Cystic ovarian follicles in the high yielding dairy cow post partum

Role of metabolic and hormonal adaptations in the pathogenesis

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<tr>
<td>A₄</td>
<td>Androstenedione</td>
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<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>BCS</td>
<td>Body Condition Score</td>
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<td>BHB</td>
<td>β-OH-butyrate</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>COD</td>
<td>Cystic Ovarian Disease</td>
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<td>COF</td>
<td>Cystic Ovarian Follicle - Cysteuz Ovariële Follikel</td>
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<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<td>CYST</td>
<td>Cystic lactations</td>
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<tr>
<td>DF</td>
<td>Dominant follicle</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<tr>
<td>DNA</td>
<td>Deoxyribo Nucleic Acid</td>
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<tr>
<td>cDNA</td>
<td>copy DNA</td>
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<tr>
<td>E₂</td>
<td>Oestradiol-17β</td>
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<tr>
<td>EDTA</td>
<td>Ethylene Diaminetetra Acetic Acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immuno-Sorbent Assay</td>
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<td>ER-β</td>
<td>Oestrogen Receptor β</td>
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<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<td>GnRH</td>
<td>Gonadotrophin-Releasing Hormone</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
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<td>IGF-2</td>
<td>Insulin-like Growth Factor 2</td>
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<tr>
<td>IGFR-1</td>
<td>Insulin-like Growth Factor Receptor 1</td>
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<tr>
<td>IGFR-2</td>
<td>Insulin-like Growth Factor Receptor 2</td>
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<tr>
<td>IR-A</td>
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<tr>
<td>IR-B</td>
<td>Insulin Receptor B</td>
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<tr>
<td>IVF</td>
<td>In Vitro Fertilization</td>
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<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
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<tr>
<td>LHR</td>
<td>Luteinizing Hormone Receptor</td>
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<tr>
<td>MMP</td>
<td>Matrix Metallo Proteinase</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>NADP+/NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
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<td>NEB</td>
<td>Negative Energy Balance</td>
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<td>NEFA</td>
<td>Non-Esterified Fatty Acids</td>
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<td>OA</td>
<td>Oleic Acid</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<td>OV</td>
<td>Ovulatory lactations</td>
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<tr>
<td>P₄</td>
<td>Progesterone</td>
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<td>P₄ supra</td>
<td>Suprabasal progesterone</td>
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<tr>
<td>PA</td>
<td>Palmitic Acid</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCOS</td>
<td>PolyCystic Ovary Syndrome</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>pp</td>
<td>post partum</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>r</td>
<td>Correlation Coefficient</td>
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<tr>
<td>RIA</td>
<td>Radio Immuno Assay</td>
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<td>RNA</td>
<td>RiboNucleic Acid</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>RT</td>
<td>Reverse Transcription</td>
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<tr>
<td>SA</td>
<td>Stearic Acid</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<td>US</td>
<td>Ultrasound</td>
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CHAPTER 1

Introduction
Over the past few decades, milk yield per dairy cow has increased considerably due to continuing genetic selection and improvement of nutrition and herd management. Simultaneously with this selection for production characteristics, dairy cow fertility has declined significantly (Lucy, 2001; Butler et al., 2003). Reproductive performance is however an important factor in determining dairy herd profitability. Economically, it is most beneficial if a cow calves each year (Dijkstraizen et al., 1997; Huirne et al., 2002). In addition, the cow’s reproductive performance plays an important role in culling decisions (Rajala-Schultz and Gröhn, 2001). So, a good reproductive performance positively affects a cow’s active herd life and plays an important role in dairy herd economics.

Fertility is a very complex process and the final outcome is the result of a close and well orchestrated interaction between hypothalamus-pituitary-ovary-uterus. The complexity of this process indicates that any factors which interfere with the functioning of one or more of the organs involved, will also influence the overall fertility outcome. The reduced fertility, observed in modern high yielding dairy cows, is most likely due to alterations at several consecutive steps in the reproductive process (Lucy, 2001). The initial step of follicle growth and development is already hampered since nearly half of the Belgian dairy herd population suffers from ovarian dysfunctions during the early postpartum period. These dysfunctions cause a delay in resumption of normal ovarian cyclicity after calving (Opsomer et al., 1998), which affects fertility and conception later on (Thatcher and Wilcox, 1973; Shresta et al., 2004). Therefore, occurrence of these dysfunctions will negatively affect the dairy cow’s reproductive performance.

