12 and C-13 is considered essential for toxicity of all trichotecenes (Mostrom and Raisbeck, 2012).
The mechanism of action of trichothecenes is linked to their ability to readily bind to eukaryotic ribosomes, in particular to the 60S ribosomal subunits, inhibiting protein synthesis (Pestka, 2010).
A large variety of effects in animals are related to tricothecenes toxicosis such as anorexia, reduced weight gain, altered nutritional efficiency and immunotoxicity (Pestka and Smolinski, 2005).

<u>DON</u>

Among the trichothecenes, DON (Figure 1.1) is the most commonly encountered worldwide (Pestka and Smolinski, 2005). DON occurs mainly in grains such as wheat, barley, and maize and less often in oats, rice, rye, sorghum

and triticale. The occurrence of DON is associated primarily with two important plant pathogens: *Fusarium graminearum* (*Teleomorph Gibberella zeae*) and *Fusarium culmorum* (Creppy, 2002). Co-occurrence with other *Fusarium* mycotoxins, including other trichotecenes, is frequently observed (EFSA, 2004).
From a chemical point of view DON is a low molecular weight sesquiterpenoid containing both 9, 10 double bond and 12, 13 epoxide group. The mechanism of action of DON is well known and is related to its ability to bind eukaryotic ribosomes and inhibit protein synthesis by blocking translation and inhibiting the elongation of peptide chains (Larsen et al., 2004; Pestka, 2010). However DON can also activate mitogen-activated protein kinases (MAPKs) that mediate selective gene expression and apoptosis, ultimately contributing to downstream pathologic sequelae (Larsen et al., 2004; Pestka et al. 2010; Li et al., 2014). DON binds to ribosomes and rapidly activates MAPKs and apoptosis in a process also known as "ribotoxic stress response" (Maresca, 2013).

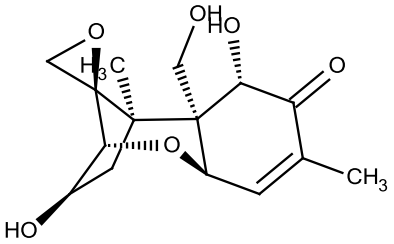

Figure 1.1. Chemical structure of Deoxynivalenol (DON).

Differences in metabolism, absorption, distribution, and elimination of DON might account for differential sensitivity among animal species (Pestka, 2007). Animal species evaluated to date are susceptible to DON according to the rank order of pigs > mice > rats > poultry ≈ ruminants (Li et al., 2014). DON undergoes significant biotrasformation into the less toxic de-epoxy DON (DOM-1) by intestinal or rumen microbe activity rather than by liver or other organs (Pestka and Smolinski, 2005; Fink-Gremmels, 2007).
The carry-over of DON into tissues or fluids of exposed animals does not appear to be significant (Richard, 2007). Indeed it has been reported that DON and DOM-1 were detected in cow's milk, but only in extremely low amounts (less than 4 ng/ml) (Pestka and Smolinski, 2005).
DON has been implicated in farm animal disease outbreaks worldwide (Morgavi et al., 2007) and may cause adverse health effects after acute, short-term, or long-term administration (Creppy, 2002). In monogastric animals after acute

administration, DON produces two characteristic toxicological effects: feed refusal and emesis (Creppy, 2002; Morgavi et al., 2007). Chronic low dose toxicity is characterised by reduced weight gain and diminished nutritional efficiency and immunologic effects (Pestka and Smolinski, 2005).
Reduced feed intake was observed in ruminants (Osweiler, 2000) and in cattle DON exposure has been correlated with decrease of milk production (Jouany and Diaz, 2005). Considering the high level of stress to which dairy cattle are subjected, these animals may be more sensitive to the effects of DON compared to beef cattle and sheep (Jouany and Diaz, 2005).
Immunosuppressive effects and kidney injury were also observed in animals after exposure to DON (Richard, 2007). DON is considered to be a major cause of economic losses due to reduced performance (Morgavi et al., 2007).

T-2 Toxin

T-2 toxin (T-2) (Figure 1.2) is produced by different *Fusarium* species, including *F. sporotrichioides, F. poae*, *F. kyushuense* and *F. langsethiae*. *F. sporotrichioides* is responsible for the T-2 production (Richard, 2007). It has been reported that T-2 is contained in corn, wheat, barley, oats, rice, rye and other crops (Richard, 2007) whereas the concentration of T-2 in grains is generally lower compared to the concentrations of DON (Fink-Gremmels, 1999).
T-2 is considered the most toxic trichothecene that can cause severe acute intoxication (Fink-Gremmels, 1999). It is highly cytotoxic, causing lesions at oral and gastrointestinal mucosa level. T-2 is responsible for pronounced pancytopenia and immuno-soppression through protein synthesis inhibition and blocking of maturation of hemopoetic cells in bone marrow (Fink-Gremmels, 1999). This toxin caused the "moldy corn toxicosis" occurred in livestock in North America and Alimentary Toxic Aleukia, a human disease that occurred when several thousand people in Russia consumed cereals that had over-wintered in the fields (Cheeke and Shull, 1985).

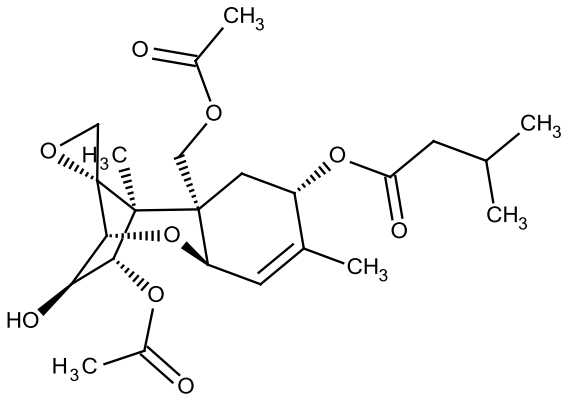

Figure 1.2. Chemical structure of T2-toxin (T-2).

1.2.2. Zearalenone

Zearalenone (ZEA) (Figure 1.3) is a phytoestrogenic compound also known as 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcylic acid μ-lactone (Zinedine et al., 2007). ZEA, produced by several *Fusarium* species (*F. culmorum*, *F. graminearum*, *F. equiseti* and *F. crookwellense*), is found to infest wheat, barley, rice, maize, and some other crops (Fink-Gremmels and Malekinejad, 2007). Also cereals products like flour, malt, soybeans and beer can be contaminated by ZEA (Zinedine et al., 2007).

Pre-harvest toxin production is the principal way of contamination, however post-harvest formation of the toxin cannot entirely be excluded, mainly when the crop is not handled and dried correctly (Fink-Gremmels and Malekinejad, 2007; Zinedine et al., 2007).

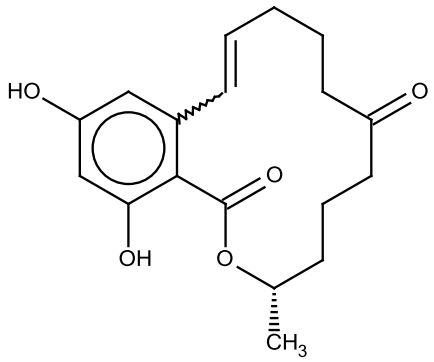

Figure 1.3. Chemical structure of Zearalenone (ZEA).

In pigs after ingestion, ZEA is rapidly absorbed from the gastrointestinal tract and metabolized mainly into α-zearalenol (α-Zol) (Figure 1.4) by liver (Malekinejad et al., 2005).
In ruminants ZEA is converted mainly into α-Zol and to less extent into β-zearalenol (β-Zol) (Figure 1.5) predominantly by rumen protozoa (Abidin and Khatoon, 2012).
ZEA and its metabolites exert their toxicity through binding to estrogens receptors and induce a syndrome described in animals as hyperestrogenism (Wang et al., 2014). Although α-Zol shows the higher binding affinity to estrogen receptors compared to ZEA and β-Zol, clinical signs of hyperoestrogenism are observed infrequently in ruminating cows. The likely explanation for this apparent discrepancy is the poor rate of absorption of the more polar α-Zol (Fink-Gremmels, 2008).
However in ruminants ZEA undergoes also hepatic metabolism: comparative experiments conducted on liver microsomes demonstrated that the ratio between α-Zol and β-Zol formation varies among animal species and that α-Zol is the most frequent metabolite in pigs whereas β-Zol seems to prevail as results of hepatic metabolism in ruminants (Malekinejad et al., 2006).

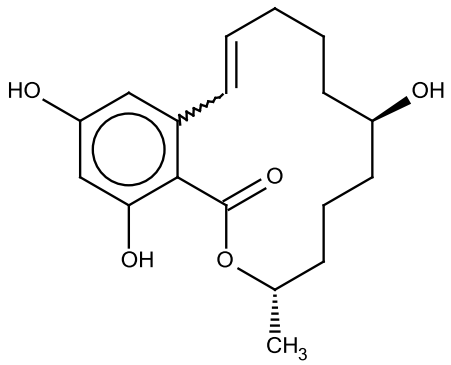

Figure 1.4. Chemical structure of α-Zearalenol (α-Zol).

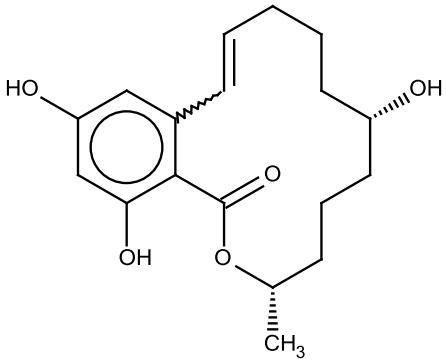

Figure 1.5. Chemical structure of β-Zearalenol (β-Zol).

The typical clinical signs of hyperestrogenism in female pigs are vulvae enlargement and vulvovaginitis, edema and swelling of the mammary gland, enlargement of the juvenile uterus, ovarian cysts, and impaired oocyte maturation (Minervini et al., 2001).
Hyperestrogenic symptoms have been reported in prepubertal heifers that after exposure to ZEA showed enlargement of mammary glands and infertility (Coppock et al., 1990). A decrease in the conception rate was also demonstrated in heifers exposed to 250 mg crystalline ZEA (Weaver et al., 1986a, 1986b). Reproductive disorders associated with exposure to ZEA, such as lower newborn percentages, infertility, increased duration of estrus and increased uterus and ovarian weight have been reported in ewes (Smith et al., 1990).
Taken together these findings suggest that also ruminants are sensitive to ZEA exposure.

1.2.3. Fumonisins

Fumonisins are produced primarily by *Fusarium verticillioides* and *Fusarium proliferatum,* however other species including *F. napiforme* and *F. nygamai* are involved in fumonisins production (Glenn, 2007). Fumonisins is found particularly in corn and corn-based products often in combination with other *Fusarium* mycotoxins, such as ZEA and DON (EFSA, 2005). Twenty-eight fumonisins have been isolated and divided into four groups known as A, B, C and P (Yazar and Omurtag, 2008). The most important as natural contaminants of cereals are the B series (fumonisin B_1, B_2 and B_3) and fumonisin B_1 (FB_1) (Figure 1.6) is the most significant in terms of toxicity and occurrence (EFSA, 2005).

It is well known that fumonisins inhibit sphingolipid biosynthesis, consequently all receptors and processes that are dependent on sphingolipids could be affected (Morgavi and Riley, 2007). FB_1 shows a species-specific toxicity and it is responsible for leukoencephalomalacia in horses (Marasas et al., 1988; Kellerman et al., 1990) pulmonary edema syndrome in pigs (Harrison et al., 1990; Osweiler et al., 1992; Colvin et al., 1993) and renal and liver injury in rodents (Diaz and Boermans, 1994; EFSA, 2005). Diet containing fumonisins ≥ 75 ppm has been associated with signs of liver injury in cattle, determining increase of serum levels of organ specific diagnostic enzymes, and increase of cholesterol and bilirubin levels (Baker and Rottinghaus, 1999; Mathur et al., 2001). FB_1 resists microbial degradation in the rumen (Caloni et al., 2000) and its oral bioavailability remains very low, even lower than in other animals (Fink-Gremmels, 2007). This could explain why in cattle severe and acute intoxications do not occur under farm conditions (Fink-Gremmels, 2007). Moreover in cattle there is no carry-over of fumonisins into milk (Richard, 2007). FB_1 is considered to be a possible carcinogen for humans and is classified by the International Agency for Research on Cancer as class 2B (IARC, 2003).

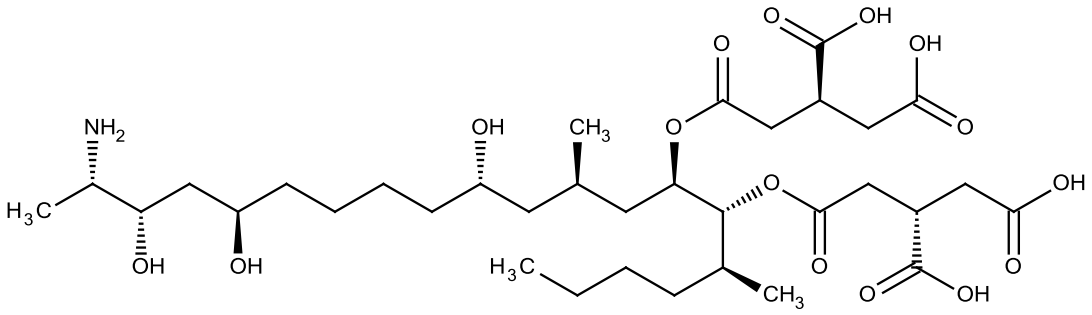

Figure 1.6. Chemical structure of Fumonisin B_1 (FB_1).

1.2.4. Fusarium mycotoxins: worldwide contamination and European guidance levels

Many data demonstrate the global contamination of cereal grains with *Fusarium* mycotoxins, particularly with trichotecenes, fumonisins and ZEA (Binder et al., 2007; Rodrigues and Naehrer, 2012). The worldwide trade of commodities could play a key role in the diffusion of mycotoxins (Placinta et al., 1999). Many attempts to investigate the worldwide contamination of mycotoxins have been made. One of the most recent was conducted between January 2009 and December 2011 on soybean/soybean meal, wheat, dried distillers grains with soluble and finished feed samples coming from Americas, Europe and Asia. ZEA, DON, fumonisins were present respectively in 45%, 59% 64% of analyzed samples (Rodrigues and Naehrer, 2012). Previously Binder et al. (2007) carried out a 2-year survey program and analyzed samples of raw materials and finished feed for detection of mycotoxins. More than half of materials collected in Europe were contaminated at levels above the limit of quantification of methods applied, whereas one third of tests on Asian-Pacific sourced samples were positive. European samples had DON, ZEA and T-2 as major contaminants; materials from Asia and the Pacific were mainly contaminated by DON, ZEA and fumonisins. These results demonstrate the high global prevalence of *Fusarium* mycotoxins in cereal grains and animal feed. The data on widespread distribution of these mycotoxins are further confirmed by other investigations (Boutigny et al., 2012; Cortinovis et al., 2012; Garrido et al., 2012; Grajewski et al., 2012).

The co-occurrence of *Fusarium* mycotoxins in cereal grains and animal feed is also a common feature of several investigations, leading to the possibility of additivity and/or synergism in the aetiology of mycotoxicosis in animals (Girgis and Smith, 2010).

Since mycotoxins are hazardous compounds both to animal and human health, guidelines and legislation are in place, or under consideration in most countries, to protect consumer and animal welfare (Edwards, 2004). The European Commission (EC) has established guidance levels for *Fusarium* mycotoxins in animal feed that varies by species reflecting their sensitivity to mycotoxins.

For fumonisins these guidance values concerning complementary and complete feeding stuffs, are 5 mg/kg for pigs, horses, rabbits and pet animals, 10 mg/kg for fish, 20 mg/kg for poultry, calves (<4 months), lambs and goat kids and 50 mg/kg for adult ruminants (>4 months) and mink (EC, 2006).

The EC has recommended guidance values for ZEA in products intended for animal feed. The guidance values for cereal/cereal products and corn by-products are 2 and 3 mg/kg, respectively (EC, 2006). The guidance values for complementary and complete feeding stuffs for piglets/gilts, sows/fattening

pigs, and calves/dairy cattle/sheep/goats are 0.1, 0.25 and 0.5 mg/kg, respectively (EC, 2006).
The EC guidance values for DON are 8 mg/kg for cereals and cereal products with the exception of corn by-products (12 mg/kg) and 5 mg/kg for complementary and complete feeding stuffs with the exception of feeding stuffs for pigs (0.9 mg/kg), and for calves (<4 months), lambs and goat kids (2 mg/kg) (EC, 2006; Pinton and Oswald, 2014).
Since data about the occurrence and toxic effects of T-2 and HT-2 toxin are scarce, there are no specific regulations or recommendations of the EC about the maximal concentrations in products intended for animal feed.

1.3. The ovarian follicle and steroidogenesis pathway in cattle

The primary functions of the ovaries are the production of female gametes (oocytes and ova) and steroidogenesis (Evans, 2012). The ovary of immature domestic mammals consists of a peripherical parenchymatous zone (cortex) containing primary oocytes, each surrounded by a single layer of supporting cells, and a central zone (medulla) containing connective tissue, nerves and blood vessels (Ball and Peters, 2004; Evans and Ganjam, 2011; Evans, 2012).
The functional and structural unit of the ovary is the follicle. Considering their stage of development, follicles are classified as primordial, primary, secondary and tertiary (antral) (Evans et al., 2007; Evans and Ganjam, 2011).
During follicular growth, the number of the cells surrounding the primary oocyte increases and with continued development a cavity or antrum is formed filled with follicular fluid. The outer layers of the follicle are the theca externa, which contains mostly fibrous tissue and the theca interna that is more cellular and contains many blood vessels. In the innermost layer there are granulosa cells (GC) (Figure 1.7) (Ball and Peters, 2004). The oocyte itself is surrounded by the zona pellucida and is suspended in the follicular fluid by a clump of cells, the cumulus oophorus. After ovulation, the cavity of the ovulated follicle is invaded by cells that are derived from the granulosa and theca interna layers of the follicle, forming a structure known as the *corpus luteum* (Ball and Peters, 2004). This structure persists on the surface of the ovary until a few days before the next ovulation, and then it begins to degenerate. Alternatively if the female is pregnant, the *corpus luteum* is maintained for the duration of the pregnancy (Ball and Peters, 2004). Granulosa and theca cells are crucial in the process of normal folliculogenesis and oocyte growth and development. They are also responsible for the delivery of nutrients to the oocyte and for the ovarian steroidogenesis (Petro et al., 2012).

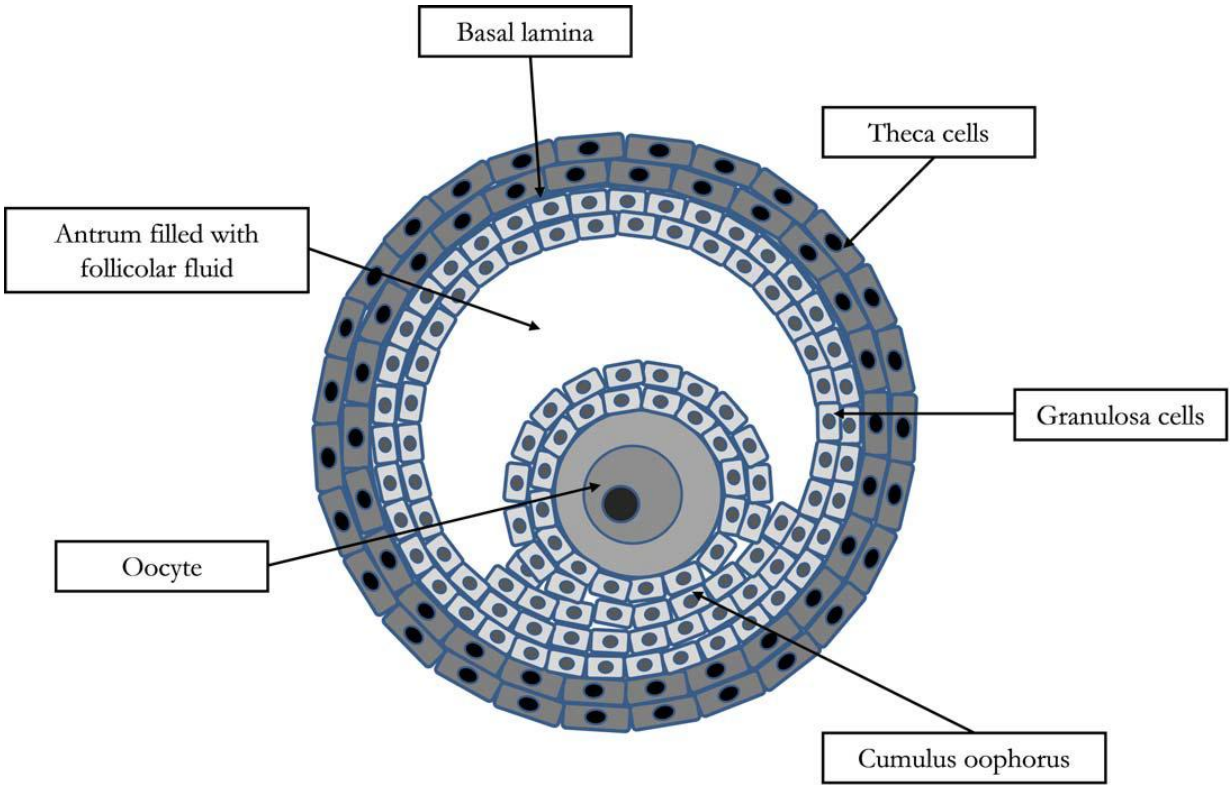

Figure 1.7. Ovarian follicle.

The ovarian steroids progesterone (P4), 17β-estradiol (E2) and androgens regulate many ovarian activity such as follicle growth, differentiation, maturation and release of viable oocytes, and formation and regression of the *corpum luteum* (Wood and Strauss, 2002).

The first and rate-limiting enzymatic step in steroidogenesis involves cholesterol, which is the common substrate for steroid biosynthesis. Cholesterol is converted into pregnenolone by P450 side chain cleavage enzyme system (P450scc, CYP11A1) (Wood and Strauss, 2002; Payne and Hales, 2008). Cholesterol in the ovary can be derived from two sources: *de novo* synthesis from acetate, or uptake from circulating lipoprotein particles (Miler, 2007). Cholesterol is carried to the outer membrane of the mitochondria and is subsequently transported to the inner mitochondrial membrane by the steroidogenesis acute regulatory protein (StAR) (Strauss et al., 1999; Stocco, 2001).

The movement of cholesterol from the outer to inner mitochondrial membrane by StAR represents the rate-limiting step in steroid production process (Christenson and Strauss, 2000; Miller, 2007). In theca cells of a growing follicle and luteal cells of the *corpum luteum*, pregnenolone is converted to P4 by 3β-hydroxysteroid dehydrogenase (3β-HSD). P4 is then converted into androgens by reactions carried out by the P450 17α-hydroxylase/17, 20 lyase ($P450_{17\alpha}$) enzyme in the theca cells. In GC P450 aromatase (P450arom, CYP19A1) converts androstenedione or testosterone to estrone (E1) and E2 respectively. $P450_{17\alpha}$ and P450arom enzymes are expressed only in the theca and GC respectively, of the growing follicles (Wood and Strauss, 2002; Payne and Hales, 2008). The steroidogenesis pathway is schematised in the Figure 1.8.

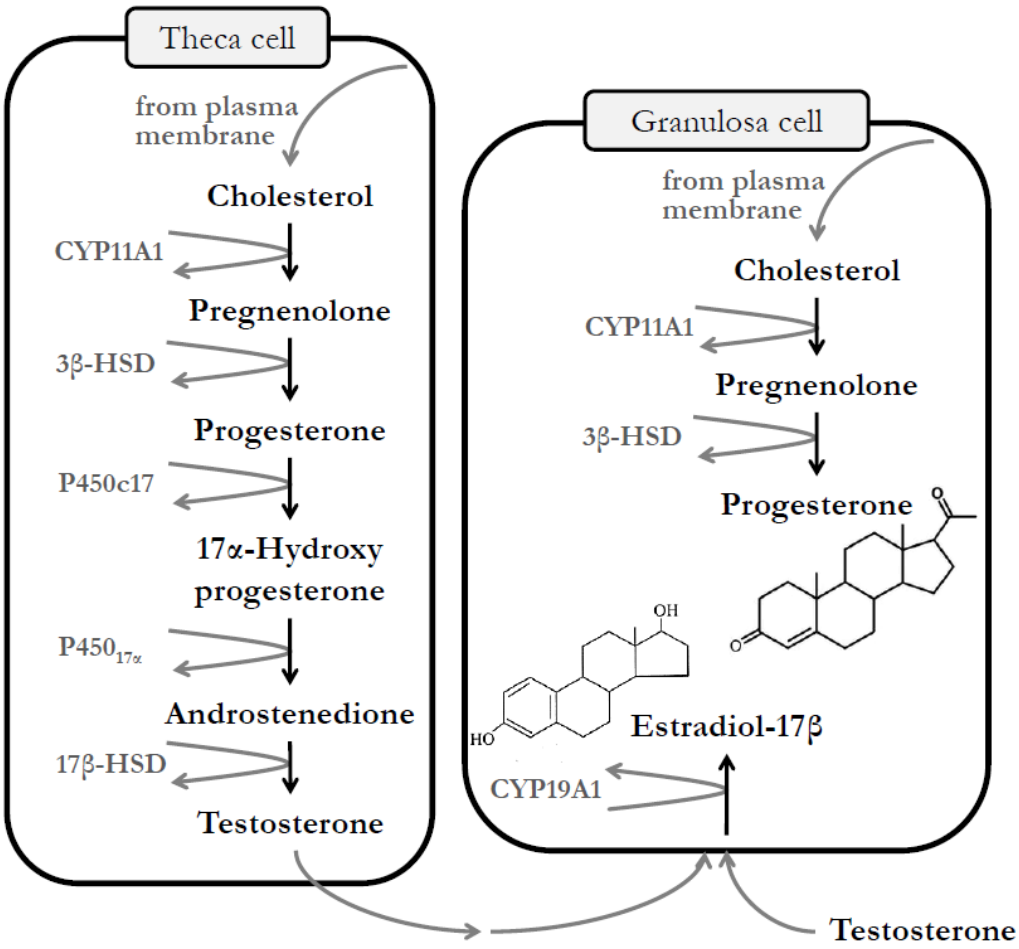

Figure 1.8. Steroidogenesis pathway

Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) are essential gonadotropins that generate and regulate the proper environment for steroid synthesis by the growing follicle. FSH increases the expression and activity of CYP19A1 in GC while LH enhances CYP11A1 expression in luteal cells resulting in increased synthesis of E2 and P4, respectively.
In theca cells of a growing follicles both CYP11A1 and $P450_{17\alpha}$ expression is increased in a LH-dependent manner resulting in augmented androgen synthesis (Wood and Strauss, 2002).
In addition there are several growth factors that modulate the action of these gonadotropins production. Insulin has a double action: firstly it mimics the actions of the gonadotropins on production of E2, P4 in GC and androgens and in theca cells; secondly increases FSH-stimulated E2 and P4 synthesis additively, while it acts synergically with LH to stimulate steroidogenesis (Franks et al., 1999). The IGFs, which show high structural similarity to insulin, also play an important role in modulating ovarian steroidogenesis. In rodent, pigs and cows IGF1 is the prevalent IGF synthetized by GC (Franks et al., 1999).
Like insulin the IGFs stimulate basal and gonadotropin-dependent steroidogenesis. Both IGF1 and IGF2 are able to stimulate basal E2, P4 release in GC and androstenedione and testosterone production in theca cells (Spicer et al., 1998; Spicer et al., 2002; Spicer and Aad, 2007), and to synergically increase FSH-stimulated E2 and P4 production in granulosa and luteal cells (Franks et al., 1999; Spicer et al., 2002).
This increased production of the ovarian steroids by IGF1 and IGF2 can be correlated to stimulated expression of several steroidogenic enzymes (Wood and Strauss, 2002). Indeed it has been demonstrated that in rat and in pig IGF1 acts synergically with FSH to increase P450scc and 3β-HSD expression and similarly in rat theca cells IGF1 increases P450scc mRNA levels and $P450_{17\alpha}$ hydroxylase activity (Franks et al., 1999).