One of the most common ovarian dysfunctions during the postpartum period is formation of a cyst after ovulation failure (Youngquist, 1986; Day, 1991b; Lopez-Diaz and Bosu, 1992; Laporte et al., 1994, Opsomer et al., 1998). In cattle, cystic ovarian follicles (COF) are generally defined as large (> 2.5 cm) anovulatory follicles that persist on the ovary for at least 10 days in the absence of a corpus luteum. Despite the condition of persistence for 10 days or more, cysts are however frequently replaced by new ones (Cook et al., 1990; Hamilton et al., 1995). Cysts most commonly occur during the first 60 to 90 days postpartum (Refsdal, 1982; Bartlett et al., 1986; Laporte et al., 1994) when they can be regarded as a transitory step from acyclicity during pregnancy to cyclicity after parturition. This is similar to horses, where polycystic ovaries are very common in the early spring when there is a transition from winter
anoestrus to the breeding season. Although the majority of these early cysts (± 60 \%) regresses spontaneously after a variable period of time (Kesler and Garverick, 1982; Refsdal, 1982; Day 1991a,b), interference with ovarian cyclicity occurs, and depending on the time postpartum, this will be accompanied by signs like anoestrus or irregular oestrus intervals. On average, the lactational incidence of COF is 10-13\% (Erb en White, 1981; Bartlett et al., 1986).

The occurrence of ovarian cysts is not limited to cows. In several species (pig, rabbit, rat, sheep) the persistence of one or more large follicles on the ovary during a prolonged period of time has been reported (Leathem, 1958; Thorsoe, 1962; Ryan and Raeside, 1991; Lopez-Diaz and Bosu, 1992). Depending on the species, cysts can be considered as being either physiological or pathological, depending on the time of occurrence. Also in women, the polycystic ovary syndrome (PCOS) is a common pathology causing anovulation (Yen, 1999) and COF is often, justified or not, compared to this syndrome. Despite the occurrence of ovarian cysts across species, there seem to be as many differences as similarities. Overall, it has become clear though that ovarian cysts are the result of a disruption/malfunctioning of the hypothalamic-pituitary-gonadal axis. The exact pathogenesis does however differ between species and remains unclear in cattle. It is generally accepted that in cows, COF have a multifactorial etiology, in which genetic, phenotypic and environmental factors are involved (reviewed by Kesler and Garverick, 1982; and by Peter, 2004). The common occurrence in high yielding dairy cows, and the phenotypic (Laporte et al., 1994; Beam, 1995; Rajala and Gröhn, 1998; Hooijer et al., 2001) and genotypic link (Hooijer et al., 2003) with milk, fat and protein yield clearly indicate that COF formation is associated with a high level of production.

A high milk yield may promote the development of COF through the metabolic adaptations that occur to sustain the animal’s high level of production. During the early postpartum period, the high yielding dairy cow has an energy deficit (negative energy balance (NEB)) due to the imbalance between energy intake through feed and energy expenditure through milk yield. This NEB is a risk factor for ovarian dysfunctions (Opsomer et al., 2000), apparently through the hormonal and metabolic adaptations which occur. These altered hormone and metabolite concentrations hamper follicle development by acting on the hypothalamic-pituitary axis and possibly on the ovaries as well (Diskin et al., 2003; Lucy, 2003). While it is obvious that a severe NEB leads to atresia of the dominant follicle (Staples et al, 1990; Beam and Butler; 1999; Butler, 2003; Diskin et al., 2003), the role of the NEB in
cyst formation remains inconclusive. International publications on the subject are limited and yield contradictory results (Huszenicza et al., 1988; Beam, 1995; Sovani et al., 2000; Zulu et al., 2002) indicating the need for further research on the role of NEB in the pathogenesis of ovarian cysts. A better understanding of how the NEB affects cyst formation will help to determine strategies for the prevention of cysts and offer opportunities to optimize dairy herd fertility and breeding programs.