1.4. Effects of *Fusarium* mycotoxins on ovarian function

1.4.1. Trichotecenes

The potential of trichothecenes to act as endocrine disruptors has not been clarified yet and it is still under investigation (Ndossi et al., 2012).

DON

DON has been implicated in reduced reproductive performance in pigs, due to its ability to impair oocyte maturation and embryo development (Alm et al.,

2002; Malekinejad et al., 2007; Alm et al., 2006; Schoevers et al., 2010) and to reduce feed intake (Tiemann and Dänicke, 2007). In the study carried out by Alm et al. (2002), DON was found to have a potent effect on *in vitro* porcine oocyte maturation, significantly decreasing the proportion of oocytes reaching metaphase II at lower concentrations than that of α-Zol and β-Zol. In a subsequent study, to evaluate DON toxicity during specific stages of oocyte meiosis, cumulus-oocyte complexes collected from ovaries of cyclic sows were exposed to 0.02, 0.2, and 2 μM DON (Schoevers et al., 2010). Exposure to the highest DON concentration inhibited cumulus expansion and induced cumulus cell death (Schoevers et al., 2010). Absence of cumulus cell expansion and induction of cumulus cell death negatively influence oocyte maturation by altering the glutathione levels in the oocyte (Qian et al., 2003). DON at all concentrations reduced metaphase II formation in line with the results obtained by Alm et al. (2002) and led to malformations of the meiotic spindle as previously observed by the same authors (Malekinejad et al., 2007). When oocytes were exposed to 2 μM DON, spindle aberrations occurred at the metaphase I stage, and oocytes maturing in the presence of 0.2 or 0.02 μM DON exhibited spindle aberrations after reaching the metaphase II stage (Schoevers et al., 2010). Spindle malformation was observed when oocytes were exposed to DON during formation of meiotic spindles at metaphase I and II, but embryo development was also reduced by DON exposure during prophase I. These results indicate that exposure to DON can adversely affect maturation of porcine oocytes by causing abnormalities of the meiotic spindles and by disturbing oocyte cytoplasmic maturation (Schoevers et al., 2010). Moreover, DON has been shown to have direct dose-dependent effects on porcine GC proliferation, steroidogenesis, and gene expression (Ranzenigo et al., 2008; Medvedova et al., 2011). In the study conducted by Ranzenigo et al. (2008), DON had a biphasic effect on cell growth with 0.034 and 0.34 μM increasing cell numbers and 3.4 μM drastically inhibiting cell numbers. DON at 0.34 and 3.4 μM inhibited P4 and E2 production induced by FSH plus IGF1 and at 3.4 μM completely blocked the FSH plus IGF1-induced CYP19A1 and CYP11A1 mRNA abundance (Ranzenigo et al., 2008). These results are confirmed by a more recent study (Cortinovis et al., 2014) where DON at 3.4 μM was found to inhibit cell proliferation and P4 and E2 release in porcine small GC. On the contrary, Medvedova et al. (2011) observed that DON at the dose of 3.4 μM increased the expression of markers of proliferation (cyclin B1 and PCNA) and stimulated P4 release in porcine GC; however, this occurred at different incubation conditions than in the studies carried out by Ranzenigo et al. (2008) and Cortinovis et al. (2014) as GC were exposed to DON in the presence of serum and exposure that lasted for 24 hours. Different from porcine GC cultured in serum-free medium that are dependent on *de novo* synthesis of

cholesterol for steroidogenesis (Baraño and Hammond, 1986), cells cultured in serum-containing medium are dependent on exogenous lipoprotein cholesterol for steroid biosynthesis (Veldhuis et al., 1984).

T-2 Toxin

The effect of oral exposure to low doses of T-2 on the ovarian function was evaluated in ewes and heifers (Huszenicza et al., 2000). The study showed that the peroral T-2 intake can significantly delay the follicle maturation, postpone the subsequent ovulation and may also possibly retard the consecutive luteinisation. As a consequence, animals inseminated during visible signs of standing heat may not conceive. Although the trial did not explain the etiopathogenesis of T-2 upon ovarian functions, it was suggested that the toxin directly deteriorates the GC and perhaps even the cells of the developing corpus luteum (Huszenicza et al., 2000). On the contrary, low-dose exposure to T-2 in mares had no effect on the length of the interovulatory interval and on the luteal and follicular phases (Juhasz et al., 2001). Similar to DON, in a subsequent *in vitro* study, the potential impact of T-2 on reproductive activity in pigs has been investigated by looking at the effects of this mycotoxin on porcine GC functions (Caloni et al., 2009). T-2 was found to have potent direct dose-dependent effects on GC proliferation and steroidogenesis. In details, T-2 strongly inhibited FSH plus IGF1-induced P4 and to a greater extent E2 production as well as cell numbers. It was concluded that these direct ovarian effects could be one mechanism whereby the presence of T-2 toxin in feedstuffs could impact reproductive performance in swine (Caloni et al., 2009).

1.4.2. Zearalenone

In cycling sows, ZEA causes several reproductive dysfunctions including nymphomania, pseudo-pregnancy, ovarian atrophy, and changes in the endometrium. ZEA causes sterility in sows by inciting a malfunction of the ovary (Mirocha et al., 1977; Minervini and Dell'Aquila, 2008). The oocyte dies in the Graafian follicles, and despite signs of estrus, there is no ovulation. ZEA acts similarly to estradiol in inhibiting the release and secretion of FSH, thus depressing the maturation of ovarian follicles during the preovulatory stage (Mirocha et al., 1977; Minervini and Dell'Aquila, 2008). The changes induced by ZEA depend on time of administration in relation to estrous cycle as well as on the dose administered (Minervini and Dell'Aquila, 2008). Chang et al. (1979) reported that concentrations of 25 to 100 mg/kg of 95% purified ZEA fed to multiparous sows after weaning or throughout the gestation period (or both) produced constant estrus, pseudopregnancy, infertility, reduced litter size,

malformation, and, probably, fetal resorption. Subsequently, Flowers et al. (1987) observed longer inter-estrous intervals in gilts receiving 20 mg of ZEA on Days 6 to 10 or Days 11 to 15 of the estrous cycle. Extended cycles were also reported in gilts fed 5 or 10 mg/kg of purified ZEA between Day 5 and 20 of the estrous cycle (Edwards et al., 1987). Luteal function was maintained due to a high plasma progesterone level recorded at Days 19 to 21 in those gilts that had extended cycles. The persistent *corpus luteum* spontaneously regressed 30 days after withdrawal of the contaminated feed (Edwards et al., 1987). These results indicated that ZEA may exhibit a luteotrophic property that prolongs the life span of the *corpus luteum* (Minervini and Dell'Aquila, 2008). In another study, a diet containing 3.61 or 4.33 mg/kg ZEA was fed at a mean daily level of 2 kg/animal to gilts from puberty to mating (Etienne and Jemmali, 1982). In 45% of these gilts, ZEA induced a pseudopregnancy state; no estrus was detected within 50 days after puberty, and *corpus luteum* developed at puberty were maintained (Etienne and Jemmali, 1982). Similar results were obtained by Young and King (1986) in pubertal gilts that consumed 6 or 9 mg/kg of purified ZEA starting the day after first estrus. A majority (88%) of the gilts became pseudo-pregnant as confirmed by plasma P4 levels and examination of their reproductive tracts (Young and King, 1986). Induced retention of *corpus luteum* by ZEA was similar to that which occurs when sows are given exogenous estrogen on Days 11 to 12 of the estrous cycle (Kidder et al., 1955; Gardner et al., 1963). Young et al. (1990) showed a linear relation between the level of ZEA in mg/kg and the length of anestrus in days. The weaning-to-estrus interval was found to increase when increased dietary ZEA was fed (Young et al., 1990). More recently, *in vitro* studies were carried out to elucidate the interaction of ZEA on gonadal cells of animals. Alm et al. (2002) investigated the influence of different doses of ZEA metabolites, α-Zol and β-Zol, on *in vitro* maturation of porcine oocytes. α-Zol and β-Zol were found to negatively affect the maturation and degeneration rates of porcine oocytes in a dose-dependent manner, but to different extents. Culture of oocytes in the presence of α-Zol for 48 hours up to the concentration of 7.5 μM resulted in a significant decrease in the maturation rate, whereas β-Zol showed a significant effect only at 30 μM (Alm et al., 2002). α-Zol had a greater effect than did β-Zol, probably by means of its greater estrogenicity (Alm et al., 2002). Consistent with these results, ZEA showed to interfere with oocyte progression through meiosis by inducing malformation of the meiotic spindles (Malekinejad et al., 2007). Importantly, ZEA-induced spindle malformations in the oocyte can result in aneuploid embryos (Malekinejad et al., 2007). In a further study, Alm et al. (2006) observed that feeding gilts with wheat naturally contaminated with DON and ZEA negatively influence the initial chromatin status of oocyte and oocyte maturation competence *in vitro*. On the contrary, no effect was observed on the expression

of the enzyme P450scc and the enzyme 3β-HSD in GC collected from ovaries of gilts fed with the contaminated diet (Alm et al., 2006). This indicates that DON and ZEA did not influence the activity of these enzymes involved in P4 synthesis. These results are not in agreement with *in vitro* studies (Tiemann et al., 2003), which show an apparent influence of ZEA on the expression of P450scc (CYP11A1) and 3β-HSD and therefore reduced P4 synthesis in cultured porcine GC. The *in vitro* addition of α-Zol and β-Zol at concentrations of 15 and 30 μM inhibited the FSH stimulated P4 synthesis (Tiemann et al., 2003). Consistent with these results, in a subsequent *in vitro* study (Ranzenigo et al., 2008) α-Zol (9.4 μM) was found to decrease abundance of CYP19A1 and CYP11A1 mRNA induced by FSH plus IGF1. However, α-Zol primarily increased P4 production induced by FSH and IGF1 (Ranzenigo et al., 2008; Cortinovis et al., 2014). E2 production exhibited a biphasic dose-response to α-Zol, with 0.094 μM increasing and 9.4 μM inhibiting FSH plus IGF1-induced E2 production (Ranzenigo et al., 2008). However Cortinovis et al. (2014) reported no effects on E2 production after exposure to α-Zol at 9.4 μM in porcine small GC. Increased P4 and decreased E2 production by porcine follicles is an indicator of follicular atresia (Guthrie et al., 1993; Pan et al., 2012). Recently, Zhu et al. (2012) reported that ZEA at high concentrations (30-120 μM) decreases the proliferation of porcine GC and causes an apoptosis and necrosis in porcine GC in a dose-dependent manner. The study revealed that ZEA leads to the loss of mitochondrial trans-membrane potential of porcine GC but increases reactive oxygen species levels of the cells. The authors concluded that ZEA induces an apoptosis and necrosis in porcine GC *via* a caspase-3- and caspase- 9 dependent mitochondrial pathways (Zhu et al., 2012). Thus, collective evidence suggests that ZEA or its metabolites, α-Zol and β-Zol, may induce atresia in porcine follicles. Ruminants are significantly less sensitive to ZEA exposure compared with pigs. However, in cows, infertility, reduced milk production, and hyperestrogenism have been associated with ZEA (D'Mello et al., 1999; Minervini and dell'Aquila, 2008). When dairy heifers were given 250 mg of ZEA (99% purified) daily over three estrous cycles, the conception rate declined from 87% to 62% (Weaver et al., 1986). Additionally, ZEA from pastures in New Zealand is a recognized cause of infertility in cattle and sheep (Towers and Sprosen, 1993). Minervini et al. (2001) reported *in vitro* a negative effect of ZEA and its derivative, α-Zol, on meiotic progression of bovine oocytes. Maturation of oocytes to metaphase II was inhibited in oocytes cultured in the presence of 94 μM of ZEA or α-Zol, with a significant increase in chromatin abnormalities, particularly after the addition of α-Zol (Minervini et al., 2001). In the study carried out by Takagi et al. (2008), oocytes were exposed to lower concentration of ZEA and no significant differences in the occurrence of metaphase I and metaphase II were observed between the control group and the group

supplemented with <0.31 μM ZEA. A significant decrease in the maturation rates of the oocytes exposed to ZEA 3.1 μM was observed; the maturation of 50% (62/124) of the examined oocytes was arrested in metaphase I (Takagi et al., 2008). Concerning the equine species, the effects of ZEA have only been reported in a few cases. In cycling mares, the effect of 10-day low-dose ZEA exposure on the reproductive parameters was studied (Juhasz et al., 2001). No effect on the length of the interovulatory intervals, luteal, and follicular phases was observed in mares fed 7 mg purified ZEA daily starting 10 days after ovulation. ZEA did not influence significantly the plasma P4 profiles and ovarian follicular activity (growth rate, maximum size of the ovulatory follicles, maximum number, and the time of first increase in the number of large follicles) (Juhasz et al., 2001). In a subsequent study, feeding oats naturally contaminated with ZEA and DON (12 and 1 mg/kg, respectively) had no relevant effects on the release of reproductive hormones, cycle length, and uterine histology in mares (Aurich et al., 2006). However, the authors stated that local ovarian effects of ZEA and DON on follicular growth cannot be excluded as the two mycotoxins tended to increase the number of growing follicles during the second half of the cycle. In addition, mares fed with the naturally contaminated oats had a high incidence of haemorrhagic *corpus luteum* and follicular hematomas, which did not occur during control cycles (Aurich et al., 2006). The *in vitro* exposure of GC collected from the ovaries of cycling mares with ZEA and its derivatives α-Zol and β-Zol induced a simultaneous increase in cell proliferation and an apoptotic process (Minervini et al., 2006). The contemporaneous presence of both processes led the authors to suggest that these mycotoxins could be effective in inducing follicular atresia, and these effects may result from both direct interaction with ERs as well as interaction with the enzymes 3-α-(β-)HSD present in the ovary and granulosa cells and involved in the synthesis and metabolism of endogenous steroid hormones (Minervini et al., 2006). Thus, studies in pigs and horses suggest that ZEA and its metabolites may induce ovarian follicular atresia.

1.4.3. Fumonisins

Though fumonisins were reported to have some adverse effects on reproduction in rats (Flynn et al., 1996), Syrian hamsters (Floss et al., 1994), and chickens (Javed et al., 1993; Bacon et al., 1995), and to have the potential to impair fertility capacity in boars (Gbore and Egbunikem, 2008; Gbore, 2009), stallions (Minervini et al., 2010) and rabbits (Ewuola and Egbunike, 2010) until now, only one study (Cortinovis et al., 2014) evaluated the direct *in vitro* effects of FB1 alone and combined with other *Fusarium* mycotoxins, on ovary. Cortinovis et al. (2014) demonstrated that in porcine small GC, FB1 had stimulatory effects on

P4 production at high dosage (14 μM) whereas at the same concentration inhibitory effects on cell growth were observed. Results on gene expression revealed that FB1 at 10 μM negatively affected CYP11A1 mRNA abundance. No significant *in vitro* interaction existed between FB1 and DON whereas a combination of FB1 and α-Zol had additive effects only on P4 (Cortinovis et al., 2014).

1.4.4. Granulosa cells as in vitro model

GC, derived from different species (swine, bovine, rat and humans), are widely used as an *in vitro* model to study the effects of contaminants (Gregoraszczuk et al., 2003, Spicer and Aad, 2007; Han et al., 2013; Mlynarczuk and Kowalik, 2013; Capcarova et al., 2014; Maruniakova et al., 2014; Tuck et al., 2014; Pizzo et al., 2015) (Figure 1.9).
In contrast with the immortalized cell lines, primary cells derive directly from tissues without subculture or transformation/transfection. In this way primary cells mimic more closely the *in vivo* situation and generate more physiologically relevant data than cell lines (Brassler et al., 2010). However, primary cells are more difficult to sustain under *in vitro* culture conditions compared with immortalized cells (Petro et al., 2012).
The use of follicular cells from domestic animals, besides laboratory rodents, has to be taken into consideration as an innovative approach in reproductive toxicology research (Petro et al., 2012).
The bovine ovarian function and oocyte characteristics are remarkably comparable with humans (Neuber and Powers, 2000; Campbell et al., 2003). Furthermore, bovine and human embryos are strongly related with respect to microtubule formation during fertilization, the timing of embryonal genome activation, metabolic requirements, interactions with the culture medium, and duration of preimplantation development (Anderiesz et al., 2000; Menezo et al., 2000; Petro et al., 2012).

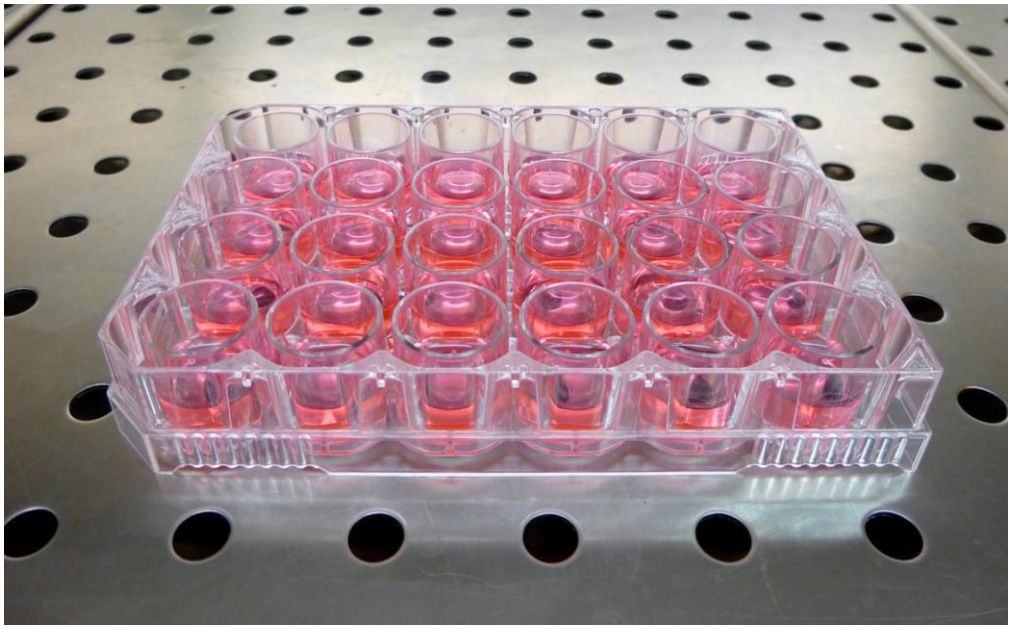

Figure 1.9. A 24-well plate with granulosa cells

1.5. References

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CHAPTER 2

Objectives

2. Objectives

It has been estimated that 25% of the world's crop production is contaminated to some extent with mycotoxins. Although the occurrence of mycotoxins exhibits often a geographic pattern, the worldwide trade with food and feed commodities allows a wide distribution of contaminated material. The global occurrence of mycotoxins is considered to be a major risk factor affecting human and animal health and the chronic exposure to low concentration of these substances is of major concern compared to the acute exposure.

Many *in vitro* studies reported the reproductive effects after exposure to *Fusarium* mycotoxins in domestic animals. The potential of deoxynivalenol (DON) and T-2 toxin to act as endocrine disruptor has been investigated *in vitro* and *in vivo* mainly in pigs whereas the effects on reproductive system of fumonisins have been scarcely studied. The role of zearalenone (ZEA) and its metabolites as endocrine disruptor is well known in many species. Due to their chemical structure they are able to bind estrogen receptors and impact the reproductive activity.

To date information about the reproductive toxicity of these mycotoxins in ruminant species is still limited and incomplete. Because of the presence of rumen, these animals are considered to be more resistant to the effects of toxic compounds. However, the detoxifying activity of the rumen is saturable and occurs only in healthy animals.

In a recent study traces of ZEA and its metabolites, such as α-zearalenol (α-Zol) and β-zearalenol (β-Zol), have been detected in follicular fluid of cattle exposed to ZEA demonstrating that both ZEA and ZEA metabolites can reach the ovaries. Therefore, cattle are actually exposed to these substances at reproductive organs level.

Granulosa cells (GC) is a well-established *in vitro* model that can be used as a predictive tool to evaluate the endocrine disruptor effects on ovarian follicle. Furthermore, in contrast with immortalized cell lines, primary cells are able to mimic more closely the *in vivo* situation.

To date only few studies have been carried out in order to determine the effects of *Fusarium* mycotoxins on reproduction in ruminants. Therefore, the purpose of the present study is to evaluate if DON and ZEA major metabolites, α-Zol and β-Zol impair cell proliferation, steroidogenesis and gene expression using primary bovine GC. For evaluating the effects on cell proliferation and estradiol and progesterone production we used GC collected from small follicles (SMGC) (chapter 3) and large follicles (LGGC) (chapter 4). The assessment of the effects of DON and ZEA metabolites on CYP19A1 and CYP11A1 mRNA abundance was carried out in SMGC (chapter 3).

Since a co-exposure to these mycotoxins can actually occurs the effects of several combinations of these mycotoxins were also investigated in both cell types.

CHAPTER 3

In vitro effects of Deoxynivalenol, α-Zearalenol and β-Zearalenol, alone and combined, on cell proliferation, steroid production and gene expression in bovine small-follicle granulosa cells

3. *In vitro* effects of Deoxynivalenol, α-Zearalenol and β-Zearalenol, alone and combined, on cell proliferation, steroid production and gene expression in bovine small-follicle granulosa cells

3.1. Abstract

Information on the potential effects of deoxynivalenol (DON) and zearalenone (ZEA), the major *Fusarium* mycotoxins, on reproduction in ruminants are few. Therefore the purpose of this study is to evaluate the impact of DON and ZEA hydroxylated metabolites, α-zearalenol (α-Zol) and β-zearalenone (β-Zol), on cell proliferation, steroidogenesis and gene expression using bovine granulosa cells (GC). GC were obtained aspirating aseptically small ovarian follicles (1 to 5 mm) (SMGC) and cultured for 2 days in medium containing 10% fetal bovine serum followed by 1 or 2 days in serum free medium without (control) or with added treatments. Cell proliferation was negatively affected after exposure to β-Zol at 31 μM and after exposure to α-Zol (3.1 μM) alone and combined with DON (3.3 μM). Steroidogenesis was differently affected by tested mycotoxins. DON and α-Zol had inhibitory effects while β-Zol at high concentration (31 μM) stimulated steroids production. DON (3.3 μM) and β-Zol (31 μM) increased SMGC CYP19A1 mRNA abundance. CYP11A1 mRNA abundance was stimulated by DON, alone and combined with α-Zol and β-Zol, whereas was inhibited by β-Zol alone. Generally mycotoxins effects on cell proliferation, steroidogenesis and gene expression were influenced by the presence or absence of IGF1. In conclusion the results obtained in this study demonstrated that DON and ZEA metabolites may impair *in vitro* cell proliferation, production and gene expression in cattle. Although more studies are needed to clarify the mechanism of action of these mycotoxins on GC, these effects should be taken into account in cattle reproductive failure.

3.2. Introduction

Mycotoxins are secondary metabolites produced by a large variety of molds which contaminate grains and feedstuffs worldwide (Park et al., 1996; D'Mello et al., 1999; Schollenberger et al., 2007).
Among the *Fusarium* species, deoxynivalenol (DON) and zearalenone (ZEA) are the major mycotoxins produced (D'Mello et al., 1999; Larsen et al., 2004). DON, produced principally by *Fusarium graminearum* (Richard, 2007), belongs to the class of mycotoxins called trichothecenes of the type B group and may co-exist with ZEA (Richard, 2007; Maresca, 2013; Pinton and Oswald, 2014).
DON has been implicated in farm animal disease outbreaks in many areas of the world and is one of the most hazardous food-associated mycotoxins (Maresca, 2013). DON is not acutely toxic to livestock however it is considered to be a major cause of economic losses since it negatively impacts performances (Morgavi and Riley, 2007). Feed refusal is the most commonly observed effect in pigs, cattle and chickens however pigs appear to be the most sensitive to DON (Morgavi and Riley, 2007).
Reproductive disorders in farm animals after exposure to DON have been also reported (Diekman and Green, 1992). It has been demonstrated that DON causes problems to the establishment of pregnancy in swine (Alm et al., 2002, 2006), whereas only few cases of intoxication after DON exposure are reported in cows. In North Europe cattle, after ingestion of a large amount of silage contaminated with DON, showed clinical signs related to laminitis and mastisis, but it was not clear if the intoxication was due to the ingestion of only DON or if feedstuffs contained also other trichothecenes (Speijers and Speijers, 2004). Little is known about the effects of DON on ruminants and the literature on this issue is still rare (Seeling et al., 2006).
Generally ruminants are considered to be more resistant to the effects of DON since in rumen fluid, DON is rapidly converted into de-epoxy DON (DOM1) that is less toxic compared to the parent compound (Fink-Gremmels, 2008). DOM1 is produced *via* intestinal or rumen microbe activity rather than by liver or other organs and it is the principal DON metabolite detected in urine and faeces of animals (Pestka, 2007).
However DOM1 production occurs only in healthy ruminants, in animals with rumen acidosis the metabolism of DON can be incomplete and it is easier to detect DON in blood (Fink Gremmels 2008 b).
ZEA is a phytoestrogenic compound (Diekman and Green, 1992) produced by several *Fusarium* species (*F. culmorum, F. graminearum, and F. sporotrichioides*) (Hussein and Brasel, 2001) and frequently found in animal feed materials (Zinedine et al. 2007). ZEA metabolism has been previously studied in rats and

it has been demonstrated that after ingestion, it is rapidly absorbed and metabolized by liver into α-Zearalenol (α-Zol) and β-Zearalenol (β-Zol) by 3-α and 3-β hydroxysteroid dehydrogenases (HSDs) (Olsen, 1981; Bielh et al. 1993). The estrogenic effects of ZEA and its derivatives are due to a close structural similarity between ZEA and estradiol (E2) (Osweiler, 2000) that allow them to bind to estrogen receptor (ER) (Parveen et al., 2009; Frizzel et al., 2011).
α-Zol has been found to have a higher binding affinity to ER, and is thus more potent than the parent compound (Upadhaya et al., 2010).
In domestic animals, metabolism of ZEA takes place in rumen, liver and intestinal mucosa (Winkler et al., 2014). Cattle are considered to be more resistant to the estrogenic effects of ZEA (Upadhaya et al., 2010).
In ruminants ZEA is converted to a large extent into α-Zol predominantly by rumen protozoa (Seeling et al., 2005), however ZEA undergoes also hepatic biotransformation and in cattle β-Zol is the dominant hepatic metabolite (Malekinejad et al., 2006).
Clinical signs of hyperoestrogenism are observed scarcely in ruminants, and only following the ingestion of highly contaminated silage, or a long-term exposure to contaminated feed materials. The likely explanation for this apparent discrepancy is the poor rate of absorption of the more polar α-Zol (Fink-Gremmels, 2008).
However, conception rates can be reduced in females (Weaver et al., 1986) while testicular atrophy was observed in bulls (Danicke et al., 2002). Enlargement of mammary glands with secretory activity were reported in prepubertal heifers which consumed highly contaminated silage (Bloomquist et al., 1982). It has also been shown (D'Mello et al., 1999) that ZEA is able to cause infertility in grazing sheep in New Zealand (Towers and Sposen, 1993).
At the moment little information is available about the effects of *Fusarium* mycotoxins on granulosa cells (GC) in cows. GC have been already proposed successfully as an *in vitro* model to evaluate the impact of contaminants on ovarian follicles (Mlynarczuk and Kowalik, 2013; Capcarova et al., 2014; Maruniakova et al., 2014; Pizzo et al., 2015). The purpose of this study was to determine, through the evaluation of cell proliferation, steroid production and gene expression, if DON, α-Zol and β-Zol have effects on bovine small GC (SMGC). Since a simultaneous exposure to DON and ZEA may be possible (Zinedine et al., 2007, Minervini and Dell'Aquila, 2008), it will be also studied if a combined presence of these mycotoxins could alter GC function.

3.3. Materials and methods

3.3.1. Reagents and hormones

Reagents were: Dulbecco's Modified Eagle Medium (DMEM), Ham's F12, fetal calf serum (FCS), Deoxynivalenol (DON), α-Zearalenol (α-Zol) and β-Zearalenol (β-Zol) obtained from Sigma Chemical Co. (St. Louis, MO); ovine FSH (Follicular Stimulating Hormone) obtained from Scripps Laboratories (San Diego, CA); IGF1 (Insulin Growth Factor 1) (recombinant human) obtained from R&D Systems (Minneapolis, MN), testosterone obtained from Steraloids (Wilton, NH).

3.3.2. Cell culture

Ovaries from non-pregnant beef cows were collected from a slaughterhouse and were treated as previously described (Spicer and Hammond, 1987; 1989; Langhout et al., 1991). Based on surface diameter small follicles (1 to 5 mm) were aspirated aseptically with an 18 gauge needle. SMGC were recovered by centrifugation at 291 x *g* for 10 min and were washed three times with 7 mL of serum free medium; at each wash, cells were separated from medium *via* centrifugation (291 x g at 4°C for 5 min). After the last centrifugation the supernatant was aspirated and replaced with 2 mL of enzyme containing medium (0.5 mg/mL of DNase and 1.25 mg/mL of collagenase) to prevent clumping of cells as previously described (Spicer et al., 2002). Numbers of viable cells were determined using the trypan blue exclusion method (Langhout et al., 1991; Tiemann et al., 2003; Spicer et al., 1993). Viable cells (2.5×10^5 in 20 to 80 µl of medium) were plated on 24 well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) in 1 ml of basal medium composed of a mixture of 1:1 DMEM and Ham's F-12 containing glutamine (2 mM), gentamicin (0.12 mM) and sodium bicarbonate (38.5 mM). Cultures were kept at 38.5° C in a humidified 95% air and 5% CO_2 environment and medium was changed every 24 h. To obtain an optimal attachment, cells were maintained in the presence of 10% FCS for the first 48 h of culture. After this time, GC were washed twice with serum free medium containing 500 ng/ml testosterone (as an estradiol precursor) and the various treatments applied in serum free medium for 48 h.

3.3.3. Determination of granulosa cells numbers and steroid concentrations

Medium was collected from individual wells and frozen at -20 °C for subsequent steroid analyses. Concentrations of progesterone (P4) and estradiol (E2) in

culture medium were determined by radioimmunoassays (RIA) as previously described (Spicer and Hammond, 1987; Spicer et al., 1990; Langhout et al., 1991). Numbers of GC, in the same wells from which medium was collected, were determined by a Coulter counter (model Z2; Beckman Coulter, Inc., Hialeah, FL), and used to calculate steroid production on ng or pg per 10^5 cell basis. Briefly, cells were gently washed twice with 0.9% saline solution (500 μL), exposed to 500 μL of trypsin (0.25% wt/vol=2.5 mg/mL) for 20 min at room temperature, and then scraped from each well and enumerated as previously described (Spicer and Hammond, 1987; Langhout et al., 1991; Ranzenigo et al., 2008). Cell aggregates were minimized by pipetting cell suspensions back and forth through a 500 μL pipette tip three to five times.

Progesterone RIA

Progesterone RIA were conducted using rabbit antiserum (X-16), which serves as the first antibody (diluted 1:3000 with assay buffer: PBS, EDTA, NaN_3, and gelatin), raised against BSA-11 glutamate derivative as described by Baraño and Hammond (1985). Goat anti-rabbit antibody (diluted 1:15 with assay buffer) was used as the second antibody (Linco Research, Inc., St. Charles, MO). [^{125}I]Iodo-progesterone (ICN Biomedicals, Costa Mesa, CA) was used as the tracer. A progesterone standard curve was prepared from a stock concentration of 80.0 ng/mL that was serially diluted with assay buffer to concentrations of 40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.625, 0.31, and 0.16 ng/mL. In duplicate, 20 μL to 100 μL of medium samples were combined with the appropriate volume of assay buffer to make a total volume of 100 μL. One hundred μL of tracer and first antibody were added and all samples were mixed and allowed to incubate at 37 °C for 1 h. Following incubation, 200 μL of second antibody were added and all samples were incubated overnight at 4 °C. The following day, 50 μL of normal rabbit serum (NRS) (diluted 1:5 with assay buffer from a 15% NRS stock) were added to all samples. Samples were centrifuged at 4 °C in a Sorvall Model RC-3 (Thermo Fisher Scientific, Inc., Miami, OK) at 1800 x *g* for 25 min. Supernatant was aspirated and precipitates were counted for 1 min using a Cobra AII Auto-Gamma counter (Packard Instrument Co., Downers Grove, IL). The intra- and interassay coefficients of variation were 7 and 13%, respectively, for the progesterone RIA.

Estradiol RIA

Estradiol RIA were conducted using anti-estradiol rabbit antibody (diluted 1:12 with assay buffer), which serves as the first antibody (Lilly Research

Laboratories, Indianapolis, IN), and goat anti-rabbit antibody (diluted 1:15 with assay buffer) which serves as the second antibody (Linco Research, Inc., St. Charles, MO). Radiolabeled estradiol (^{125}I-estradiol) was used as the tracer (ICN Biomedicals, Costa Mesa, CA). The assay buffer was the same as the progesterone RIA buffer described above. An estradiol dose response curve was prepared from a stock concentration of 256 pg/100 μL that was serially diluted to 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 pg/100 μL using assay buffer. In duplicate, sample media was added at either 50 or 100 μL and (if needed) combined with assay buffer to make a total volume of 100 μL. Two hundred μL of tracer were added to all samples, along with 100 μL of first antibody. All tubes were mixed and allowed to incubate for 1 h at 37 °C. Following this incubation, 200 μL of second antibody were added and the assay allowed to incubate at 4 °C overnight. The following day, assay tubes were centrifuged, supernatant aspirated, and precipitate counted as described for the progesterone RIA. The intra- and interassay coefficients of variation were 8% and 17%, respectively, for the estradiol RIA.

3.3.4. RNA analysis

RNA extraction

At the end of the treatment period, cells from two replicate wells were lysed in 500 μL of TRIzol® reagent and RNA was extracted as previously described (Voge et al., 2004; Aad et al., 2006). Briefly, 250 μL TRIzol® reagent was added to all wells and cells were lysed by repeated pipetting and then combined with their respective replicates. Combined wells were then transferred to 1.5 mL eppendorf tubes. Each treatment containing 4 wells generated 2 replicate samples of RNA. Cell lysates were incubated in TRIzol® reagent for approximately 5 min at room temperature, then 100 μL of chloroform was added to each sample followed by a 15 s vortex. After approximately a 2 min incubation at room temperature, samples were centrifuged at 3500 x *g* for 30 min at 4 °C using eppendorf centrifuge 5417C (Brinkmann Instruments, Westbury, NY). The upper aqueous phase of each sample was then transferred to a fresh eppendorf tube and RNA was precipitated using 250 μL isopropanol. Samples were incubated at room temperature for 10 min and then centrifuged at 3500 x *g* for 10 min at 4 °C. The RNA pellets were washed after discarding the supernatant with 500 μL of 70% ethanol and allowed to dry at room temperature. The RNA pellets were suspended in 16.5 μL of DEPC-treated water. RNA was quantitated by spectrophotmetry at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Aliquots of 1.5 μL of RNA were used to determine the concentration in ng/μL as well as the purity given as a ratio of 260/280 nm where values between 1.8

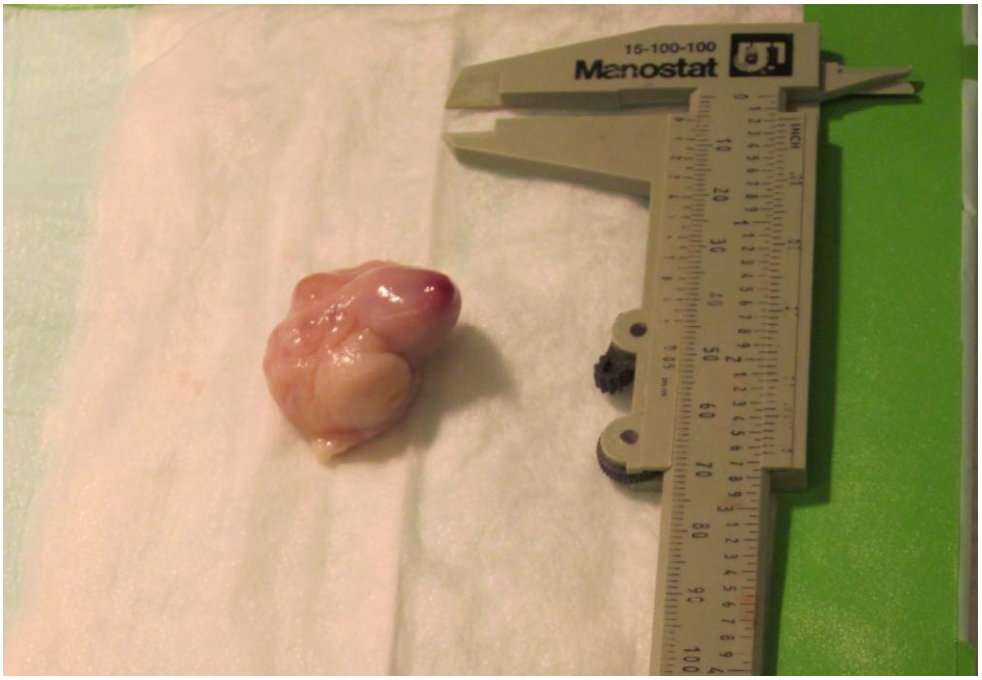


Figure 3.1. Measurement of follicle diameters (longitudinal and transversal planes) with a caliper. Only follicles with a mean diameter $\leq$ 5 mm were used to collect small granulosa cells

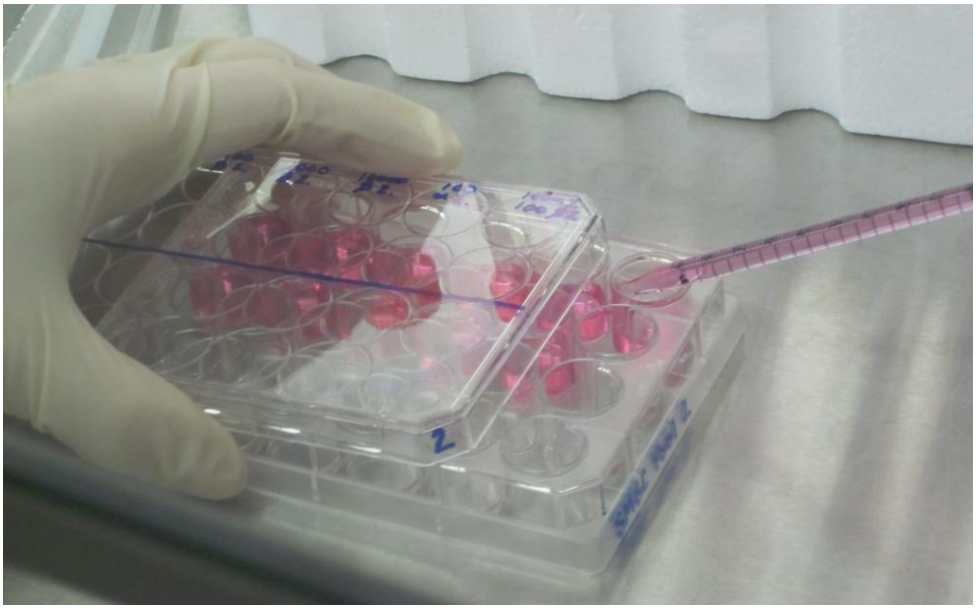


Figure 3.2. Daily medium change.

and 2.2 were acceptable. RNA was then diluted to 10 ng/μL in DEPC-treated water and stored at -80 °C until used for quantification of target gene expression. Just prior to use, an RNA aliquot was thawed on ice for 3-5 min.

Quantitative RT-PCR

The target gene primers (forward, reverse) and probe sequences for aromatase enzyme (CYP19A1; Accession U92245) were TGCCAAGAATGTTCCT TACAGGTA, CAGAGTGACCTTCATCATGACCAT and CATTTGGCTTT GGGCCCCGG, respectively; and for P450 side-chain cleavage enzyme (CYP11A1; Accession NM 214427) were CTCCGTGACCCTGCAGAGATAC, ATAGACGGCCACTTGTACCAATG and TTGGTTCTTCGAGATTACA TGATTCCTGCC, respectively.
A BLAST search (www.ncbi.nlm.nih.gov/BLAST) was also conducted to insure the specificity of the designed primers and probe and to assure that they were not designed from any homologous regions coding for other genes. The differential expression of target gene mRNA granulosa cells was quantified using the one-step multiplex real-time RT-PCR reaction for Taqman® Gold RT-PCR Kit (Applied Biosystems, Foster City, CA) as previously described (Spicer and Aad, 2007; Ranzenigo et al., 2008). Briefly, based on preliminary optimization results, 100 ng of total RNA was amplified in a total reaction volume of 25 μL consisting of 200 nM forward primer, 200 nM reverse primer and 100 nM fluorescent (FAM/TAMRA) probe for each target gene, 50 nM of 18S rRNA primers and 100 nM of the 18S rRNA VIC-labeled probe, along with 12.5 μL of TaqMan Master Mix without uracil N-glycosylase, and 1 U Multiscribe with RNase inhibitor (Applied Biosystems). Thermal cycling conditions were set to 30 min at 50 ºC for reverse transcription, 95 ºC for 10 min for AmpliTaq Gold activation, and finished with 55 cycles at 95 ºC for 15 s for denaturing and 60 ºC for 1 min for annealing and extension. All samples were run in duplicate. The 18S rRNA values were used as internal controls to normalize samples for any variation in amounts of RNA loaded, and relative quantification of target gene mRNAs was expressed using the comparative threshold cycle method as previously described (Voge et al., 2004; Aad et al., 2006). Briefly, the ΔCt was determined by subtracting the 18S Ct value from the target unknown value. For each target gene, the ΔΔCt was determined by subtracting the higher ΔCt (the least expressed unknown) from all other ΔCt values. Fold changes in target gene mRNA abundance were calculated as being equal to $2^{-\Delta\Delta Ct}$.

3.3.5. Experimental design

Experiment 1 was designed to determine the dose response of DON and α-Zol and their interaction at low dosages on bovine GC proliferation and

steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and then treated for 48 h in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of DON (0, 0.1, 0.33, and 3.3 μM) and α-Zol (0, 0.09, 0.31, 3.1 μM). A concomitant treatment with DON and α-Zol at 0.1 and 0.09 μM respectively was also carried out. After 48 h of treatment, medium was collected for P4 and E2 determination, and cells were counted. Doses of FSH and IGF1 were selected based on previous studies (Ranzenigo et al., 2008; Spicer et al., 2002). FSH was added to all treatments, because IGF1 alone does not have effects on steroid production (Spicer et al., 2002; Ranzenigo et al., 2008).

Experiment 2 was carried out to determine the effects of β-Zol alone and combined with α-Zol on GC proliferation and to evaluate resulting steroidogenesis in presence and absence of IFG1 (30 ng/mL). FSH (30 ng/mL) was added to all treatments. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h, with treatments change after first 24 hours, in serum-free medium containing FSH and IGF1. β-Zol was tested at 0.31, 3.1 and 31 μM. The effects of a concomitant exposure to β-Zol (0.31 μM) and α-Zol (0.31 μM) were also evaluated. After 48 h the cells were counted and medium was collected for P4 and E2 determination.

Experiment 3 was performed to evaluate the effects of β-Zol alone and combined with DON in presence of FSH and with or without IGF1 on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing FSH (30 ng/mL) with or without IGF1 (30 ng/mL). β-Zol was tested at 3.1 and 31 μM and concomitant treatments with DON at 3.3 μM and β-Zol at 3.1 and 31 μM were also performed. After 48 h the cells were counted and medium was collected for P4 and E2 determination.

Experiment 4 was carried out to determine the effects of α-Zol alone and combined with DON in presence of FSH but without IGF1 on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing FSH (30 ng/mL). α-Zol was tested at 3.1 μM and the effects derived from a concomitant treatment with α-Zol at 3.1 μM and DON at 3.3 μM were also evaluated. After 48 h the cells were counted and medium was collected for P4 and E2 determination.

Experiment 5 was designed to evaluate the effects of β-Zol, at low concentration (0.09 μM), in presence of FSH and IGF1 on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free

medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL). After 48 h the cells were counted and medium was collected for P4 and E2 determination.
Experiment 6 was designed to determine the effects of DON (3.3 μM), α-Zol (3.1 μM) and β-Zol (31 μM), with FSH in presence or absence of IGF1, on CYP11A1 and CYP19A1 mRNA abundance. The potential effects caused by a co-exposure to DON (3.3 μM) and α-Zol (3.1 μM) and to DON (3.3 μM) and β-Zol (31 μM) on FSH plus IGF1-induced CYP11A1 and CYP19A1 mRNA abundance were also evaluated. Doses of DON, α-Zol and β-Zol were selected considering the results obtained in the previous experiments. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 24 h in serum-free medium containing FSH (30 ng/mL) with or without IGF1 (30 ng/mL). After 24 h of treatment medium was aspirated and cells were lysed for RNA extraction.

3.3.6. Statistical analysis

Experimental data are presented as the least squares means ± SEM of measurements from replicated culture wells. Each experiment was performed three times with different pools of granulosa cells collected from two to six ovaries for each pool and each treatment replicated three times within each experiment. For mRNA experiments, treatments were applied in quadruplicate culture wells with each mRNA sample being obtained from two wells. Treatment effects and interactions were assessed using the GLM procedure of the Statistical Analysis System (SAS, 1999). Main effects were treatment, experiment and their interaction when data from more than one experiment were analyzed. Steroid production was expressed as ng or pg/10^5 cells per 24 h, and cell numbers determined at the end of the experiment were used for this calculation. Mean differences in steroid production and mRNA abundance between treatments were determined using the Fisher's protected least significant difference (LSD) procedure (Ott, 1977).

3.4. Results

3.4.1. Experiment 1: dose-response of DON and a-Zol, alone and combined, on cell proliferation and E2 and P4 production in presence of FSH plus IGF1

Experiment 1 was carried out to determine the effects of DON and α-Zol, alone and combined, on cell proliferation and steroid production in presence of IGF1. Cell proliferation was not affected after exposure to all mycotoxin treatments ($P > 0.05$) (Figure 3.3). E2 production was inhibited by all concentrations of DON and α-Zol tested. The highest inhibitory effect was observed after exposure to DON at 3.3 μM by 98% ($P < 0.0001$). Mycotoxins, when combined, affected E2 release more (52%) ($P < 0.0001$) than how DON ($P < 0.0001$) and α-Zol ($P < 0.001$) did alone (36% and 22% respectively) compared to the control (Figure 3.4 A). P4 production was dramatically reduced (90%) after exposure to DON at the highest concentration (3.3 μM) ($P < 0.0001$) while DON at 0.33 μM and α-Zol at 0.31 μM had minor inhibitory effects on P4 release (by 39% and 34% respectively) ($P < 0.001$). No effects on P4 production were reported after concomitant treatments with mycotoxins ($P > 0.05$) (Figure 3.4 B).

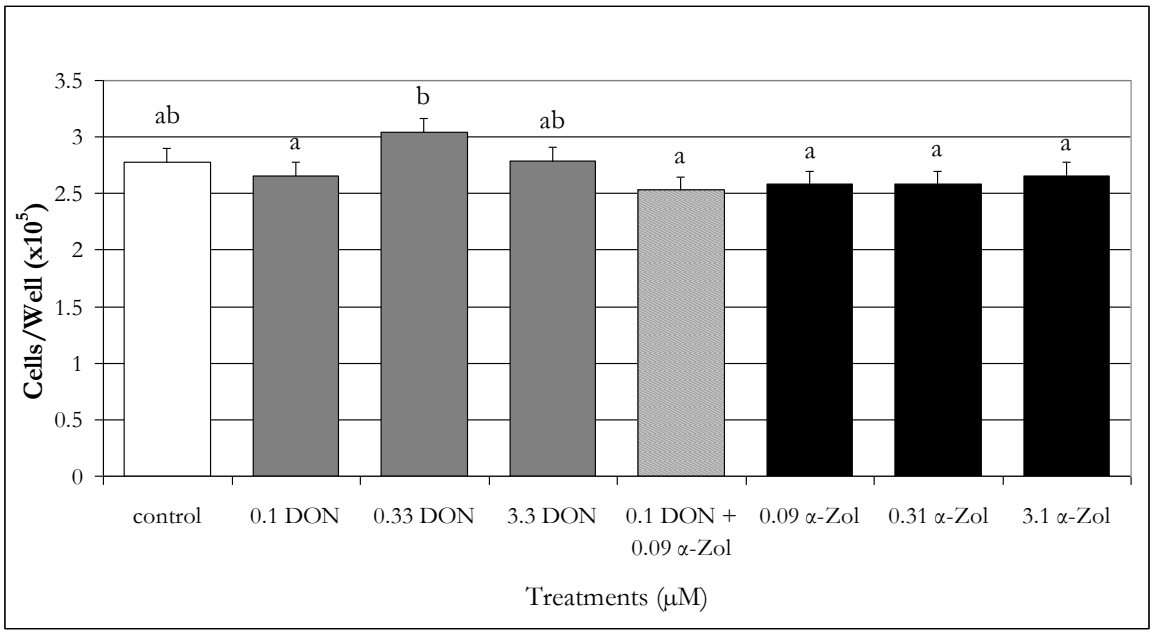
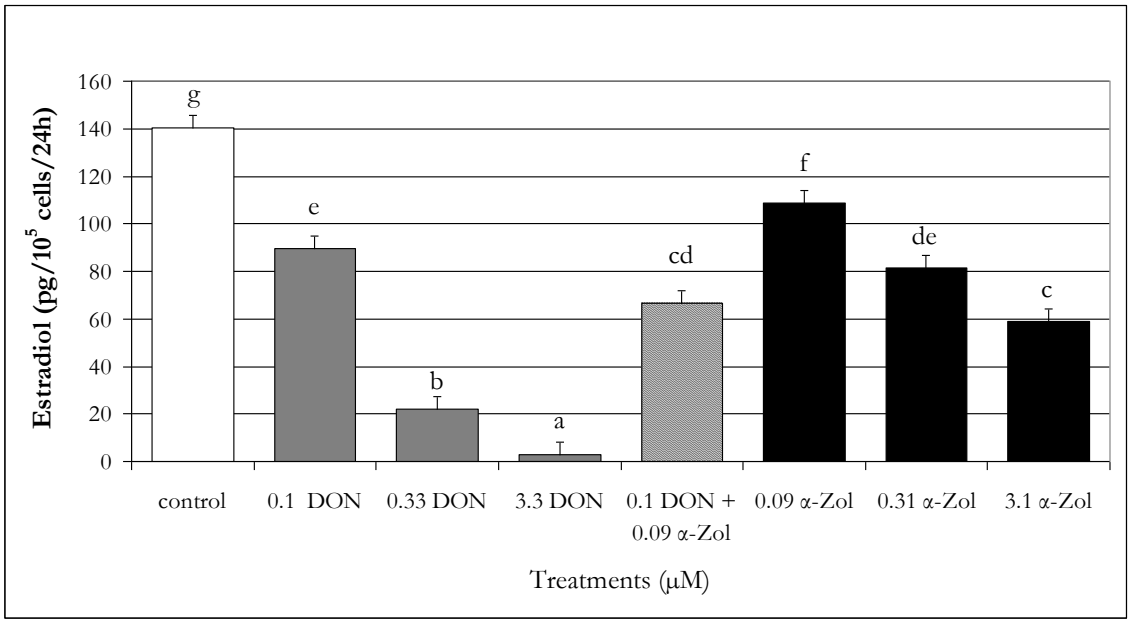


Figure 3.3. Effects of DON and α-Zol, alone and combined, on numbers of granulosa cells from bovine small follicles (Experiment 1). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of DON and α-Zol. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-b) differ ($P < 0.05$).

(A)



(B)

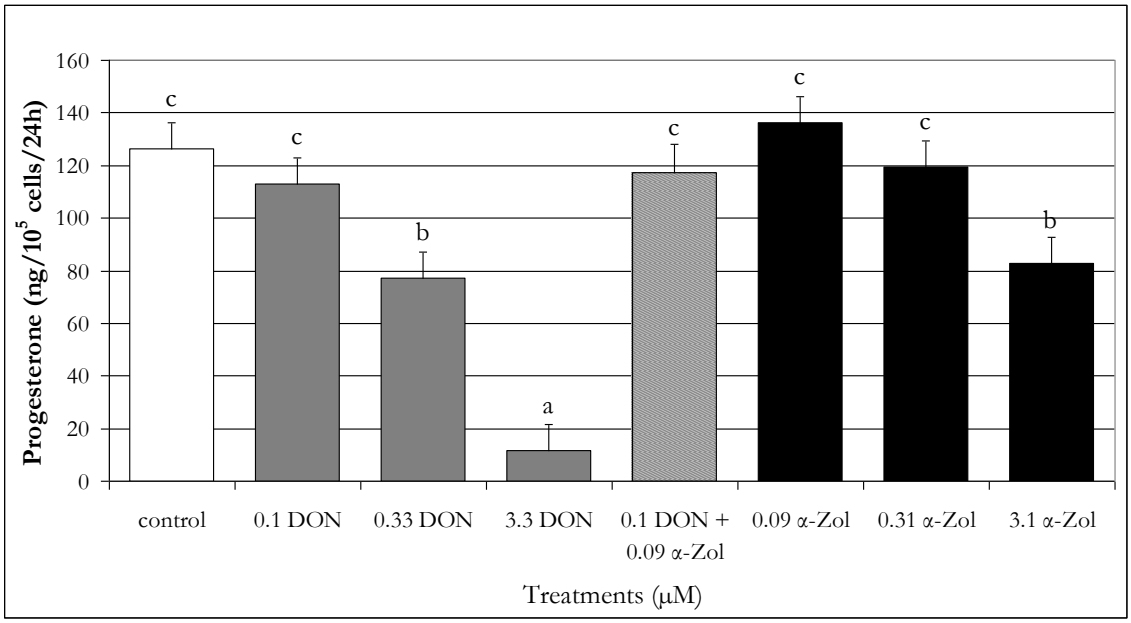


Figure 3.4. Effects of DON and α-Zol, alone and combined, on FSH plus IGF1-induced estradiol (Panel A) and progesterone (Panel B) production by granulosa cells from bovine small follicles (Experiment 1). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of DON and α-Zol. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-f) differ ($P < 0.05$).

3.4.2. Experiment 2: dose response of β-Zol alone and combined with a-Zol, on cell proliferation and steroidogenesis with and without IGF1

Experiment 2 was carried out to assess the effects of β-Zol alone and combined with α-Zol on cell proliferation and steroid production in presence or absence of IGF1. In presence of IGF1 cell proliferation decreased after exposure to β-Zol at 3.1 ($P < 0.05$) and 31 μM ($P < 0.0001$). All the other treatments had no effects compared to the control ($P > 0.05$) (Figure 3.5). α-Zol and β-Zol, when combined, had inhibitory effect on E2 production ($P < 0.05$) by 46% compared to the control. The other treatments had no effects on E2 and P4 release ($P > 0.05$) (Figure 3.6 A and B). In absence of IGF1 cell number dramatically decreased compared to the control ($P < 0.0001$) only after exposure to the highest concentration of β-Zol (31 μM) (Figure 3.7). E2 and P4 release were strongly stimulated ($P < 0.0001$) by β-Zol at 31 μM, whereas all the other treatments had no effects on steroid production ($P > 0.05$) (Figure 3.8 A and B).

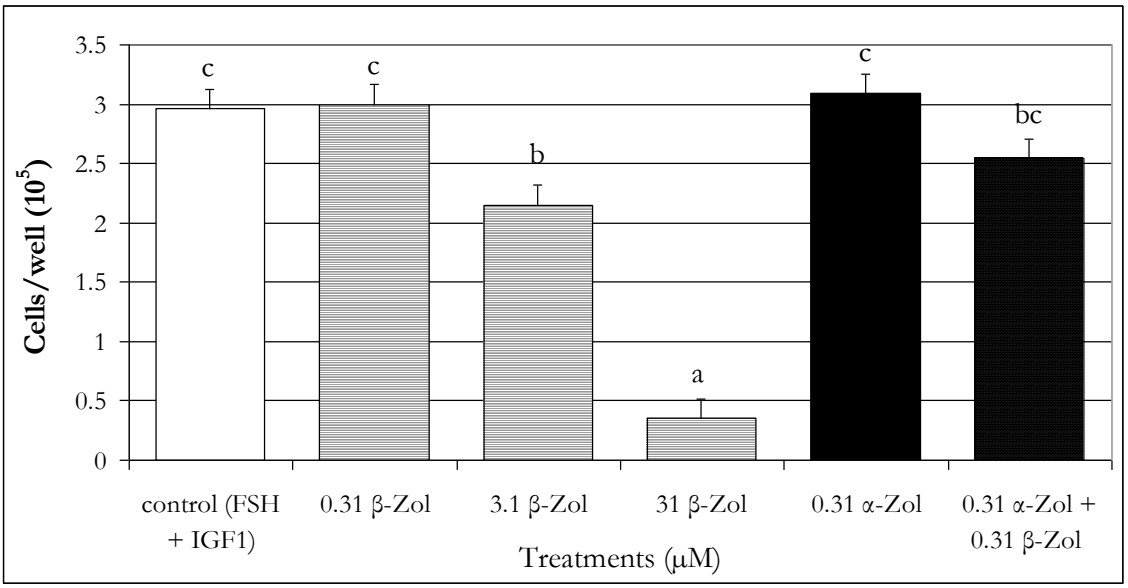


Figure 3.5. Effects of β-Zol, alone and combined with α-Zol, on numbers of granulosa cells from bovine small follicles (Experiment 2). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of β-Zol and α-Zol. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-c) differ ($P < 0.05$).

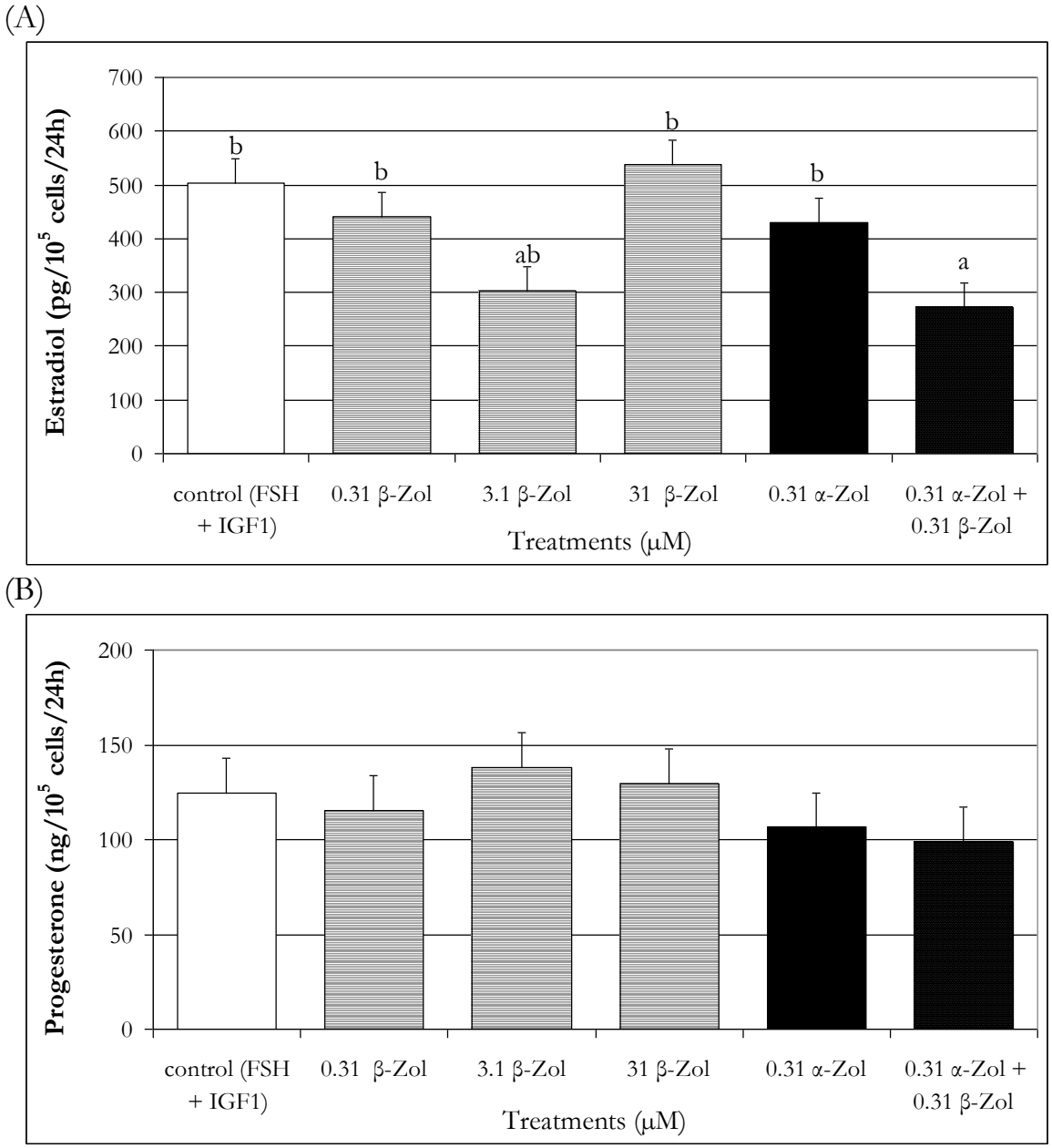


Figure 3.6. Effects of β-Zol, alone and combined with α-Zol, on FSH plus IGF1-induced estradiol (Panel A) and progesterone (Panel B) production by granulosa cells from bovine small follicles (Experiment 2). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of β-Zol and α-Zol. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-b) differ ($P < 0.05$).

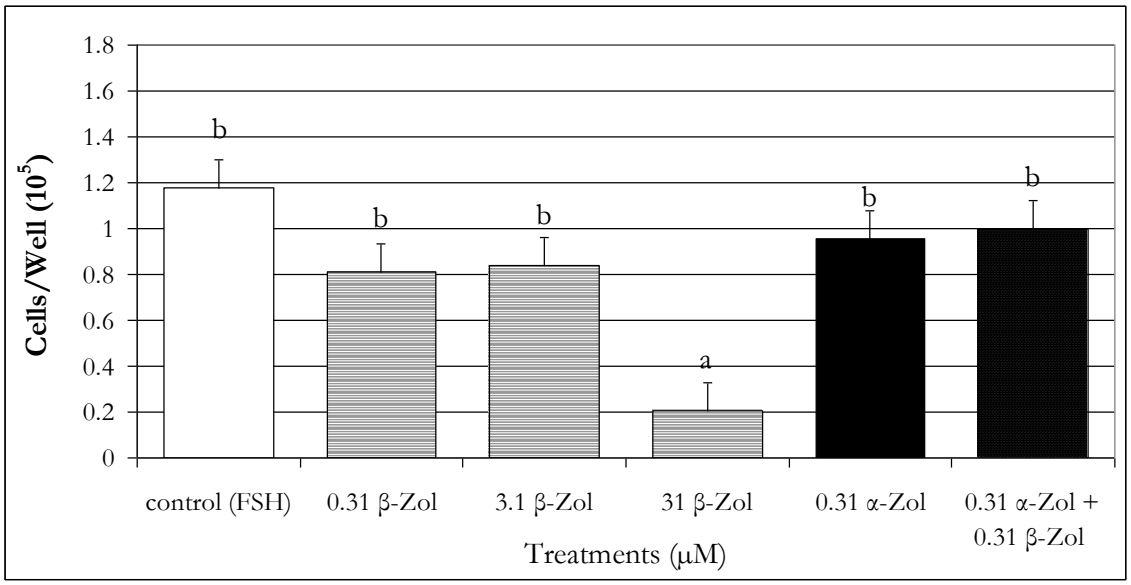


Figure 3.7. Effects of β-Zol, alone and combined with α-Zol, on numbers of granulosa cells from bovine small follicles (Experiment 2). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) with or without the various doses of β-Zol and α-Zol. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-b) differ ($P < 0.05$).

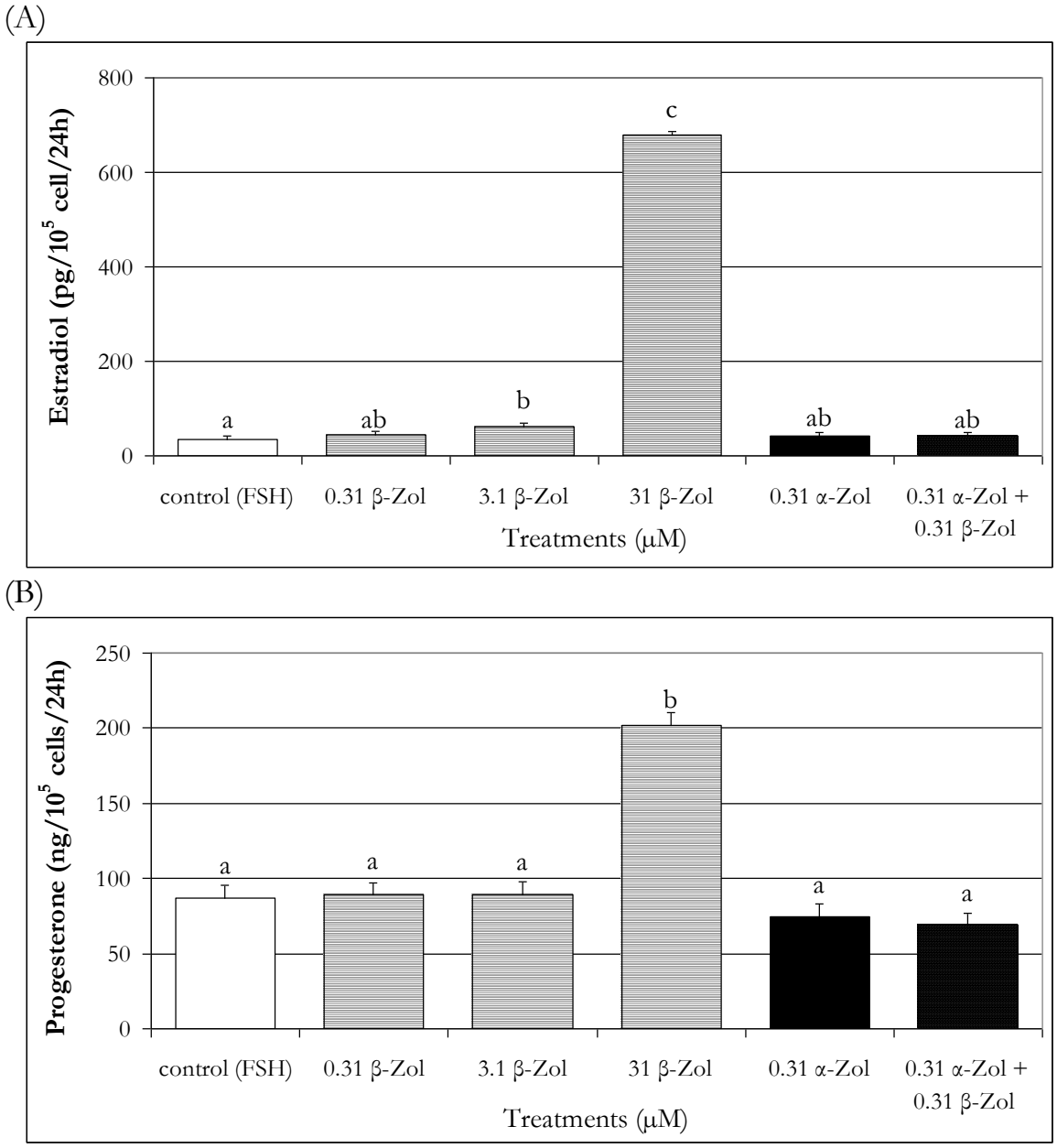


Figure 3.8. Effects of β-Zol, alone and combined with α-Zol, on estradiol (Panel A) and progesterone (Panel B) production in granulosa cells from bovine small follicles (Experiment 2). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) with or without the various doses of β-Zol and α-Zol. Values are means from three separate experiments (*n = 9*). Means without a common letter (a-c) differ ($P < 0.05$).

3.4.3. Experiment 3: dose response of β-Zol, alone and combined with DON, on cell proliferation and steroidogenesis with and without IGF1

Experiment 3 was conducted to determine the effects of β-Zol alone and combined with DON with and without IGF1. In presence of IGF1 β-Zol at 31 μM alone and combined with DON decreased cell growth by 52% and 63% respectively ($P < 0.05$). The other treatments did not affect cell proliferation compared to the control ($P > 0.05$) (Figure 3.9). E2 production was strongly decreased after exposure to DON alone and combined with β-Zol at 3.1 μM ($P < 0.05$) all the other treatments had no effects compared to the control ($P > 0.05$) (Figure 3.10 A). P4 release was strongly inhibited after exposure to DON alone and combined with β-Zol ($P < 0.001$). The other treatments had no effects on P4 production ($P > 0.05$) (Figure 3.10 B). In absence of IGF1, β-Zol at 31 μM alone and in combination with DON inhibited cell number by 58% and 45% respectively ($P < 0.05$). The other treatments did not affect cell proliferation compared to the control ($P > 0.05$) (Figure 3.11). At 31 μM β-Zol alone strongly stimulated E2 production ($P > 0.05$) and in combination with DON showed a synergic stimulatory effect on E2 release ($P < 0.0001$) (Figure 3.12 A). P4 production was affected only after exposure to β-Zol at 31 μM that increase P4 release by 90% compared to the control ($P < 0.05$). All the other treatments had no effects on P4 release ($P > 0.05$) (Figure 3.12 B).

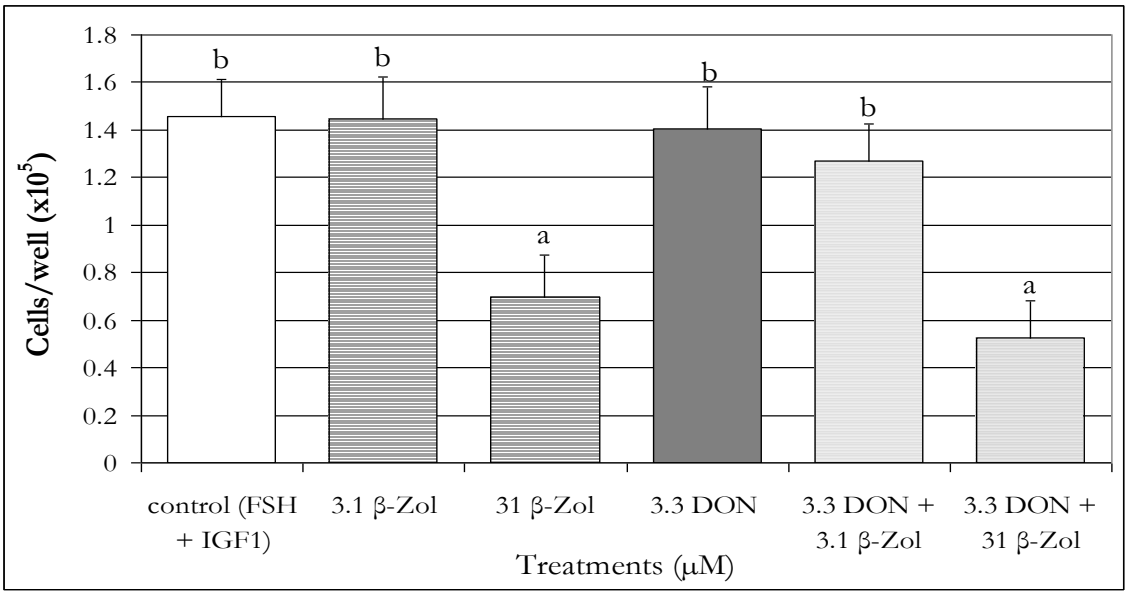


Figure 3.9. Effects of β-Zol, alone and combined with DON, on numbers of granulosa cells from bovine small follicles (Experiment 3). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of β-Zol and DON. Values are means from three separate experiments (*n = 9*). Means without a common letter (a-b) differ ($P < 0.05$).

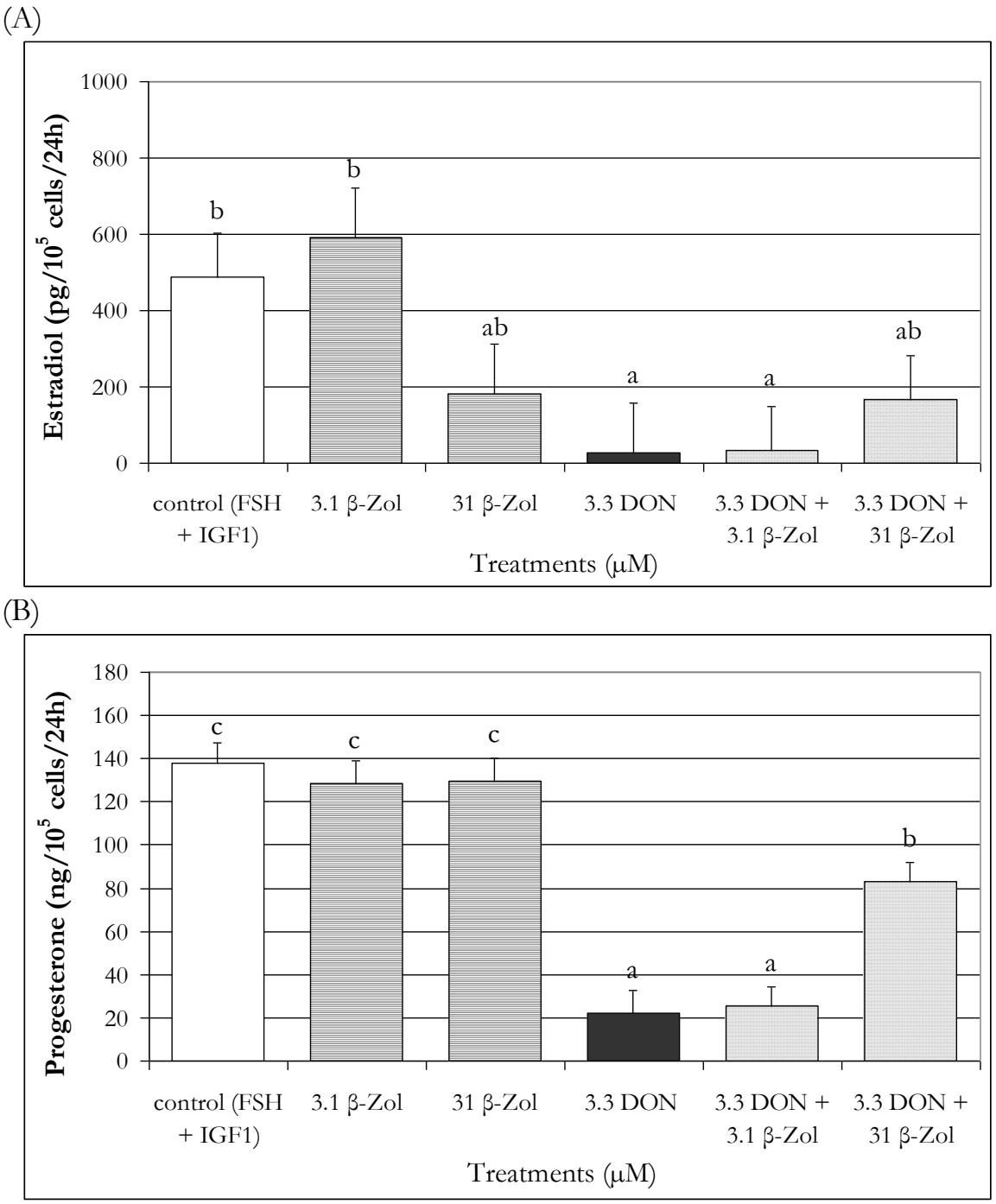


Figure 3.10. Effects of β-Zol, alone and combined with DON, on estradiol (Panel A) and progesterone (Panel B) production in granulosa cells from bovine small follicles (Experiment 3). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of β-Zol and DON. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-c) differ ($P < 0.05$).

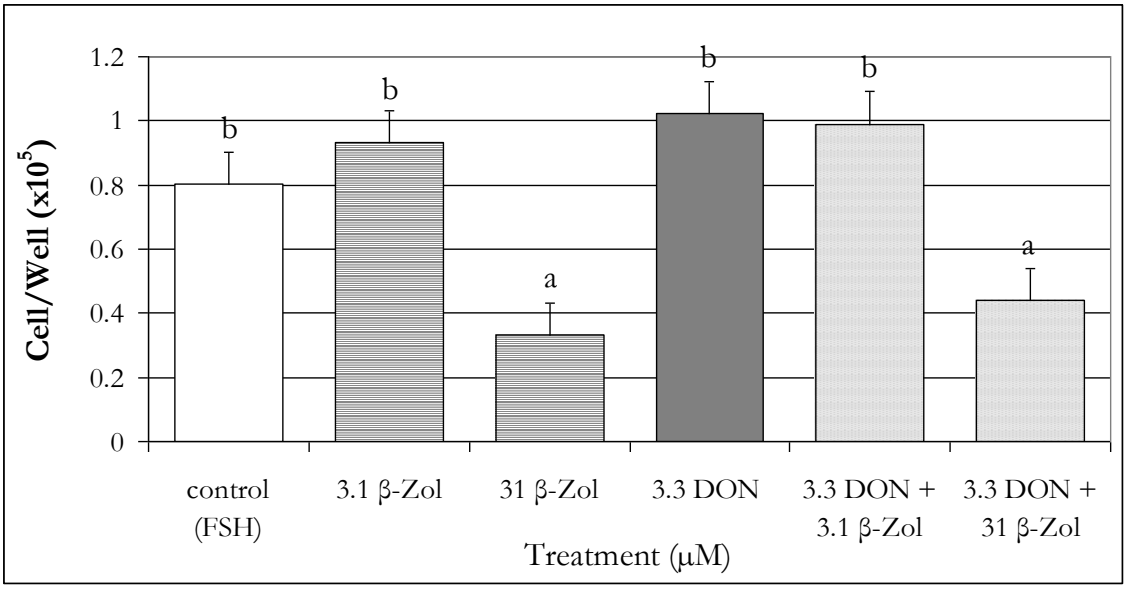


Figure 3.11. Effects of β-Zol, alone and combined with DON, on numbers of granulosa cells from bovine small follicles (Experiment 3). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) with or without the various doses of β-Zol and DON. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-b) differ ($P < 0.05$).

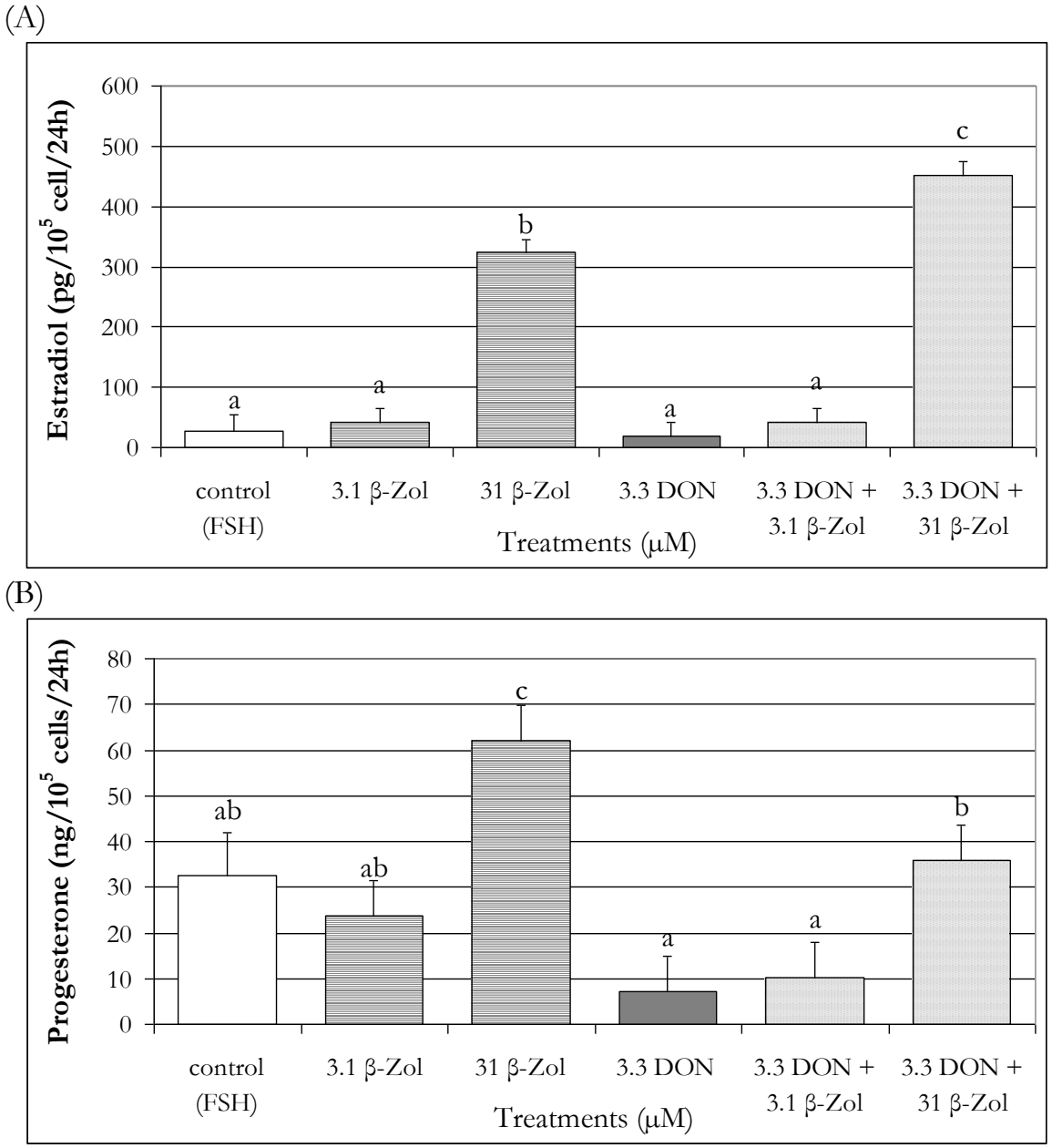


Figure 3.12. Effects of β-Zol, alone and combined with DON, on estradiol (Panel A) and progesterone (Panel B) production in granulosa cells from bovine small follicles (Experiment 3). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) with or without the various doses of β-Zol and DON. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-c) differ ($P < 0.05$).

3.4.4. Experiment 4: dose response of a-Zol alone and in combination with DON without IGF1

Experiment 4 was conducted to assess the effects of α-Zol alone and combined with DON on cell proliferation and steroidogenesis without IGF1. As reported in the experiment 3, DON alone, in absence of IGF1, showed no significant effects on cell proliferation and steroidogenesis ($P > 0.05$). Cell proliferation after exposure to α-Zol and α-Zol combined with DON was significantly reduced compared to the control by 14% ($P < 0.05$) and 61% respectively ($P < 0.0001$) (Figure 3.13). α-Zol alone had no effects on E2 and P4 production ($P > 0.05$) while, when in combination with DON, strongly increased E2 and P4 release by 142% and 89% respectively compared to the control ($P < 0.0001$) (Figure 3.14 A and B).

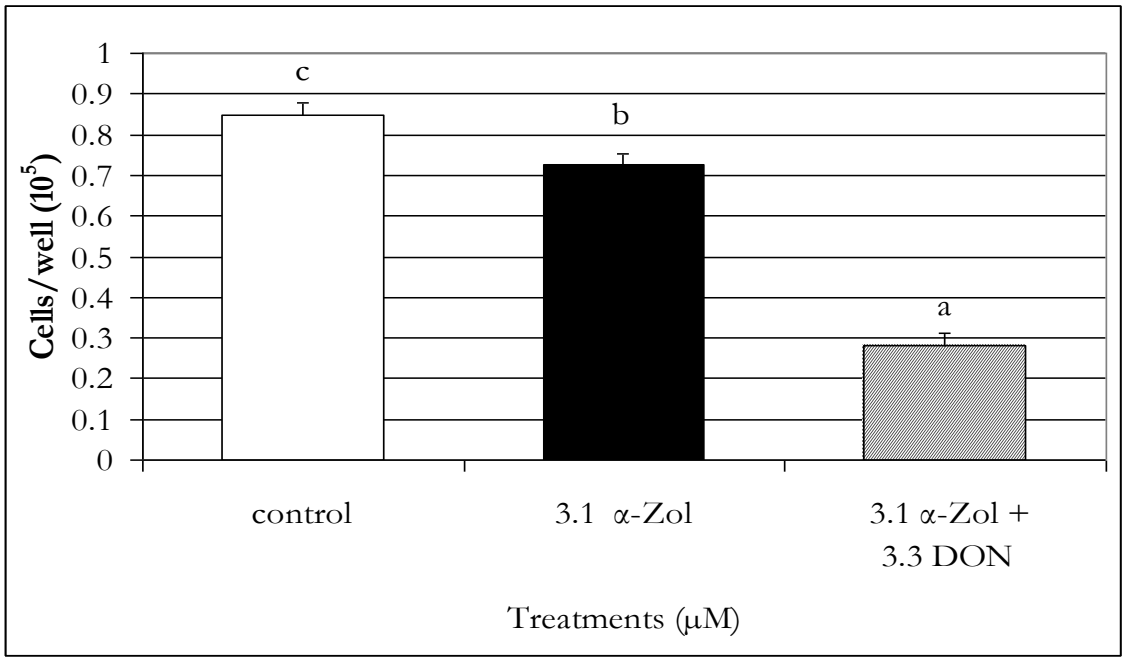


Figure 3.13. Effects of α-Zol, alone and combined with DON, on numbers of granulosa cells from bovine small follicles (Experiment 4). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) with or without the various doses of α-Zol and DON. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-c) differ ($P < 0.05$).

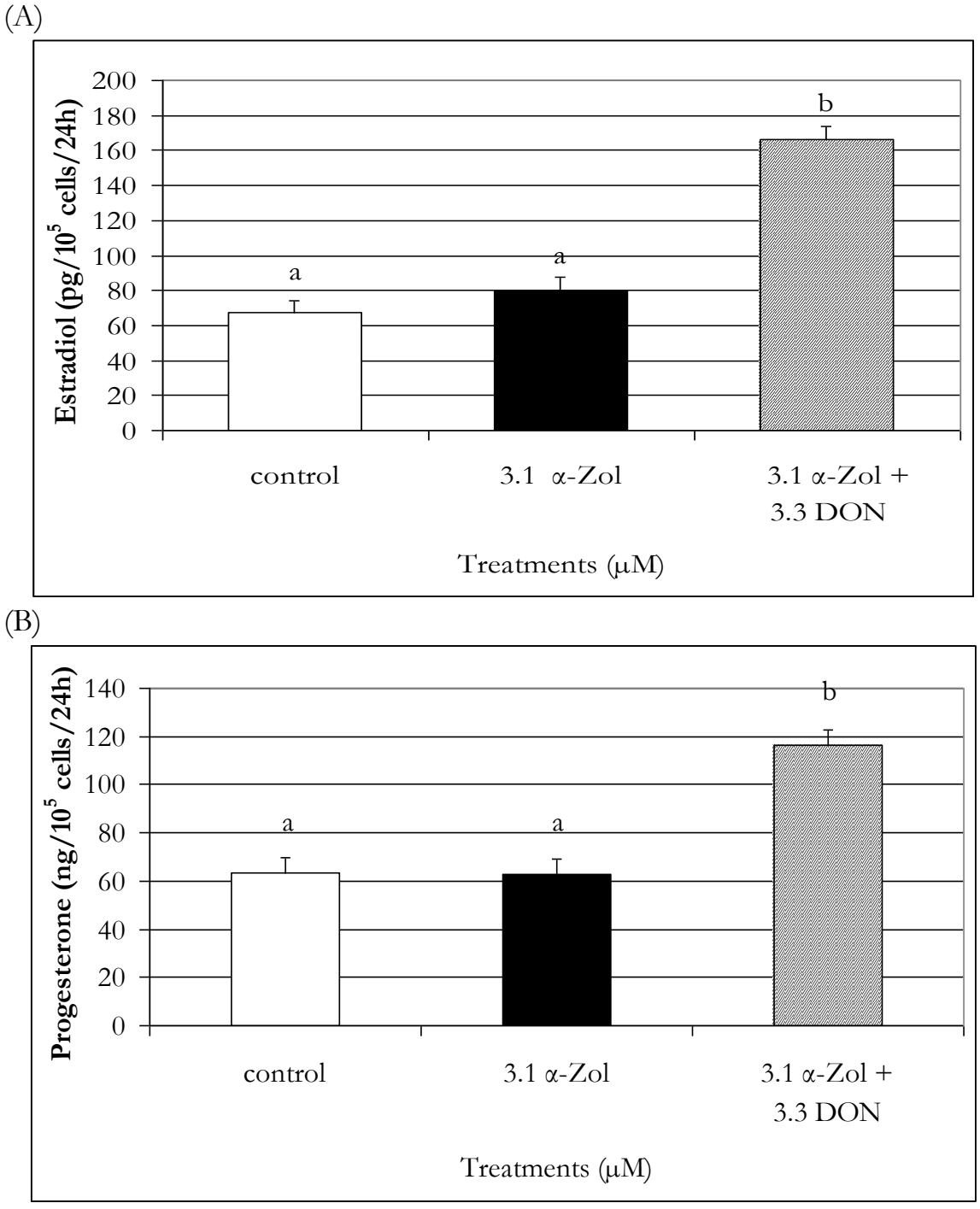


Figure 3.14. Effects of α-Zol, alone and combined with DON, on estradiol (Panel A) and progesterone (Panel B) production in granulosa cells from bovine small follicles (Experiment 4). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) with or without the various doses of α-Zol and DON. Values are means from three separate experiments (*n = 9*). Means without a common letter (a-b) differ ($P < 0.05$).

3.4.5. Experiment 5: dose response of β-Zol at low concentration in presence of IGF1

Experiment 5 was carried out to determine the effects of β-Zol at low concentration on cell proliferation and steroid production in presence of IGF1. No effects were observed on cell proliferation and steroidogenesis ($P > 0.05$) after exposure to low dosage of β-Zol (Figures 3.15 and 3.16).

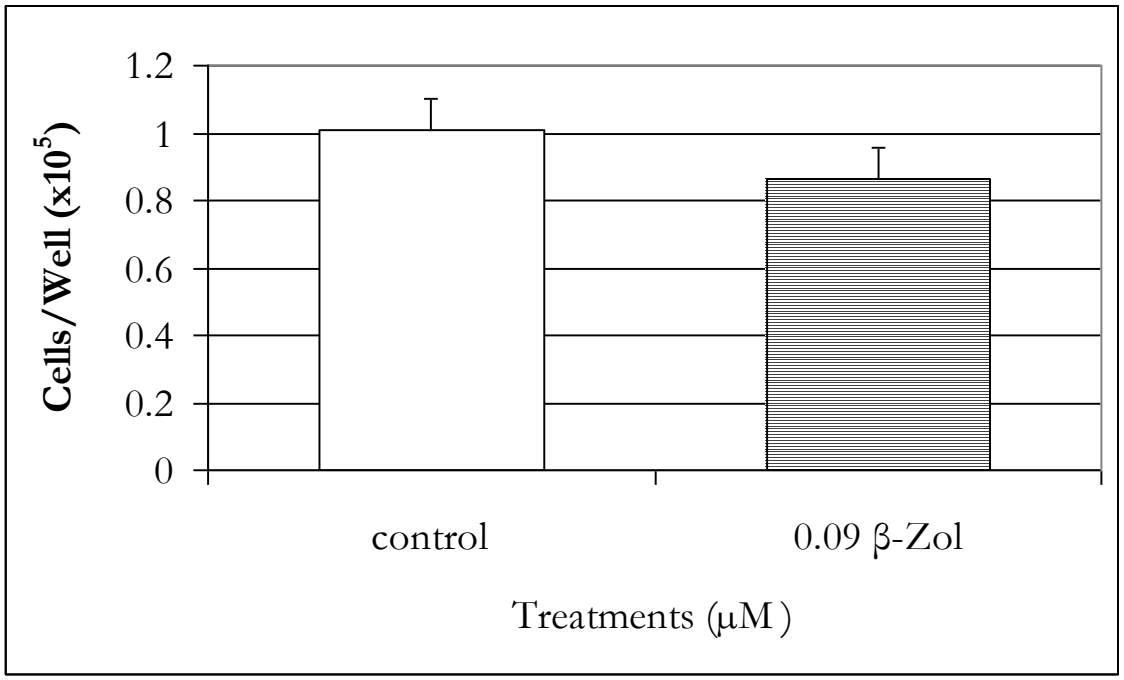
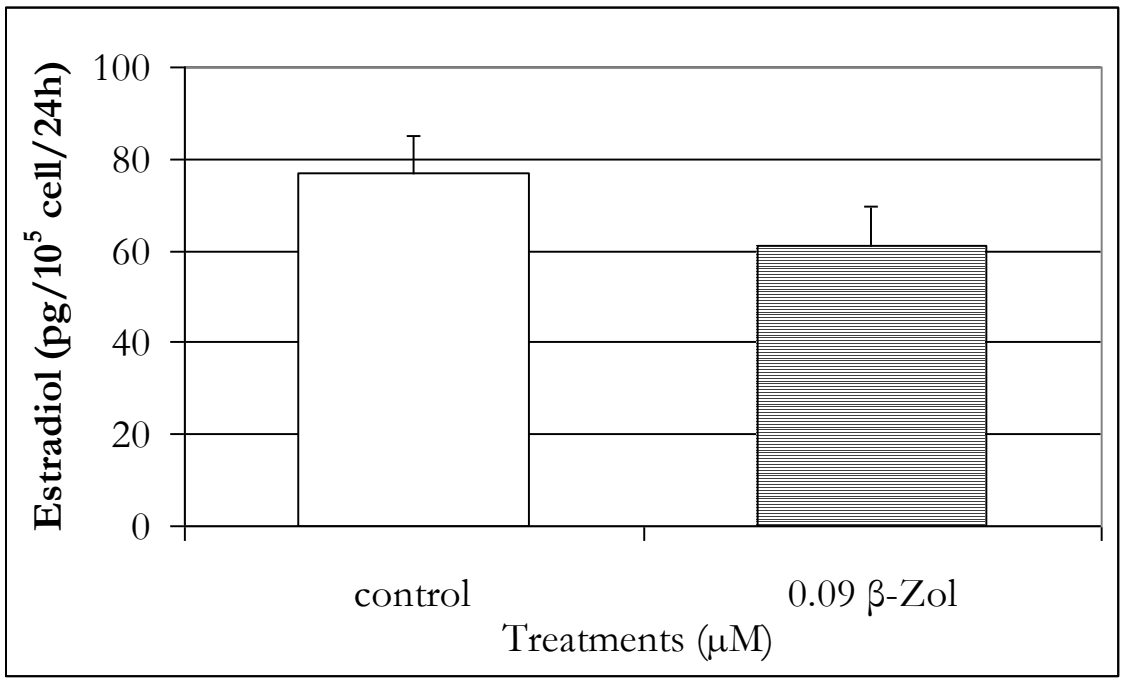


Figure 3.15. Effects of β-Zol on numbers of granulosa cells from bovine small follicles (Experiment 5). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without β-Zol. Values are means from three separate experiments (*n = 9*).

(A)



(B)

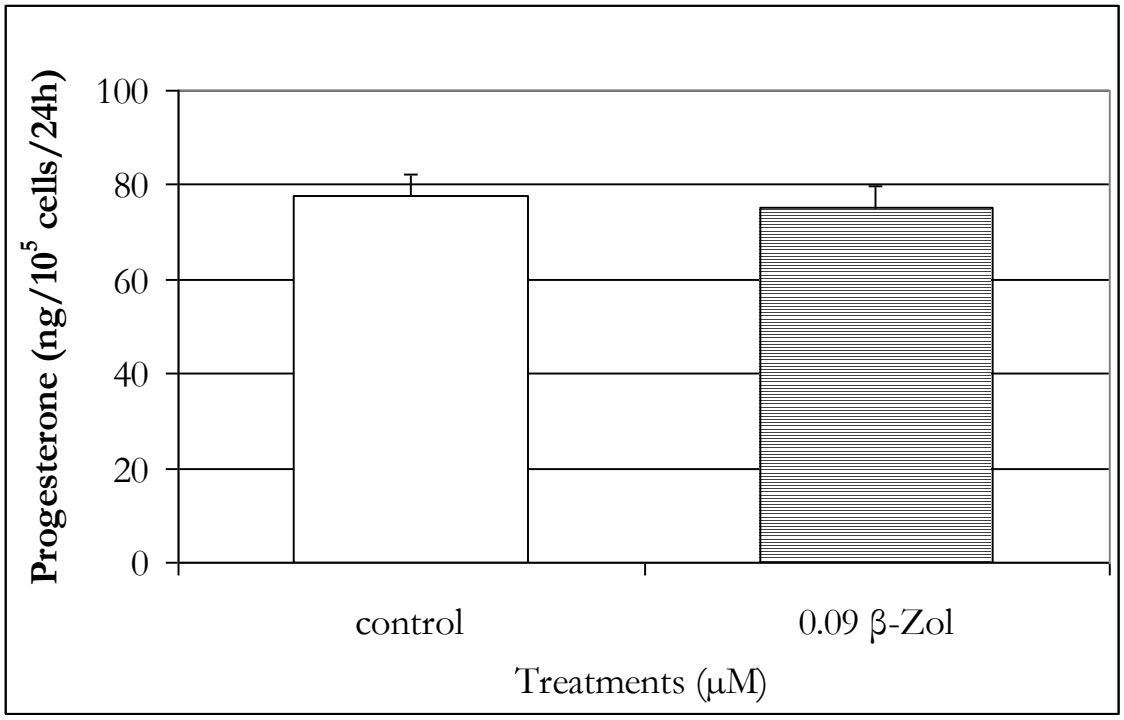


Figure 3.16. Effects of β-Zol on estradiol (Panel A) and progesterone (Panel B) production in granulosa cells from bovine small follicles (Experiment 5). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without β-Zol. Values are means from three separate experiments ($n = 9$).

3.4.6. Experiment 6: dose response of DON, α-Zol and β-Zol on small granulosa cells CYP19A1 and CYP11A1 mRNA with and without IGF1

Experiment 6 was conducted to assess the effects of DON, α-Zol and β-Zol, alone and combined, on CYP19A1 and CYP11A1 mRNA abundance with and without IGF1. In presence of IGF1 DON stimulated CYP11A1 mRNA abundance ($P < 0.05$) (Figure 3.17 A) whereas α-Zol and β-Zol alone had no effects but when combined with DON stimulatory effects (by 63 and 72% respectively) were reported ($P < 0.05$) (Figure 3.17 A). CYP19A1 mRNA abundance was strongly stimulated by β-Zol at 31 μM ($P < 0.001$) whereas all the other treatments had no effects ($P > 0.05$) (Figure 3.17 B). In absence of IGF1 DON showed a stimulatory effect on CYP19A1 mRNA abundance ($P < 0.05$) whereas had no effect ($P > 0.05$) on CYP11A1 mRNA abundance (Figure 3.18 A and B). β-Zol inhibited basal GC CYP11A1 mRNA abundance by 61% ($P < 0.001$) (Figure 3.18 A) while strongly stimulated CYP19A1 mRNA abundance ($P < 0.001$) (Figure 3.18 B). α-Zol had no effects on basal GC CYP11A1 and CYP19A1 mRNA abundance ($P > 0.05$).

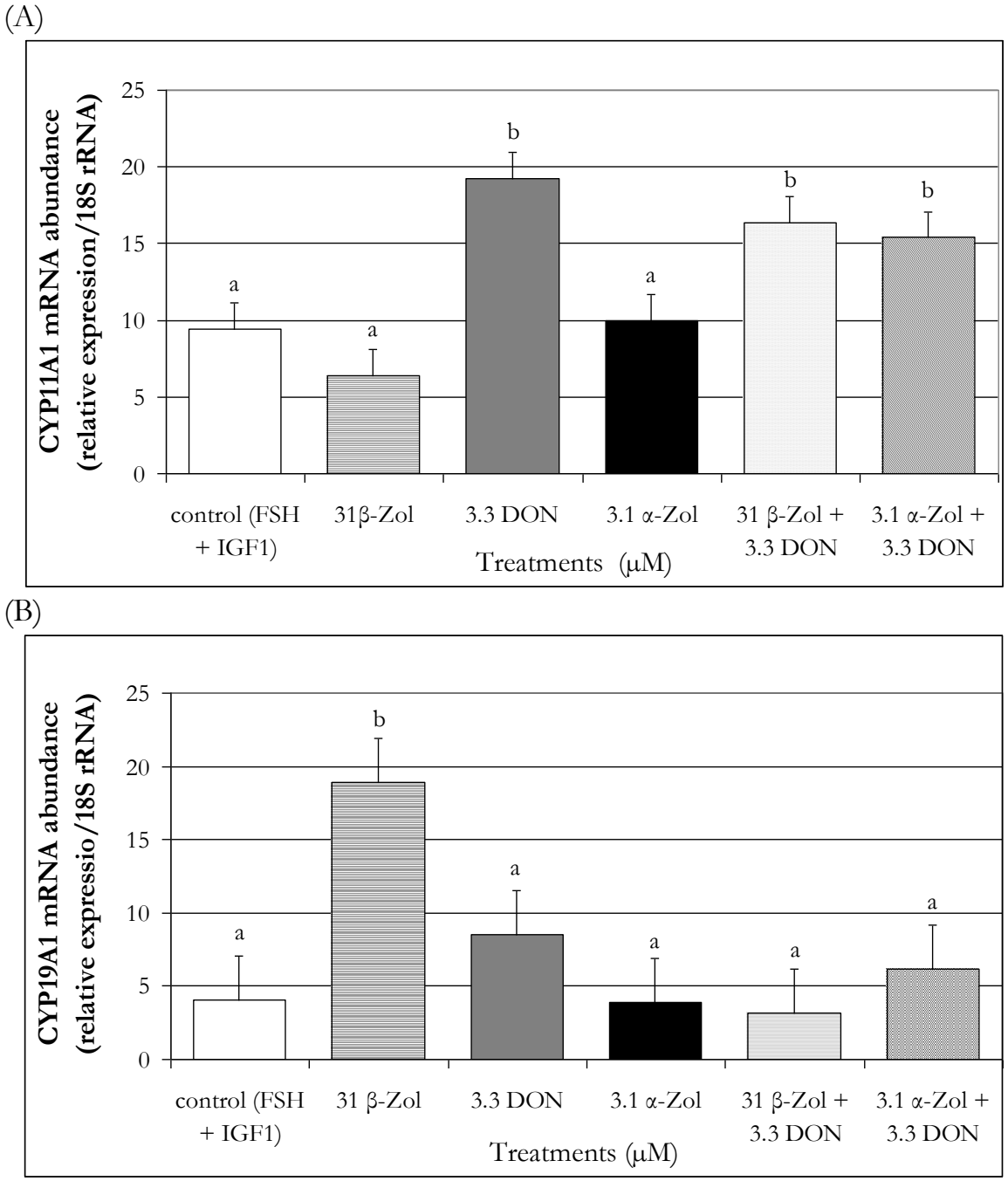


Figure 3.17. Effects of β-Zol, DON and α-Zol, alone and combined, on CYP11A1 (Panel A) and CYP19A1 (Panel B) abundance in granulosa cells from bovine small-follicles (Experiment 6). Granulosa cells were cultures for 3 days as described in Material and Methods. During the last day of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of β-Zol, DON and α-Zol. Values are means from three separate experiments (*n = 6*). Means without a common letter (a-b) differ ($P < 0.05$).

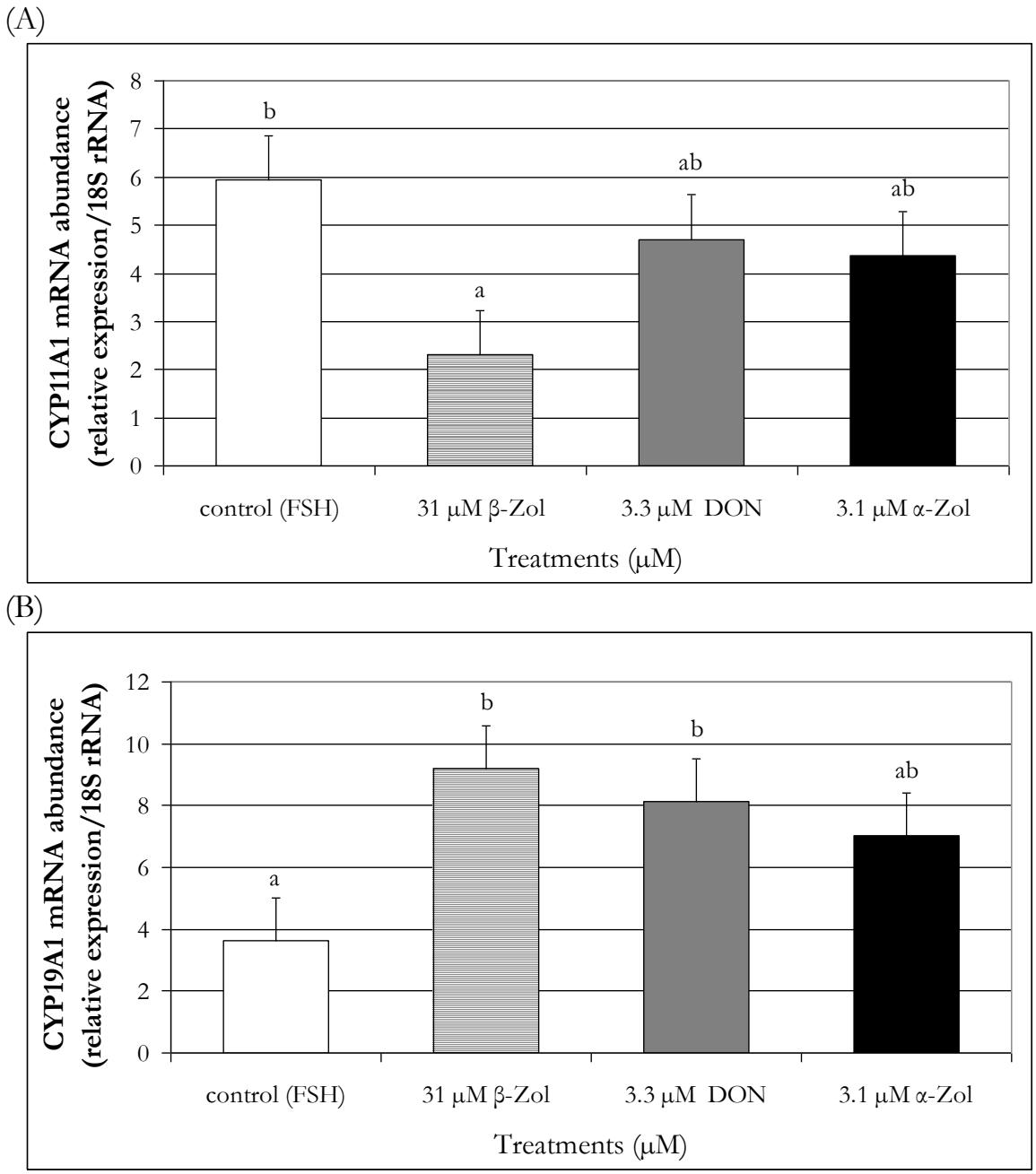


Figure 3.18. Effects of β-Zol, DON and α-Zol on CYP11A1 (Panel A) and CYP19A1 (Panel B) abundance in granulosa cells from bovine small-follicles (Experiment 6). Granulosa cells were cultures for 3 days as described in Material and Methods. During the last day of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) with or without the various doses of β-Zol, DON and α-Zol. Values are means from three separate experiments (*n = 6*). Means without a common letter (a-b) differ ($P < 0.05$).

3.5. Discussion

Fusarium mycotoxins have been implicated in poor reproductive performance in domestic animals (Malekinejad et al., 2007; Gbore and Egbunikem, 2008; Ranzenigo et al., 2008, Caloni et al., 2009; Schoevers et al., 2010). Although infertility, reduced milk production and hyperestrogenism in ruminants have been associated with exposure to ZEA (D'Mello et al., 1999; Minervini and Dell'Aquila, 2008) the possible reproductive effects of *Fusarium* mycotoxins in cattle are still unclear. The function of GC is essential in the process of normal folliculogenesis and oocyte growth and development. Substances that interfere with the function of GC compromise the oocyte's survival and normal follicle growth (Petro et al., 2012).

In the present study we exposed bovine primary GC from small follicles to DON and ZEA metabolites in order to understand if these mycotoxins have direct effects on cell proliferation, steroids production and gene expression. Information on combined toxic effects of mycotoxins is generally limited (Wan et al, 2013). Since the co-occurrence of *Fusarium* mycotoxins in cereal grains and animal feed is a common feature of several surveys leading to the possibility of toxicological interaction (Zinedine et al., 2007, Minervini and Dell'Aquila, 2008), to consider possible additive or synergistic effect of these mycotoxins is mandatory. In the present study the effects of different combinations of *Fusarium* mycotoxins (DON, α-Zol and β-Zol) on cell proliferation, steroidogenesis and gene expression in bovine SMGC were also evaluated.

In the present study, DON was found to not alter cell proliferation in SMGC at concentration ranging from 0.1 to 3.3 μM (Experiments 1 and 3). Previously the effects of DON on cell proliferation in GC were evaluated only in porcine species and the results found were in disagreement with ours on bovine. Ranzenigo et al. (2008) tested DON on porcine GC and found to inibit cell proliferation inhibition at 3.3 μM; while at minor concentration (0.03 and 0.33 μM) DON increased cell number. More recently, Medvedova et al. (2011) reported that DON increased cell proliferation at 0.33 and 3.3 μM in porcine GC. However Medvedova et al. (2011) exposed GC at different incubation condition compared to Ranzenigo et al. (2008).

The potential toxic effect of DON on cell proliferation was evaluated also in other cell types than GC. It has been demonstrated that DON is able to reduce significantly cell numbers in cultured porcine endometrial cells (Tiemann et al., 2003 a), in porcine whole-blood cells (Luongo et al., 2008) and in intestinal porcine epithelial cell lines (Diesing et al., 2011). Differential pattern of influence of DON on proliferation in comparison to the present study could be due to

different animal species involved, cell types used and experimental designs (e.g. concentration and duration of exposure).
Steroidogenesis is a complex process involving many enzymes and can be altered at any level leading to changes in the rate of production and concentration of hormones (Ndossi et al., 2012). Ovarian steroids are critical for many uterine functions such as establishment and maintenance of the pregnancy (Wood and Strauss, 2002). In the present study we evaluated the effects of DON on E2 and P4 levels in SMGC. Our results revealed that *in vitro* exposure to DON, in presence of FSH and IGF1, affects E2 and P4 production in SMGC (Experiments 1 and 3). In particular DON was found to inhibit E2 release at concentrations ranging from 0.1 to 3.3 μM and P4 production at 0.33 and 3.3 μM. Although we used GC from bovine species our results are in agreement with a previous study (Ranzenigo et al., 2008) conducted on porcine SMGC that evaluated the effects on P4 and E2 production after exposure to DON.
On the contrary a more recent research (Medvedova et al., 2011) tested the effects of DON on P4 production in porcine SMGC and found that DON stimulated P4 release at 3.3 μM. However, this occurred at different exposure and incubation conditions that may explain the difference among these studies.
DON is known to be a potent protein synthesis inhibitor by interfering with the 60S ribosomal subunit and determining translational inhibition (Pestka, 2010; Pinton and Oswald, 2014). Hormone production requires *de novo* synthesis of several enzymes which catalyzes the different steps in the pathways of steroid biosynthesis (Hanukogl, 1992). The ability of DON to negatively interfere with ribosomal activity may explain our findings. However, in absence of IGF1, the effects of DON on steroidogenesis were negligible (Experiment 3). IGF1 is known to be a potent inducer of the steroidogenesis pathway (Spicer and Chamberlain, 1998; Spicer et al., 2002) and its presence in cell culture medium increased P4 and E2 in control cells in our experiments (Experiments 1 and 3). One possible explanation of our results is that in absence of IGF1 the rate synthesis of proteins involved in hormone production is likely to be minimal (Mani et al., 2010; Monga et al., 2011) and therefore DON effects cannot be observed. Otherwise it could be postulated that DON may interfere with molecular pathways activated or blocked by IGF1.
In the present study quantitative Real Time RT-PCR was used to determine the potential of DON and ZEA metabolites to modulate the expression of important genes in steroidogenesis in SMGC.
We found that DON at 3.3 μM significantly up-regulated CYP11A1 mRNA abundance while CYP19A1 mRNA was increased but not at statistical level (Experiment 6). Interestingly previous studies (Gray and Pestka, 2007; Gray et al., 2008) demonstrated that DON promotes stability of several mRNAs interfering with post-transcriptional processes and avoiding their rapid

degradation. This phenomenon might explain the increase of CYP enzymes mRNA levels after DON exposure although the observed reductions of steroids release. SMGC cultured with FSH but in absence of IGF1 and exposed to DON showed a significant increase in CYP19A1 mRNA level while no effects were observed on CYP11A1 compared to cells cultured in presence of only FSH (Experiment 6). The up-regulation of CYP19A1 mRNA is likely related to the ability of DON to stabilize mRNA as explained above. Monga et al (2011) demonstrated that FSH is able to increase CYP19A1 mRNA while the effects on CYP11A1 are minor. Therefore the presence of FSH in culture medium may induce CYP19A1 gene transcription and the resulting mRNA is stabilized by the presence of DON while the induction of FSH on CYP11A1 was weak making the effects of DON not clearly visible.
However, additional experiments are needed to clarify the effects of DON on CYP enzymes involved in steroidogenesis; the protein quantification of CYP11A1 and CYP19A1 will be of particular interest as well as the evaluation of DON effects on other cytochromes involved in the steroid production pathway.

In the present study the *in vitro* effects of ZEA major metabolites were also evaluated. It is well known that effects of α-Zol and β-Zol are mediated by the bind to intracellular estrogen receptors (ER) since these mycotoxins have sufficient structural similarity to the endogenous estrogens (Parveen et al., 2009). Based on our results, α-Zol had no effects on cell proliferation (Experiments 1 and 2) whereas was found to inhibit both E2 and P4 production in SMGC at concentration ranging from 0.09 to 3.1 μM in presence of IGF1 (Experiment 1). These findings on cell proliferation and steroidogenesis are in agreement with a previous study (Frizzel et al., 2011) conducted on H295R cells. Moreover, accordingly with Ranzenigo et al. (2008), in presence of IGF1 cell proliferation was not affected (Experiments 1 and 2). On the contrary effects on E2 production at low concentration of α-Zol (0.09 μM) are discordant: Ranzenigo et al. (2008) showed an increase in E2 production whereas in the present study α-Zol was found to reduce E2 release. These findings suggest a species-specific response to α-Zol at low concentration.
Our results on gene expression in presence of IGF1 demonstrated that α-Zol had no effects on CYP11A1 and CYP19A1 mRNA abundance (Experiment 6). The lack of effects at mRNA abundance level and the simultaneous alteration of hormones production might lead to the conclusion that α-Zol in presence of IGF1 act on steroid release through a different mechanism not involving CYP11A1 and CYP19A1 mRNA abundance.
On the contrary, in absence of IGF1, α-Zol at 3.1 μM had inhibitory effects on cell numbers whereas did not impair steroidogenesis (Experiment 4). Our data on steroidogenesis are confirmed by a previous study (Minervini et al. 2001) that

determined the effects of high concentration of α-Zol (ranging from 0.93 to 93 μM) on E2 production in absence of IGF1 in bovine GC. Minervini et al. (2001) found that only the highest concentration of α-Zol (93 μM) was able to induce E2 production whereas the other treatments had no effects. In the present study in absence of IGF1 effects on CYP11A1 and CYP19A1 mRNA abundance were not observed confirming the lack of effects on steroid production after exposure to α-Zol. However, Tienmann et al. (2003 b) showed that α-Zol treatment at 5 μM, was able to increase the protein expression of CYP11A1 in porcine GC although no differences at mRNA level were observed as well as increase in hormone production. This leads to the conclusion that the lack of effects on mRNA abundance does not exclude the possibility of interference of α-Zol on protein expression of cytochromes P450.

To date only few studies were carried out in order to assess the *in vitro* effects and the mechanism of action of the β-epimer. In the present study β-Zol (at 31 μM) had inhibitory effects on cell numbers both in presence and absence of IGF1 (Experiment 2 and 3) while stimulated E2 and P4 production only when IGF1 was not present (Experiment 2 and 3). Our results on cell proliferation are in agreement with previous studies in porcine granulosa and endometrial cells by Tiemann et al. (2003 a,b) that demonstrated the cytotoxic effects of β-Zol. Minervini et al. (2006) tested different concentrations of β-Zol on equine GC and demonstrated that this mycotoxin did not affect cell numbers at concentration lower than 1 μM. In agreement with Minervini et al. (2006) the lowest concentration used in the present study (0.09 μM) had no effects on cell growth (Experiment 5). In relation to E2 and P4 release similar effects were already reported by Frizzel et al. (2011) on H295R cells although the authors did not provide an explanation for steroid alteration. Interestingly in our study CYP19A1 which regulates the conversion of testosterone to estrogen was up-regulated in absence of IGF1 (Experiment 6), supporting the increase of E2 production. However the mechanism of action of β-Zol on steroid production needs further clarification.

Generally our results suggested that α-Zol and β-Zol might act on GC through different mechanisms of action and at different levels as confirmed by a recent study (Molina Molina et al., 2014). On the other hand it has been already postulated that ZEA and ZEA metabolites are able to exert estrogenic effects also independently of ER activation through a non-genomic mechanism (Tiemann et al., 2003 a). Moreover, the present study confirmed previous results on ZEA metabolites (Minervini et al., 2006; Frizzel et al., 2011) that reported the stronger estrogenic potency of α-Zol compared to β-Zol. Indeed the highest binding affinity to ER has been reported with α-Zol, followed by ZEA and least with β-Zol (Malekinejad et al., 2006). Considering our results, at the same concentration (0.09, 0.31 and 3.1 μM) α-Zol had several effects on cell

proliferation and steroidogenesis whereas β-Zol did not affect GC activity (Experiments 2, 3 and 5). Indeed the effects of β-Zol were shown only when it was tested at very high concentration (31 μM).
Previously Ranzenigo et al. (2008) investigated the combined effects of DON (0.03 μM) and α-Zol (0.09 μM) on SMGC in pigs while in the present study the effects of DON were evaluated at 0.1 μM and α-Zol at 0.09 μM (Experiment 1) in bovine GC. Results on cell proliferation were consistent with findings of Ranzenigo et al. (2008) whereas our findings on E2 and P4 production were in disagreement. The concentrations of exposure and the different species used in the experiments could explain the differences found in the results. Generally our results showed that combine exposure to DON and α-Zol (at low concentration and in presence of IGF1) had significant additive inhibitory effects on E2 production (Experiment 1). When DON and α-Zol are combined in absence of IGF1 and at high concentrations had additive stimulatory effects on steroidogenesis while reduced cell numbers (Experiment 4). The difference between results obtained in experiments 1 and 4 could be explained by the presence or absence of IGF1 and its ability to stimulate steroidogenesis in GC as already explained. ZEA metabolites, when combined and in presence of IGF1, showed synergic inhibitory effects on E2 release whereas had not effects on P4 and cell proliferation (Experiment 2). On the contrary Tatay et al. (2014) evaluated the effects of a mixture of α-Zol and β-Zol (at concentration higher than 6 μM) using ovarian CHO-K1 cells and demonstrated that a combination of these mycotoxins decreased significantly cell numbers.
In experiment 3 combined effects of DON and β-Zol were investigated. After exposure to DON at 3.3 μM combined with β-Zol at 3.1 μM the resulting effects were comparable to those obtained after exposure to DON alone. However β-Zol at high concentration (31 μM) when combined with DON had antagonist effects to DON increasing the hormone production.
Based on our results, the effects on CYP11A1 mRNA abundance (Experiment 6) after exposure to α-Zol or β-Zol combined with DON are more similar to the effects observed after exposure to DON alone. Therefore these effects are likely due to the presence of DON rather than to a real interaction occurring between the two mycotoxins.
Although the mechanism of interaction among mycotoxins is still not clear this study demonstrated that co-exposure to mycotoxins may cause combined effects on GC.
In the present study it was found that the effects of DON, α-Zol and β-Zol, alone and combined, vary among the presence or absence of IGF1. FSH and IGF1 are able to stimulate cell proliferation, steroidogenesis and various genes encoding enzymes involving in steroid production pathway in bovine GC and that IGF1 increased hormones production much more that FSH alone (Spicer et

al., 2002; Mani et al., 2010; Monga et al., 2011). Previous studies reported different effects caused by various mycotoxins on porcine GC depending on the presence or absence of IGF1 in culture medium (Ranzenigo et al., 2008; Caloni et al., 2009; Cortinovis et al., 2014).

All these results taken together suggest that these mycotoxins might interfere with molecular pathways altered by IGF1. However, the mechanism by which *Fusarium* mycotoxins affect bovine GC function will require further elucidation.

In this pilot study the *in vitro* effects of different concentration of DON, α-Zol and β-Zol alone or in combination in bovine SMGC were evaluated. Results demonstrated that DON, α-Zol and β-Zol affect cell proliferation and steroidogenesis, and seem to support the hypothesis that these fusariotoxins may influence gene expression of enzymes involved in steroidogenesis.

3.6. References

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CHAPTER 4

Individual and combined effects of Deoxynivalenol and α-Zearalenol on cell proliferation and steroidogenesis in bovine large-follicle granulosa cells

4. Individual and combined effects of Deoxynivalenol and α-Zearalenol on cell proliferation and steroidogenesis in bovine large-follicle granulosa cells

4.1. Abstract

In the present study we evaluated the effects of fusariotoxins deoxynivalenol (DON) and the zearalenone (ZEA) hydroxylated metabolite, α-zearalenol (α-Zol) on cell proliferation and steroidogenesis using bovine large granulosa cells (LGGC). Bovine ovaries were collected from a slaughterhouse and ovarian follicles (8 to 22 mm) were aspirated aseptically. LGGC were cultured for 2 days in medium containing 10% fetal bovine serum followed by 1 or 2 days in serum free medium without (control) or with treatments. At the end of each experiment, steroid levels were determined *via* radioimmunoassay. Three different experiments were performed using different dosages of DON and α-Zol and in different combinations. The results obtained demonstrated that both mycotoxins are able to affect normal LGGC function. DON strongly inhibited progesterone (P4) and estradiol (E2) production mainly at high dose. α-Zol alone and in combination with DON impacted cell proliferation increasing cell growth. This study demonstrates that α-Zol and DON can impact bovine GC function, however further studies will be required to better understand the mechanism of the action of these fusariotoxins.

4.2. Introduction

Deoxynivalenol (DON) and zearalenone (ZEA) are the major mycotoxins produced by *Fusarium* species (D'Mello et al., 1999; Larsen et al., 2004) which contaminate feedstuffs mostly in the northern hemisphere (Tiemann et al., 2003; Larsen et al., 2004). A simultaneous exposure to DON and ZEA may be possible since some *Fusarium* species are able to produce both compounds (Zinedine et al., 2007, Minervini and Dell'Aquila, 2008).
DON exerts its toxicity by binding to ribosomes that leads to the activation of various protein kinases, the modulation of gene expression, the inhibition of protein synthesis and cell toxicity. This mechanism is also known as "ribotoxic stress" (Maresca, 2013). DON is a mycotoxin also implicated in the reproductive disorders in livestock (Diekman and Green, 1992). In ruminants, after ingestion, DON is converted by rumen-associated bacteria (Seeling et al., 2006; Fink Gremmels 2008a; Maresca, 2013) into its non-toxic metabolite de-epoxy DON (DOM1) (Maresca, 2013), and this chemical transformation could explain the high tolerance of this species to DON exposure. However, DOM1 production occurs only in healthy ruminants because in animals with various rumen diseases the metabolism of DON is incomplete and DON can be detected in blood easily (Fink Gremmels, 2008a). Only few cases of intoxication related to DON exposure are reported in cows. In North European cattle, after ingestion of a large amount of silage contaminated with DON, clinical signs related to laminitis and mastisis were demonstrated, but it was not clear if the intoxication was due to the ingestion of DON alone or if other tricothecenes were also present in feedstuffs (Speijers and Speijers, 2004). However, knowledge about the effects of DON on ruminants is limited and the literature on this issue is still rare (Seeling et al., 2006).
ZEA is frequently present in maize, wheat, oats and barley (Zinedine et al., 2007). Because of their chemical structures, ZEA and its metabolites are able to bind to estrogen receptors and so exert estrogenic effects (Parveen et al., 2009; Frizzell et al., 2011). In pigs, estrogenic effects of ZEA are well documented. In particular, immature gilts seem to be the most sensitive to this mycotoxin (Park et al., 1996; D'Mello et al., 1999; Zinedine et al., 2007) because their endogenous endocrine system is still undeveloped (Minervini and Dell'Aquila, 2008). After exposure to ZEA young gilts show hyperemia, vulvar edema, vaginal and rectal prolapse (Minervini and Dell'Aquila, 2008). Ruminants are considered to be more resistant to the effects of ZEA and metabolites (Fink-Gremmels, 2008; Upadhaya et al., 2010). Clinical signs of hyperestrogenism caused by intoxication with ZEA are rarely observed in cows and generally they are induced by a high

contamination of silage or by long term exposure to contaminated feedstuffs (Bloomquist et al., 1982; Coppock et al., 1990). After feeding heifers with a diet containing ZEA, conception rate decreased from 87% to 62% (D'Mello et al., 1999). Also, ZEA has been shown to cause infertility in grazing sheep in New Zealand (Towers and Sposen, 1993). Enlargement of mammary glands with secretory activity were reported in prepubertal heifers which consumed *Fusarium*-contaminated silage (Bloomquist et al., 1982). Presently, little information is available about the effects of *Fusarium* mycotoxins on granulosa cells (GC) activity in cows. Therefore, the purpose of this study is to determine, through the evaluation of cell proliferation and steroid production, if DON and α-Zol have effects on bovine large-follicle granulosa cells (LGGC). Because DON and ZEA metabolites can contaminate commodities coincidently, combined presence of DON and α-Zol on GC function was also evaluated.

4.3. Materials and methods

4.3.1. Reagents and Hormones

Reagents were: Dulbecco's Modified Eagle Medium (DMEM), Ham's F12, fetal calf serum (FCS), Deoxynivalenol (DON) and α-zearalenol (α-Zol) obtained from Sigma Chemical Co. (St. Louis, MO); purified ovine FSH (FSH activity: 175 x NIH-FSH-S1 U/mg) obtained from Dr. A. F. Parlow, National Hormone & Pituitary Program (Torrance, CA); recombinant human IGF1 obtained from R&D Systems (Minneapolis, MN), and testosterone obtained from Steraloids (Wilton, NH).

4.3.2. Cell Culture

Ovaries from non pregnant beef cows were collected from a slaughterhouse and were treated as previously described (Langhout et al., 1991; Spicer et al., 2002). Based on surface diameter, large follicles (8 to 22 mm) were aspirated aseptically with an 18 gauge needle (Figure 4.1). GC were recovered by centrifugation at 291 x g for 10 min and were washed twice with 7 mL of serum free medium; at each wash, cells were separated from medium *via* centrifugation (291 x g at 4°C for 5 min). After the last centrifugation the supernatant was aspirated and replaced with 2 mL of enzyme containing medium (0.5 mg/mL of DNase and 1.25 mg/mL of collagenase) to prevent clumping of cells as previously described (Spicer et al., 2002). Cell viability averaged 57 + 5% and was determined using the trypan blue exclusion method (Langhout et al., 1991; Tiemann et al., 2003).

Viable cells (1.0 x 10^5 in 40 to 70 µl of medium) were placed on 24 well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) in 1 ml of basal medium composed of a mixture of 1:1 DMEM and Ham's F-12 containing 10% FCS, glutamine (2 mM), gentamicin (0.12 mM) and sodium bicarbonate (38.5 mM) (Figure 4.2). Cultures were kept at 38.5° C in a humidified 95% air and 5% CO_2 environment and medium was changed every 24 h. To obtain an optimal attachment, cells were maintained in the presence of 10% FCS for the first 48 h of culture. After this time, GC were washed twice with serum-free medium (0.5 ml) and the various treatments applied in serum-free medium (1.0 ml) containing 500 ng/ml testosterone (as an estradiol precursor) for 24 or 48 h.

4.3.3. Determination of granulosa cells numbers and steroid concentrations

Medium was collected from individual wells and frozen at -20 ºC for subsequent steroid analyses. Concentrations of progesterone and estradiol in culture medium were determined by radioimmunoassays (RIA) as previously described (Spicer and Hammond, 1987; Spicer et al., 1990; Langhout et al., 1991). Numbers of granulosa cells in the same wells from which medium was collected were determined by a Coulter counter (model Z2; Beckman Coulter, Inc., Hialeah, FL), and used to calculate steroid production on ng or pg per 10^5 cell basis. Briefly, cells were gently washed twice with 0.9% saline solution (500 µL), exposed to 500 µL of trypsin (0.25% wt/vol=2.5 mg/mL) for 20 min at room temperature, and then scraped from each well and enumerated as previously described (Spicer and Hammond, 1987; Langhout et al., 1991; Ranzenigo et al., 2008). Cell aggregates were minimized by pipetting cell suspensions back and forth through a 500 µL pipette tip three to five times.

Progesterone RIA

Progesterone RIA were conducted using rabbit antiserum (X-16), which serves as the first antibody (diluted 1:3000 with assay buffer: PBS, EDTA, NaN_3, and gelatin), raised against BSA-11 glutamate derivative as described by Baraño and Hammond (1985). Goat anti-rabbit antibody (diluted 1:15 with assay buffer) was used as the second antibody (Linco Research, Inc., St. Charles, MO). [^{125}I]Iodo-progesterone (ICN Biomedicals, Costa Mesa, CA) was used as the tracer. A progesterone standard curve was prepared from a stock concentration of 80.0 ng/mL that was serially diluted with assay buffer to concentrations of 40.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.625, 0.31, and 0.16 ng/mL. In duplicate, 20 µL to 100 µL of medium samples were combined with the appropriate volume of assay buffer to make a total volume of 100 µL. One hundred µL of tracer and first antibody were added and all samples were mixed and allowed to incubate at 37 °C for 1 h. Following incubation, 200 µL of second antibody were added and all samples

were incubated overnight at 4 °C. The following day, 50 μL of normal rabbit serum (NRS) (diluted 1:5 with assay buffer from a 15% NRS stock) were added to all samples. Samples were centrifuged at 4 °C in a Sorvall Model RC-3 (Thermo Fisher Scientific, Inc., Miami, OK) at 1800 x *g* for 25 min. Supernatant was aspirated and precipitates were counted for 1 min using a Cobra AII Auto-Gamma counter (Packard Instrument Co., Downers Grove, IL). The intra- and interassay coefficients of variation were 7 and 13%, respectively, for the progesterone RIA.

Estradiol RIA

Estradiol RIA were conducted using anti-estradiol rabbit antibody (diluted 1:12 with assay buffer), which serves as the first antibody (Lilly Research Laboratories, Indianapolis, IN), and goat anti-rabbit antibody (diluted 1:15 with assay buffer) which serves as the second antibody (Linco Research, Inc., St. Charles, MO). Radiolabled estradiol (^{125}I-estradiol) was used as the tracer (ICN Biomedicals, Costa Mesa, CA). The assay buffer was the same as the progesterone RIA buffer described above. An estradiol dose response curve was prepared from a stock concentration of 256 pg/100 μL that was serially diluted to 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 pg/100 μL using assay buffer. In duplicate, sample media was added at either 50 or 100 μL and (if needed) combined with assay buffer to make a total volume of 100 μL. Two hundred μL of tracer were added to all samples, along with 100 μL of first antibody. All tubes were mixed and allowed to incubate for 1 h at 37 °C. Following this incubation, 200 μL of second antibody were added and the assay allowed to incubate at 4 °C overnight. The following day, assay tubes were centrifuged, supernatant aspirated, and precipitate counted as described for the progesterone RIA. The intra- and interassay coefficients of variation were 8% and 17%, respectively, for the estradiol RIA.

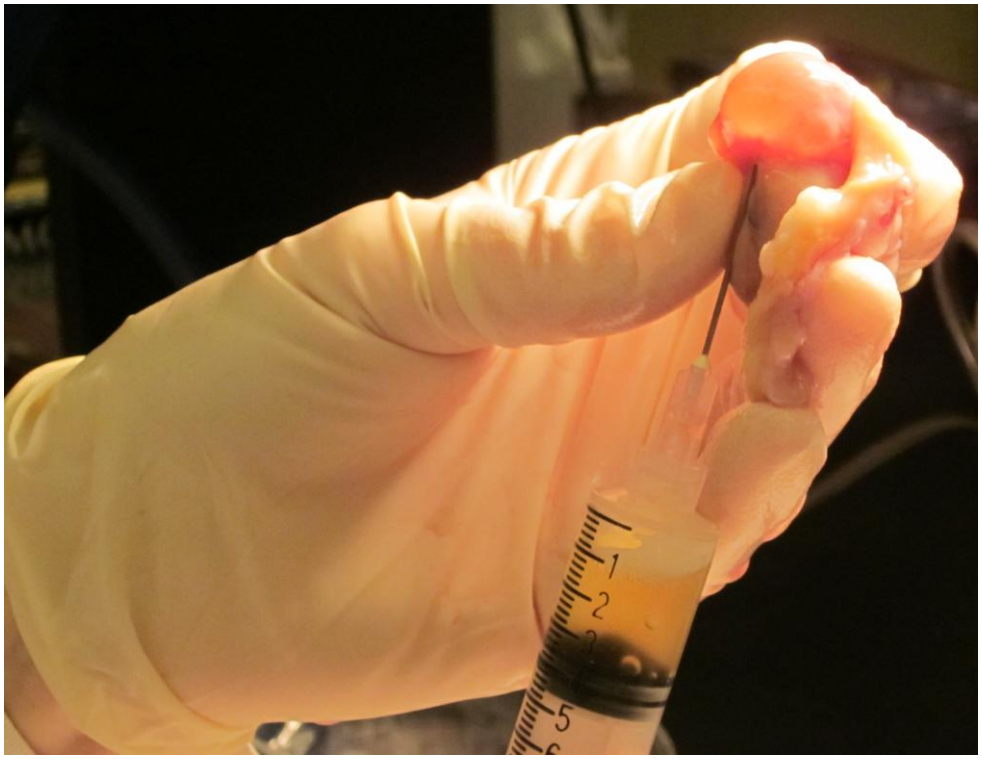


Figure 4.1. Follicular fluid was aspirated aseptically from bovine large follicles (8 to 2 mm).

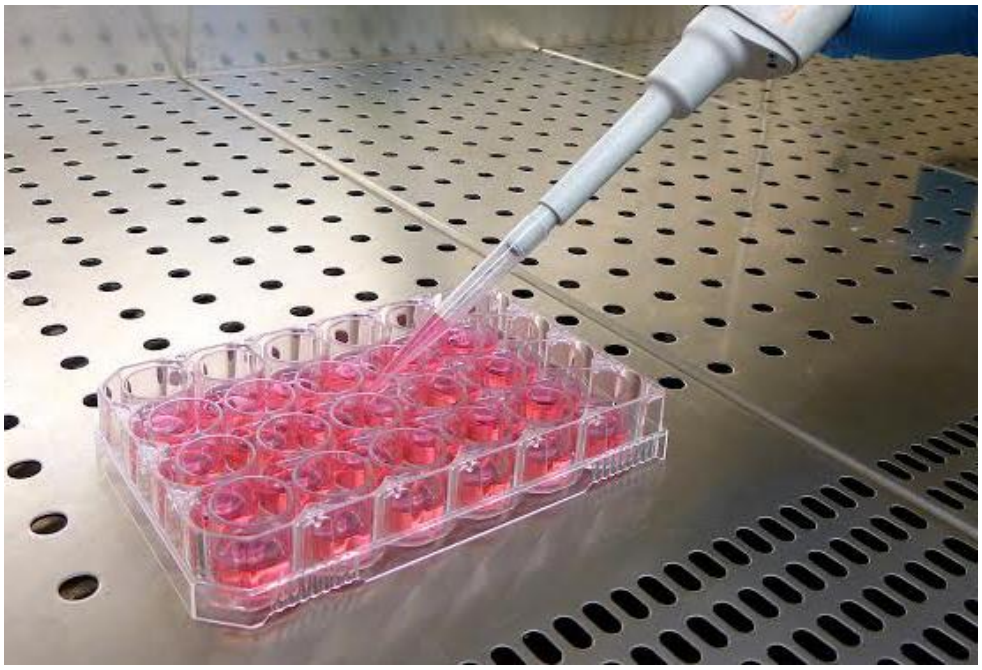


Figure 4.2. Cell seeding. Viable cells were placed into Falcon multiwell plates containing 1 mL of medium.

4.3.4. Experimental design

Experiment 1 was designed to determine the dose response of DON and α-Zol and their interaction at low dosages on bovine GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and then treated for 48 h in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of DON (0, 0.1, 0.33, and 3.3 μM) and α-Zol (0, 0.09, 0.31, 3.1 μM). A concomitant treatment with DON and α-Zol at 0.1 and 0.09 μM respectively was also carried out. After 48 h of treatment, medium was collected for P4 and E2 determinations, and cells were counted. Doses of FSH and IGF1 were selected based on previous studies (Ranzenigo et al., 2008; Spicer et al., 2002). FSH was added to all treatments, because IGF1 alone does not have an effect on steroid production (Spicer et al., 2002; Ranzenigo et al., 2008).
Experiment 2 was designed to evaluate the time course effects of DON and α-Zol induced on GC proliferation and to determine resulting steroidogenesis in presence FSH and IGF1. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 24 and 48 h, with treatments change after first 24 hours, in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of DON (i.e., 0, 3.3 μM) and α-Zol (i.e., 0, 0.09, 0.31, μM). After 24 and 48 h the cells were counted and after 48 hours medium was collected for P4 and E2 determinations. The mycotoxin doses were selected based on the results of experiment 1.
Experiment 3 was designed to evaluate the effects of DON and α-Zol and their combination, at high dosages, with FSH but without IGF1 on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 24 and 48 h in serum-free medium containing FSH (30 ng/mL) with or without the various doses of DON (i.e., 0, 3.3 μM) and α-Zol (i.e., 0, 3.1 μM) and their combination at 3.3 μM and 3.1 μM, respectively. After 24 and 48 h the cells were counted and after 48 h medium was collected for P4 and E2 determination.

4.3.5. Statistical analysis

Experimental data are presented as the least squares means ± s.e.m. of measurements from replicated culture wells. Each experiment was performed three times with different pools of GC collected from 5 to 8 ovaries for each pool. Treatment effects and interactions were assessed using the GLM procedure of the Statistical Analysis System (SAS) for Windows (version 9.2, SAS Institute Inc., Cary, NY). Main effects were treatment, experiment, and their

interaction when data from more than one experiment were analyzed. Each well was a replicate and each experiment contained three replicates per treatment. Steroid production was expressed as ng or pg/105 cells per 24 h and cell numbers at the termination of the experiment were used for this calculation.

4.4. Results

4.4.1. Experiment 1: dose response of DON and a-Zol on cell proliferation and E2 and P4 production in the presence of FSH plus IGF1

The highest dose of DON (3.3 μM) decreased cell growth by 22% ($P < 0.05$) whereas α-Zol at the lowest treatment (0.09 μM) stimulated cell proliferation by 31%, compared to the control ($P < 0.05$). The combination of DON and α-Zol at 0.1 and 0.09 μM respectively increased cell proliferation by 45% compared to the control ($P < 0.001$). All the other doses tested did not affect cell proliferation ($P > 0.05$) (Figure 4.3). DON at 0.33 and 3.3 μM strongly inhibited E2 production by 68% ($P < 0.001$) and by 96% ($P < 0.0001$) respectively while at the lowest dose (0.1 μM) had no effect compared to the control ($P > 0.05$) (Figure 4.4 A). The highest dose of DON also inhibited P4 production (by 40%) compared to the controls ($P < 0.001$) (Figure 4.4 B). All tested doses of α-Zol (0.09, 0.31, 3.1 μM) and the combination of the two mycotoxins had no effect on E2 and P4 production ($P > 0.05$) (Figure 4.4 A and B).

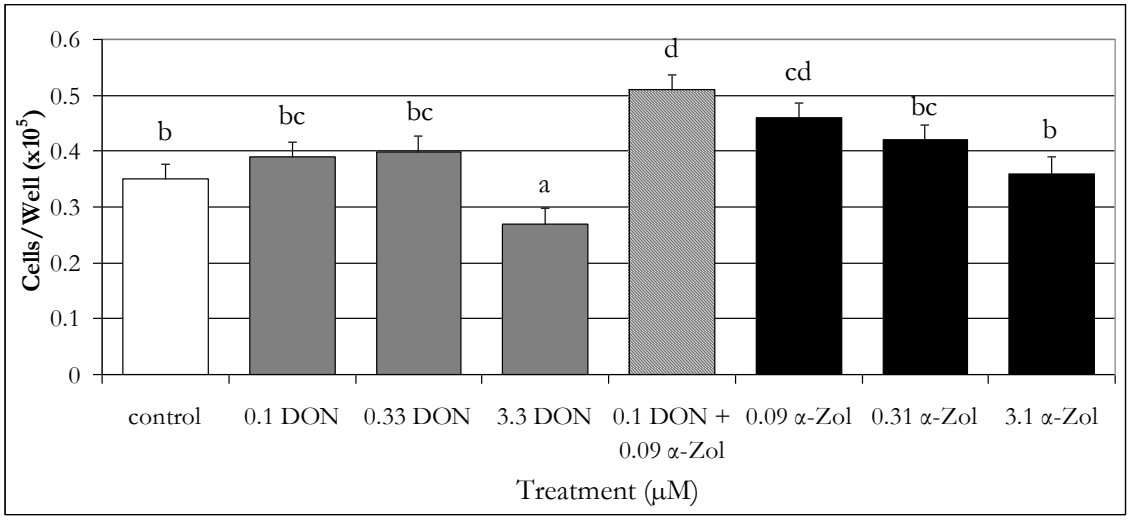


Figure 4.3. Effects of DON and α-Zol, alone and combined, on numbers of granulosa cells from bovine large follicles (Experiment 1). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of DON and α-Zol. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-d) differ ($P < 0.05$).

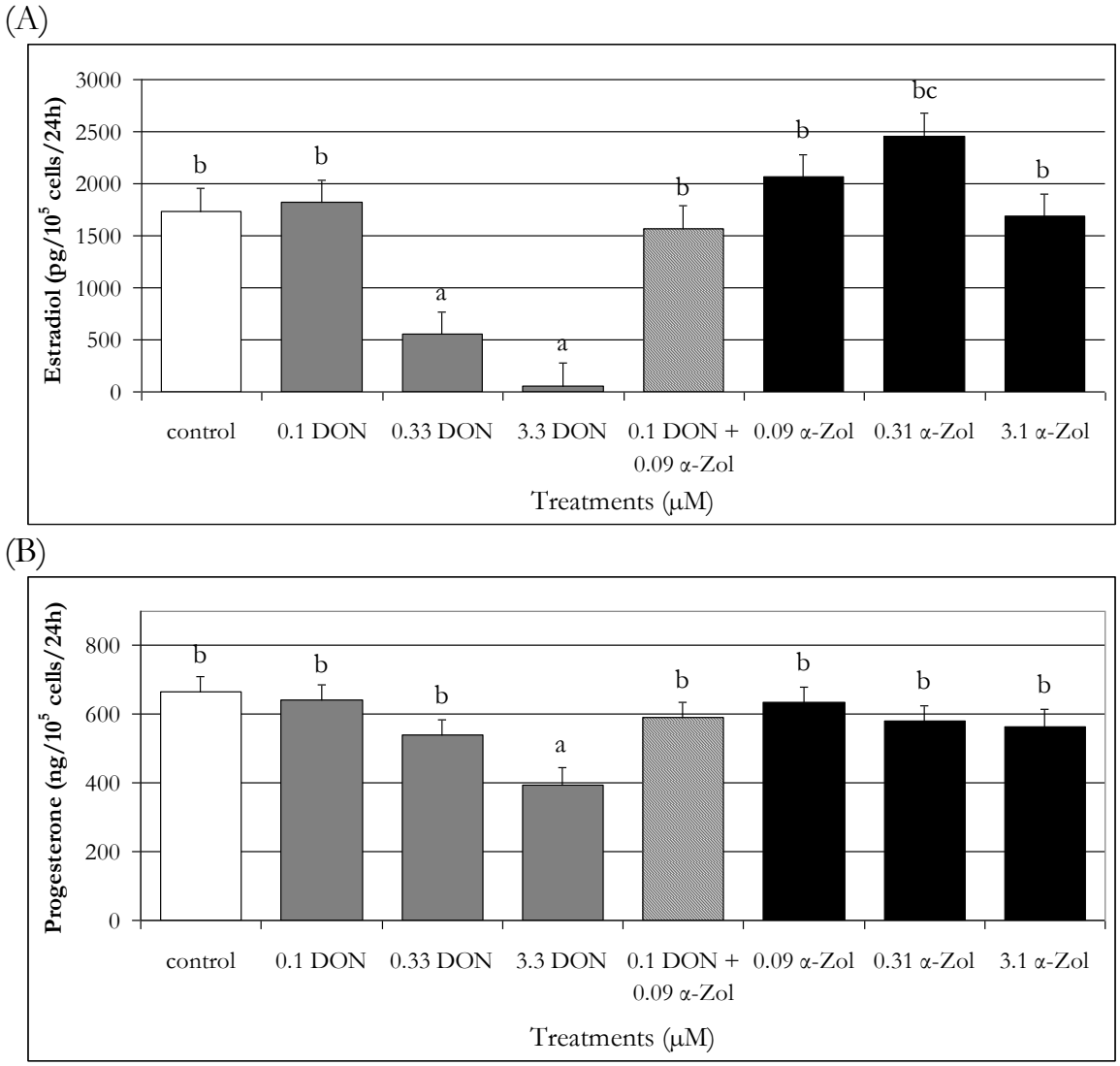


Figure 4.4. Effects of DON and α-Zol, alone and combined, on FSH plus IGF1-induced estradiol (Panel A) and progesterone (Panel B) production by granulosa cells from bovine large follicles (Experiment 1). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of DON and α-Zol. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-d) differ ($P < 0.05$).

4.4.2. Experiment 2: Time course response (effects on day 1 and day 2) of DON and a-Zol on cell number and P4 and E2 production in presence of FSH and IGF1

In presence of FSH and IGF1 cell number increased ($P < 0.05$) between days 0 to 2. At day 1 cell growth was not affected after treatment with α-Zol whereas at day 2 α-Zol at 0.09 and 0.31 μM stimulated cell growth (by 28 and 57% respectively) but in the first case the increase was not statistically significant ($P > 0.05$). DON had no effect ($P > 0.05$) on cell numbers on day 1 and day 2 (Figure 4.5) but strongly inhibited E2 ($P < 0.05$) (Figure 4.6 A) and P4 ($P < 0.0001$) (Figure 4.6 B) production by 89% and 84%, respectively compared to controls. However, treatment with α-Zol at 0.09 and 0.31 μM had no significant effect ($P > 0.05$) on steroid production (Figure 4.6 A and B).

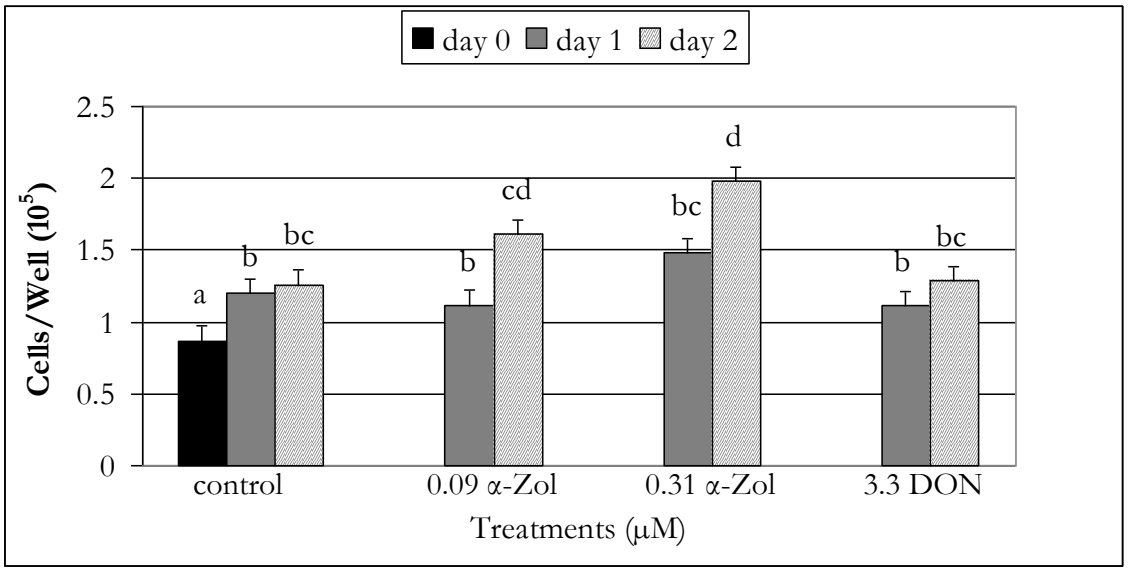


Figure 4.5. Effects of α-Zol and DON on day 0, day 1 and day 2 on numbers of granulosa cells from bovine large follicles (Experiment 2). Values are means from three separate experiments (*n = 9*). Means without a common letter (a-d) differ ($P < 0.05$).

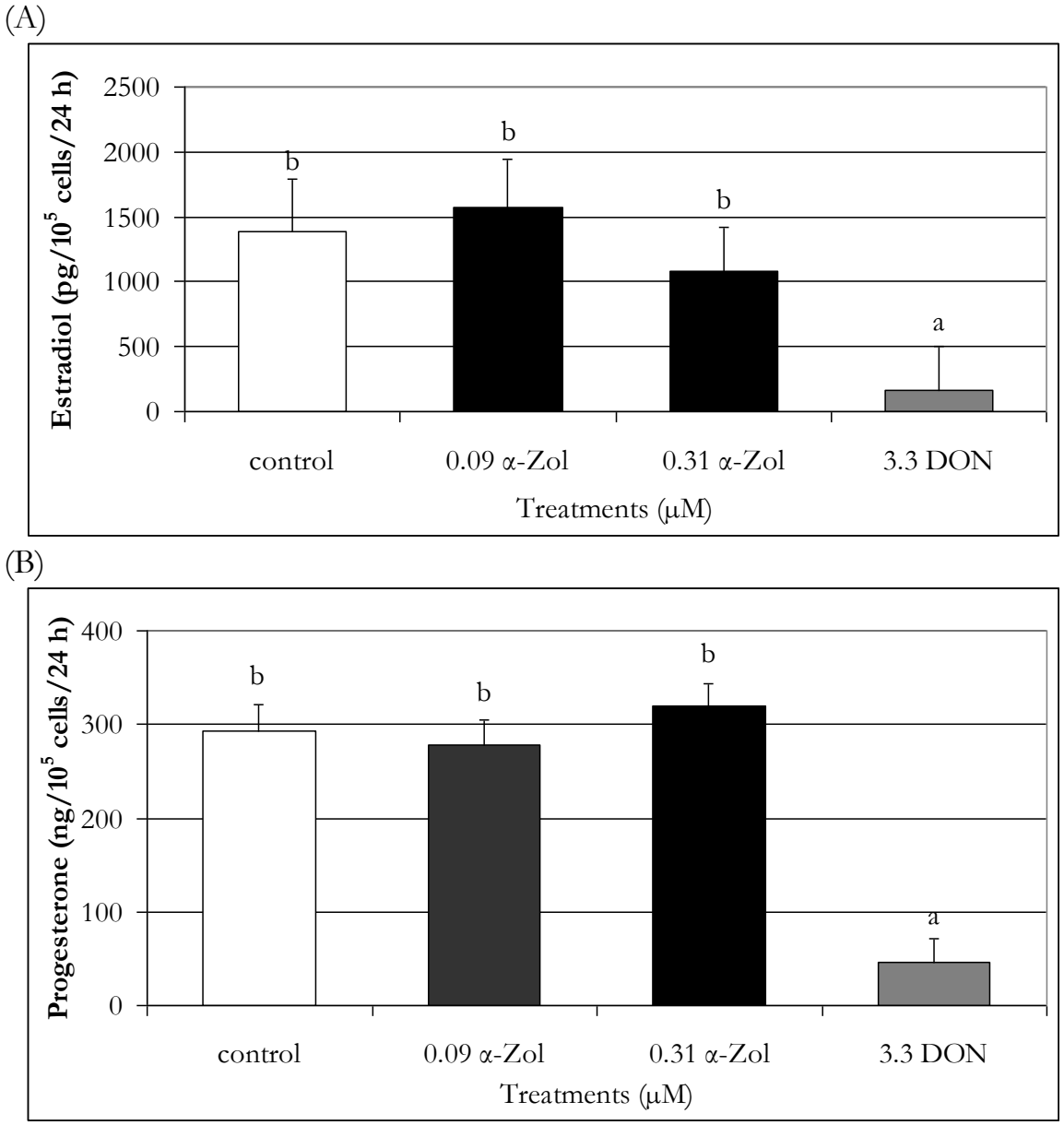


Figure 4.6. Effects of α-Zol and DON, on FSH plus IGF1-induced estradiol (Panel A) and progesterone (Panel B) production by granulosa cells from bovine large follicles (Experiment 2). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of DON and α-Zol. Values are means from three separate experiments ($n = 9$). Means without a common letter (a-b) differ ($P < 0.05$).

4.4.3. Experiment 3: Time course response (effects on day 1 and day 2) on cell number and P4 and E2 production in presence of FSH

In presence of only FSH, cell growth significantly decreased between days 0 to 2. At day 1 only the combination of the two mycotoxins stimulated cell growth by 20% compared to the control ($P < 0.05$). At day 2 only α-Zol at 3.1 μM had effect on cell proliferation increasing cell number by 24% compared to the control ($P < 0.05$) (Figure 4.7). After exposure to α-Zol and DON, alone and combined, E2 production was not significantly affected ($P > 0.05$) (Figure 4.8 A). P4 release decreased after exposure to α-Zol alone ($P < 0.01$) and combined with DON ($P < 0.01$), whereas DON alone had no effect on P4 release compared to the control ($P > 0.05$) (Figure 4.8 B). However E2 and P4 showed a trend to decrease (not statistically significant, $P > 0.05$) after exposure to DON alone.

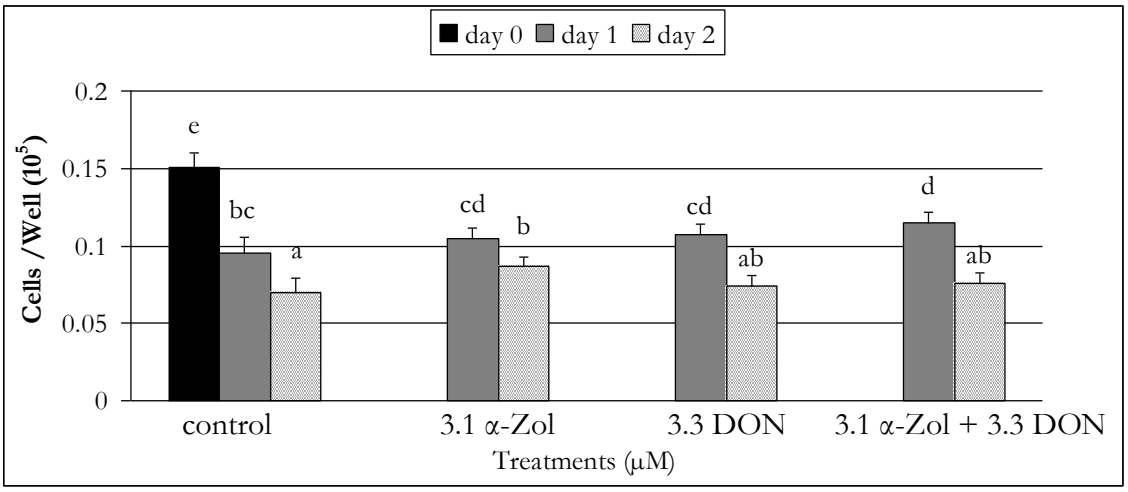
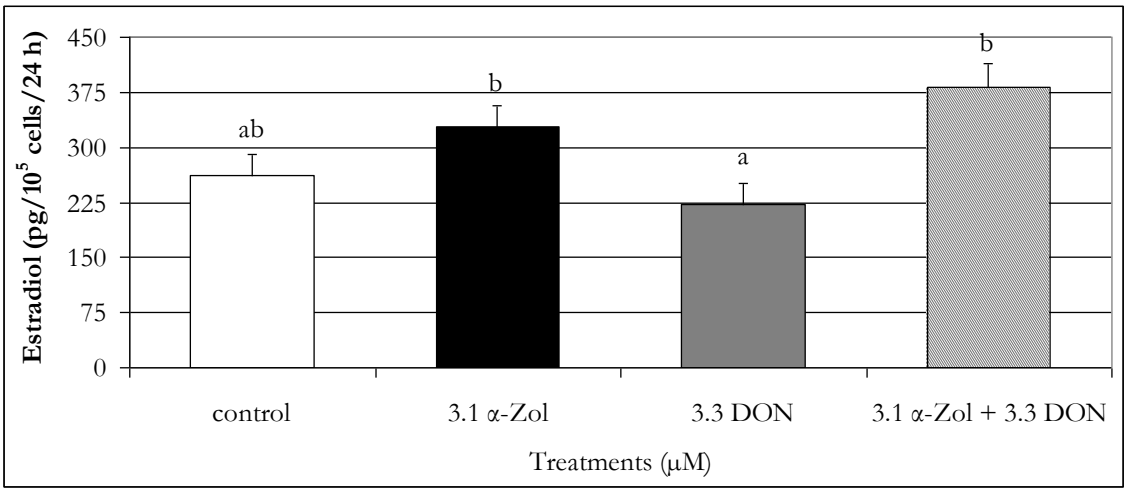


Figure 4.7. Effects of α-Zol and DON, alone and combined, on day 0, day 1 and day 2 on numbers of granulosa cells from bovine large follicles (Experiment 3). Values are means from three separate experiments (n = 9). Means without a common letter (a-e) differ ($P < 0.05$).

(A)



(B)

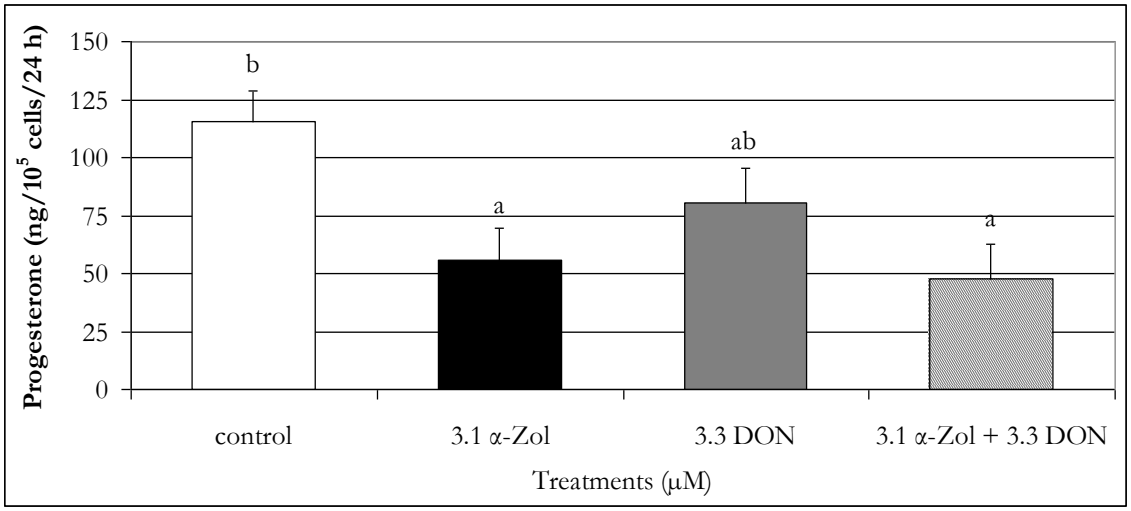


Figure 4.8. Effects of α-Zol and DON, alone and combined, on estradiol (Panel A) and progesterone (Panel B) production in granulosa cells from bovine large follicles (Experiment 3). Granulosa cells were cultures for 4 days as described in Material and Methods. During the last 2 days of culture, cells were treated in serum-free medium containing FSH (30 ng/mL) with or without the various doses of DON and α-Zol. Values are means from three separate experiments (*n = 9*). Means without a common letter (a-b) differ ($P < 0.05$).

4.5. Discussion

Ruminants have always been considered to be less susceptible to the adverse effects of mycotoxins compared to monogastric animals due to the ability of rumen microflora to detoxify many mycotoxins (Fink Gremmels, 2008b). However this may vary between different chemical classes of mycotoxins: rumen protozoa effectively transform DON into its less toxic metabolite (DOM1) but it also converts ZEA mainly into α-Zol which shows higher affinity for estrogen receptor compared to the parent compound (Fink Gremmels, 2008a).
In addition, the rumen microflora could be modified by some mycotoxins because of their antimicrobial properties (Danicke et al., 2005; Fink Gremmels, 2008b). In case of incompetent rumen microflora some mycotoxins have the ability to cross the rumen barrier unchanged and to become bioavailable through absorption *via* the duodenum (Fink Gremmels, 2008b).
The present study aimed to elucidate direct effects of DON and α-Zol on bovine primary LGGC. In support of the fact that cattle can actually be exposed to mycotoxins at the reproductive organ level, a recent study by Takagi et al. (2008) demonstrated that both ZEA and its metabolites, α-Zol and β-Zol, were detected in bovine ovarian follicular fluid by using liquid chromatography-tandem mass spectrometry. In our results DON strongly inhibited both E2 and P4 production only in presence of IGF1 (Experiments 1 and 2) whereas when IGF1 was not present (Experiment 3) DON showed a weak inhibitory trend (non-significant) on steroid release. Many studies (Spicer and Chamberlain, 1998; Spicer et al., 2002) reported *in vitro* effects of IGF1 on GC and demonstrated that IGF1 has strong stimulatory effects on the release of E2 and P4 and on cell proliferation. Our data on steroidogenesis are in agreement within those experiments performed in presence of IGF1 (experiments 1 and 2), while some differences can be noticed when IGF1 was not present (experiment 3). The *in vitro* effects of DON on steroidogenesis have been previously investigated in pigs. Ranzenigo et al. (2008) exposed porcine GC to concentrations of DON similar to ours (0.03, 0.31 and 3.3 μM) and found that DON strongly inhibited E2 and P4 production at 0.33 and 3.3 μM in presence of IGF1 and FSH. A more recent study (Medvedova et al., 2011) demonstrated that DON at 3.3 μM increased P4 production in gilts in absence of IGF1, however this occurred at different incubation conditions than in the study carried out by Ranzenigo et al. (2008). It is well established that DON binds to the eukaryotic 60S ribosomal subunit, leading to protein synthesis inhibition, and rapidly activates mitogen-activated protein kinases (MAPKs) *via* a pathway known as the "ribotoxic stress response" (Pestka, 2007 and 2010). Although the mechanism by which DON decreases E2 and P4 release needs further investigation, it can be postulated that

DON, inhibiting protein synthesis, makes difficult the formation of steroid hormones that requires *de novo* proteins synthesis (Hanukogl, 1992).
In the present study the *in vitro* effects of α-Zol, one of the major ZEA metabolites, on steroidogenesis and cell proliferation were also investigated. Based on our findings, α-Zol had no effect on steroidogenesis (Experiments 1 and 2) in presence of IGF1 and FSH, but when IGF1 was not present (experiment 3) α-Zol showed an inhibitory effect on P4 production at a high concentration (3.1 μM). Our results are in agreement with findings of a previous study (Tiemann et al., 2003) conducted on porcine GC that demonstrated that in presence of only FSH, α-Zol was able to inhibit P4 production. It is well known that, due to their chemical structures, ZEA and its metabolites are able to bind estrogen receptor (Parveen et al., 2009, Frizzell et al., 2011). However, a different mechanism of action has been postulated (Minervini et al., 2001; Tiemann et al., 2003; Fink-Gremmels and Malekinejad, 2007).
Tiemann et al. (2003) demonstrated that derivatives of ZEA, such as α-Zol, are able to reduce also the intracellular levels of CYP11A1 and 3β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) transcripts, although the precise mechanism is still unclear. CYP11A1 catalyzes the conversion of cholesterol to pregnenolone while 3β-HSD catalysed the transformation of pregnenolone to P4 and they are normally stimulated by the presence of IGF1 and FSH (DeMoura et al., 1997). It has also been postulated that α-Zol may decrease FSH-stimulated P4 accumulation by binding and changing the FSH receptor conformation and/or by interrupting G-protein-GTP-mediated pathways (Tiemann et al., 2003) that normally leads to the increase of intracellular cAMP content (Richards, 1994; Conti, 2002). These events stimulate CYP11A1 gene transcription and therefore P4 production (Brentano et al., 1992). Consistent with this hypothesis, in our experiments in the absence of IGF1, α-Zol was able to affect FSH-induced P4 production reducing the amount of P4 released by GC (experiment 3).
We found that α-Zol had no effect on E2 production both with and without IGF1 on bovine LGGC. To our knowledge, no studies have reported the effects of *Fusarium* mycotoxins on bovine LGGC in the literature; however some information is available on bovine small granulosa cells (SMGC). Minervini et al. (2001) exposed bovine small follicle mural GC and found that E2 production was significantly increased only after exposure to α-Zol at 30000 ng/ml (90 μM), a dose 30-fold higher than those we used in our experiments. Ranzenigo et al. (2008) studied the effects of α-Zol on porcine SMGC and demonstrated that α-Zol had biphasic effects on FSH plus IGF1-induced E2 release. However, different cell cultures conditions and differences in metabolism between cell types complicate the possibility to compare our results to other studies. The role

of α-Zol on the release of E2 and P4 in bovine species needs to be further investigated.

Data on cell proliferation will also require further clarification. In experiment 1, DON at highest concentration and in the presence of IGF1 and FSH had inhibitory effects on cell growth, whereas at the same concentration had no effect on cell proliferation in experiment 2 and 3. In comparison, α-Zol at 0.09 μM had stimulatory effects on cell growth (experiments 1 and 2), although in experiment 2 the increase induced by α-Zol was not statistically significant, whereas at high concentration showed a stimulatory effect only in absence of IGF1 (experiment 3). Thus, further research is needed to clarify the effects of these mycotoxins on cell proliferation in cattle.

Since a co-exposure to DON and ZEA is possible (Zinedine et al., 2007, Minervini and Dell'Aquila, 2008), we studied the effects of a concomitant treatment with DON and α-Zol. Experiment 1 showed that low concentration of the two mycotoxins in presence of IGF1 and FSH had no effect on steroidogenesis whereas an increase of cell growth was observed compared to the control. Experiment 3 investigated the effects of a co-exposure at high dose in the absence of IGF1. The 1-day cell proliferation was increased by the co-presence of the two mycotoxins whereas 2-day cell growth did not statistically differ from the control. P4 production was inhibited by the combined treatments compared to the control but was not significantly different compared with either DON alone or α-Zol alone. Particularly for experiment 3, co-exposure of DON and α-Zol showed effects on steroidogenesis more similar to those observed after exposure to α-Zol alone. This fact could lead to the conclusion that the effects observed after co-exposure to DON and α-Zol are due to the action of α-Zol alone rather than to a real interaction occurring between the two mycotoxins.

The present study demonstrated that *in vitro* exposure to DON and α-Zol, individually or in combination, affected cell proliferation and steroidogenesis in bovine LGGC suggesting a possible involvement of these fusariotoxins on reproductive disorders in cattle.

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CHAPTER 5

General Discussion

5. General discussion

Mycotoxin contamination is estimated to be a relevant cause of economic loss and health effects in domestic animals (Gbore, 2009, Gbore et al., 2012). There are many studies on the effects of *Fusarium* mycotoxins on growth rates and metabolism in livestock but less research has been focused on the effects of *Fusarium* mycotoxins on reproduction (Gbore and Egbunike, 2008). The role of zearalenone (ZEA) and its metabolites as endocrine disruptors is well-established in many domestic animals whereas the action of deoxynivalenol (DON) on reproductive system needs further elucidation. In comparison to monogastric species, ruminants are generally considered more resistant to the adverse effects caused by contamination of feeds with mycotoxins because of the presence of rumen (Fink-Gremmels, 2008). However in cows, infertility, reduced milk production, and hyperestrogenism have been associated with exposure to ZEA (D'Mello et al., 1999; Minervini and dell'Aquila, 2008). Only few *in vivo* and *in vitro* studies were carried out in order to clarify the effects of DON and ZEA on reproduction in cattle (Weaver et al., 1986 a,b; Minervini et al., 2001; Takagi et al., 2008). In the present study the *in vitro* effects of DON and ZEA metabolites on reproductive function in cattle were investigated. We evaluated if DON and ZEA metabolites can impact cell proliferation, steroidogenesis (estrogen, E2 and progesterone, P4) and gene expression (CYP11A1 and CYP19A1 mRNA abundance) using primary GC from small-follicle (< 5 mm) (SMGC) (chapter 3) and cell proliferation and steroidogenesis using primary GC from large-follicle (8 to 22 mm) (LGGC) (chapter 4).

Based on our results on cell proliferation DON had no effects on cell growth in SMGC (chapter 3), however a slight trend to reduce cell number was demonstrated in LGGC (chapter 4). Results on steroidogenesis in SMGC and in LGGC are in agreement and demonstrated that DON is able to reduce dramatically E2 and P4 levels mainly at high concentration (3.3 μM) and in presence of IGF1. On the contrary when IGF1 was not present, DON had no effects on hormone production. Our *in vitro* results of DON on steroidogenesis are in agreement with previous study by Ranzenigo et al. (2008) while Medvedova et al. (2011) reported different effects. However the culture conditions applied in Medvedova et al. (2011) were different compared to Ranzenigo et al. (2008) and our studies. It is well known that DON inhibits protein synthesis by interfering with the 60S ribosomal subunit and determining translational inhibition (Pestka, 2010; Pinton and Oswald, 2014). Since hormone production requires *de novo* synthesis of several enzymes which catalyzes the different steps in the pathways of steroid biosynthesis (Hanukogl, 1992) the inhibition of protein synthesis by DON may explain our results. The lack of

effects of DON in absence of IGF1 needs further investigations. Since IGF1 is a potent inducer of the steroidogenesis pathway (Spicer and Chamberlain, 1998; Spicer et al., 2002) in its absence the rate of proteins synthesis involved in hormone production could be minimal (Mani et al., 2010; Monga et al., 2011) and therefore DON effects cannot be observed. It can be also postulated that DON could interfere with molecular pathways activated or blocked by IGF1.

CYP11A1 and CYP19A1 are enzymes involved in the steroidogenesis pathway and convert cholesterol into pregnenolone and testosterone into estrogens respectively (Wood and Strauss, 2002; Payne and Hales, 2008). We found that DON at 3.3 μM in presence of IGF1 significantly up-regulated CYP11A1 mRNA abundance while CYP19A1 mRNA was increased but not at statistical level. Interestingly previous studies (Gray and Pestka, 2007; Gray et al., 2008) demonstrated that DON is able to promote stability of several mRNAs interfering with post-transcriptional processes and avoiding their rapid degradation. This phenomenon might explain the increase of CYP enzymes mRNA levels after DON exposure although the observed reductions of steroids release.

SMGC cultured with FSH but in absence of IGF1 and exposed to DON showed a significant increase in CYP19A1 mRNA level while no effects were observed on CYP11A1 compared to cells cultured in presence of only FSH. The up-regulation of CYP19A1 mRNA is likely related to the ability of DON to stabilize mRNA as explained above. Monga et al (2011) demonstrated that FSH is able to increase CYP19A1 mRNA while the effects on CYP11A1 are minor. It can be postulated that the presence of FSH in culture medium may induce CYP19A1 gene transcription and the resulting mRNA is stabilized by the presence of DON while the induction of FSH on CYP11A1 was weak making the effects of DON not clearly visible.

In the present study the *in vitro* effects of α-Zol, one of the major ZEA metabolites, on cell proliferation, steroidogenesis and gene expression were also investigated in SMGC (chapter 3) and in LGGC (chapter 4).

Based on our results α-Zol had no effects on cell proliferation whereas was found to inhibit both E2 and P4 production in SMGC at concentration ranging from 0.09 to 3.1 μM in presence of IGF1. Our results on gene expression in presence of IGF1 demonstrated that α-Zol had no effects on CYP11A and CYP19A1 mRNA abundance in SMGC. The lack of effects at mRNA abundance level and the simultaneous alteration of hormone production might lead to the conclusion that α-Zol in presence of IGF1 acts on steroid release through a different mechanism. On the contrary in absence of IGF1, α-Zol at 3.1 μM had inhibitory effects on cell numbers whereas did not impair steroidogenesis in SMGC. In absence of IGF1 effects on CYP11A1 and CYP19A1 mRNA abundance were not observed confirming the lack of effects

on steroid production after exposure to α-Zol. However, Tienmann et al. (2003) showed that α-Zol treatment at 5 μM, was able to increase the protein expression of CYP11A1 in porcine GC although no differences at mRNA level were observed as well as increase in hormone production. This leads to the conclusion that the lack of effects on mRNA abundance does not exclude the possibility of interference of α-Zol on protein expression of cytochromes P450.
In LGGC α-Zol had no effect on steroidogenesis in presence of IGF1 and FSH, but when IGF1 was not present α-Zol showed an inhibitory effect on P4 production at a high concentration (3.1 μM).
As already demonstrated (Gregoraszczuk et al., 2003; Shimizu et al., 2012), the differences in responsiveness of SMGC vs. LGGC may be due to the intrinsic difference between the two cell types and their differentiation state. In the present study SMGC and LGGC were isolated from small and large ovarian follicles respectively, selected on the basis of their diameter. This size classification was based on previous observations indicating that: 1) follicles ≥ 8 mm in diameter have much greater androstenedione and E2 concentrations than small follicles (Stewart et al., 1996; Spicer et al., 2001), 2) follicles that are destined to ovulate average 10 ± 2 mm surface diameter and selection of dominant follicles occurs at about 8 mm in diameter (Beg and Ginther, 2006), and 3) similar classifications have been used previously to inventory follicles during bovine estrous cycles (McNatty et al., 1984; Spicer et al., 1986).
Moreover, many studies (Spicer et al., 1993; Spicer et al., 2002; Spicer et al., 2011) reported difference responses and sensitivity between SMGC and LGGC to the action of FSH and IGF1. Spicer et al. (1994) demonstrated that GC from large follicles have a greater number of IGF1 receptors than cells from small follicles (Spicer et al., 1994) and that LGGC are able to produce more E2 and P4 compared to SMGC (Spicer et al., 2002).
Considering our finding it can be postulated that α-Zol is able to interfere with GC altering different cell parameters and that the types and the intensity of the responses strictly depend on the differentiation state of the cells. However, the role of α-Zol and its mechanism of action on cell growth, E2 and P4 release and gene expression in bovine GC need to be further investigated.
To date only few studies were carried out in order to assess the *in vitro* effects and the mechanism of action of β-Zol. In the present study the effects of β-Zol were investigate only in SMGC (chapter 3). We demonstrated that β-Zol at 31 μM had inhibitory effects on cell numbers both in presence and absence of IGF1 while stimulated E2 and P4 production only when IGF1 was not present. Our results on cell proliferation are in agreement with previous studies (Tiemann et al., 2003 a,b; Minervini et al. 2006) and similar effects on steroidogenesis were reported by Frizzel et al. (2011) on H295R cells. Interestingly in our study CYP19A1 was up-regulated in absence of IGF1, supporting the increase of E2

production. However the mechanism of action of β-Zol on steroid production needs further clarification.
Generally our results suggested that α-Zol and β-Zol might act on GC through different mechanisms of action and at different levels as confirmed by a recent study (Molina Molina et al., 2014). α-Zol and β-Zol tested at the same concentrations (0.09, 0.31 and 3.1 μM) showed that α-Zol impacted GC proliferation and steroid production whereas β-Zol had no effects. Therefore, the present study confirmed the stronger estrogenic potency of α-Zol compared to β-Zol as previously reported (Malekinejad et al., 2006; Minervini et al., 2006; Frizzel et al., 2011).
Since the co-occurrence of mycotoxins in cereal grains and animal feed is a common feature of several survey raising the possibility of toxicological interactions (Zinedine et al., 2007, Minervini and Dell'Aquila, 2008), SMGC and LGGC were exposed to DON, α-Zol and β-Zol at different combinations. Previously Ranzenigo et al. (2008) investigated the combined effects of DON (0.03 μM) and α-Zol (0.09 μM) on SMGC in pigs. In our study on SMGC (chapter 3) effects on cell proliferation were consistent with findings of Ranzenigo et al. (2008) whereas results on E2 and P4 production were in disagreement. However, the concentrations of exposure and the different species used in the experiments could explain the differences found in the results. Generally our results showed that combine exposure to DON and α-Zol (at low concentration and in presence of IGF1) had significant additive inhibitory effects on E2 production in SMGC (chapter 3) whereas in LGGC (chapter 4) had no effect on steroidogenesis but increased cell growth.
When DON and α-Zol were combined in absence of IGF1 and at high concentration had additive stimulatory effects on steroidogenesis and reduced cell numbers in SMGC (chapter 3) whereas in LGGC (chapter 4) increased the 1-day cell proliferation and inhibited P4 production.
Some of the differences obtained in these experiments could be explained by the presence of IGF1 and its known ability to stimulate steroidogenesis in GC (Spicer and Chamberlain, 2002). Moreover, the different capacity to respond to the stimuli between SMGC and LGGC due to their rate of differentiation could be a further explanation.
α-Zol and β-Zol, when combined and in presence of IGF1, showed synergic inhibitory effects on E2 release whereas had not effects on P4 production and cell proliferation. Our results differ from Tatay et al. (2014) which reported cell number decrease of CHO-K1 cells exposed to a mixture of α-Zol and β-Zol at higher doses.
After exposure to DON at 3.3 μM combined with β-Zol at 3.1 μM the resulting effects were comparable to those obtained after exposure to DON alone.

However β-Zol at high concentration (31 μM) when combined with DON had antagonist effects to DON increasing the hormone production.
Based on our results, the effects on CYP11A1 mRNA abundance after exposure to α-Zol or β-Zol combined with DON are more similar to the effects observed after exposure to DON alone. Therefore, these effects are likely due to the presence of DON rather than to a real interaction occurring between the two mycotoxins.
Although the mechanism of action of these interactions is still not clear and needs further investigation, this study demonstrated that the co-exposure to mycotoxins may cause combined effects that may be additive, synergistic and antagonist.
In conclusion, the results of this thesis demonstrated that *Fusarium* mycotoxins, alone and combined, had direct effects on bovine GC. Under farm conditions, chronic exposure to low doses of mycotoxins is a common feature, making the effects on target organs difficult to be detected (Korosteleva et al., 2009). For this reason, changes in animal reproductive performance are possible subtle signs of exposure to low levels of mycotoxins in feed (Morgavi and Riley, 2007). Therefore, the effects here reported should be taken into consideration in reproductive failures in cattle.

5.1. References

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CHAPTER 6

Summary

6. Summary

The global occurrence of mycotoxins is considered to be the major risk affecting human and animal health. Fusarium mycotoxins, such as deoxynivalenol (DON), T-2 toxin, zearalenone (ZEA) and fumonisins, are the major mycotoxins occurring throughout the world in cereal grains and animal feed.
Previous studies have reported the effects on reproductive system caused by exposure to Fusarium mycotoxins in domestic animals.
However, so far there is a lack of information on the potential effects of these mycotoxins on reproduction in ruminants.
Therefore, the purpose of this study is to evaluate the impact of DON and ZEA major hydroxylated metabolites, α-zearalenol (α-Zol) and β-zearalenone (β-Zol), on bovine reproductive activity using primary granulosa cells (GC) derived from small (SMGC) and large (LGGC) ovarian follicles.
SMGC and LGGC show differences in responsiveness to FSH and IGF1, due to their differentiation state. In particular LGGC have a greater number of IGF1 receptors and are able to produce more steroid hormones compared to SMGC.
Two different studies were carried out.
In the first study the effects of DON, α-Zol and β-Zol, alone and combined, on cell proliferation, steroidogenesis and gene expression were evaluated on SMGC. Bovine ovaries were collected from a slaughterhouse and SMGC were obtained aspirating aseptically small ovarian follicles (1 to 5 mm). SMGC were cultured for 2 days in medium containing 10% fetal bovine serum followed by 1 or 2 days in serum free medium without (control) or with added treatments. At the end of the experiments, numbers of GC were determined using a Coulter counter and the concentration of estradiol (E2) and progesterone (P4) was evaluated *via* radioimmunoassay. For RNA experiments, SMGC were lysed, RNA extracted and RNA quantity determined spectrophotometrically. Real-time RT-PCR was used to clarify the effects of DON, α-Zol and β-Zol on gene expression of P450scc (CYP11A1) and aromatase (CYP19A1). Cell proliferation was negatively affected after exposure to β-Zol at 31 μM and after exposure to α-Zol (3.1 μM) alone and combined with DON (3.3 μM). Steroidogenesis was differently affected by tested mycotoxins: DON and α-Zol had inhibitory effects while β-Zol at high concentration (31 μM) stimulated steroid production. DON (3.3 μM) and β-Zol (31 μM) increased GC CYP19A1 mRNA abundance. CYP11A1 mRNA abundance was stimulated by DON, alone and combined with α-Zol and β-Zol, whereas was inhibited by β-Zol alone. Results on cell proliferation, steroidogenesis and gene expression were affected by the presence or absence of IGF1.

The second study was conducted to evaluate the impact of DON and α-Zol, on cell proliferation and steroidogenesis of bovine LGGC. LGGC were obtained aspirating aseptically large ovarian follicles (8 to 22 mm) and were cultured as described above. At the end of each experiment, cell numbers were evaluated using a Coulter counter and steroid levels were determined via radioimmunoassay. Three different experiments were performed using different dosages of DON and α-Zol and in different combinations. DON strongly inhibited P4 and E2 production mainly at high dose (3.3 μM). α-Zol alone and in combination with DON impacted cell proliferation increasing cell growth.

In conclusion this thesis demonstrates that DON and ZEA metabolites have direct effects on *in vitro* bovine GC impairing cell proliferation, steroid production and gene expression and also provides information on the interaction between DON and ZEA metabolites in GC.

These results could be useful to clarify and to better understand the possible involvement of these Fusarium mycotoxins on reproductive disorders in cattle.

CHAPTER 7

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