Besides a role for metabolism in the pathogenesis, genetics seem to be involved as well. As mentioned before, COF and production are genetically linked (Hooijer et al., 2003) and within the dairy herd population, certain animal lines are genetically predisposed to develop COF (Kirk et al., 1982; Cole et al., 1986; Hooijer et al., 2001). Knowledge on the genetic background of COF is however still very limited. The heritability is rather low (0.07 to 0.12) and there is no clear indication which gene(s) is(are) involved. In addition, cyst formation is unpredictable and an animal does not necessarily develop a cyst in every lactation. Therefore, it is difficult to study differences in gene expression at cow level. However, at the level of the ovary, studying gene expression in cystic follicles in comparison to normal dominant follicles could be valuable. Although this approach does not demonstrate a cause-effect relationship, it may indicate which genes are likely candidates to be involved in the pathogenesis of cysts and could be of interest for future research. In women, this approach has helped to elucidate which genes are linked to PCOS (Franks et al., 1997; Urbanek et al., 1999).

Up to ovulatory size the development of COF and normal follicles appears very similar (Beam, 1995; Calder et al., 2001), so differences in gene expression, if present, are most likely subtle. To be able to detect such differences, the use of fine-scale, accurate and specific quantification methods like real-time PCR is necessary (Ding and Cantor, 2004). This method does however require the selection of a certain number of candidate genes. Based on the importance of LH, insulin and the insulin-like growth factors for follicular growth and ovulation (Robker et al., 2000; Butler, 2003; Butler et al., 2004), the genes encoding for their receptors, are likely candidates to be involved in COF formation.

Identification of the gene(s) that promote the development of COF, would make it possible to genetically screen bulls and cows for COF prior to their use in artificial insemination programs and other assisted reproductive technologies.
References

- Erb HN, White ME, 1981. Incidence rates of cystic follicles in Holstein cows according to 15-day and 30-day intervals. Cornell Vet 71, 326-331


Aims of the study
The first aim of the present thesis was to gain more insight in the role of the negative energy balance in cyst formation, and more specifically how elevated metabolite concentrations may affect follicular growth and development at the ovarian level. The second aim was to determine whether gene transcription levels of receptors for certain hormones, important for follicular growth and development, differ between cystic follicles and normal dominant follicles, and could therefore be likely candidates to be involved in the pathogenesis of COF.

In order to achieve these scientific aims, the following experiments were performed:

1. Determination of hormonal and metabolic profiles of high yielding dairy cows prior to ovarian cyst formation or first ovulation post partum.

2. A study of the effect of NEB-associated non-esterified fatty acid concentrations on follicular cell function and viability in vitro.


4. Determination of the gene transcription levels of insulin receptor isoforms A and B, insulin-like growth factor receptors I and II, and luteinizing hormone receptor in cystic ovarian follicles and normal dominant follicles.
Aetiology and pathogenesis of Cystic Ovarian Follicles in dairy cattle: a review
Cystic ovarian follicles (COF) are an important cause of subfertility in dairy cattle, as they extend the calving interval (Lee et al., 1988; Borsberry and Dobson, 1989, Fourichon et al., 2000). Prolongation of the calving interval and treatment costs of COF result in economic loss for the dairy farmer. In most of the literature, COF are referred to as Cystic Ovarian Disease (COD). However, this terminology should be revised since the emphasis on cystic follicles has shifted over time. In the 1940’s, the presence of cystic follicles on the ovaries was mainly associated with nymphomania and a bull-like appearance in cows (Cassida et al., 1944; Garm, 1949), which are clear clinical signs of a state of “disease”. Over the past decades, dairy herd management and economics have evolved to a situation in which normal functioning of the ovaries in the postpartum period is utterly important. During this period, cystic follicles are rather common, and generally occur without obvious clinical signs. Normal ovarian cyclicity is however delayed and these cysts should therefore be regarded as COD, despite the absence of signs of disease in the majority of cases. In addition, after a variable period of time cysts can become non-steroidogenic and then they no longer interfere with cyclicity (Dobson et al., 2000; Noble et al., 2000). Consequently, at the time the non-steroidogenic cyst is observed, no other clinical abnormalities are present.

Conclusively, in the present-day dairy herd health programs “cysts” are often diagnosed in the absence of clear clinical signs. Therefore the term “Cystic Ovarian Disease” does no longer seem appropriate and should be replaced by the term “Cystic Ovarian Follicle(s)” which does not necessarily implicate a state of disease. In this review, we will therefore use COF instead of COD. We prefer to use COF instead of “ovarian cysts”, because the former term indicates that it is the ovarian follicle(s) and not any other ovarian tissue that becomes cystic.

**Definition**

Cystic ovarian follicles develop when one or more follicles fail to ovulate and subsequently do not regress but maintain their growth and steroidogenesis. They are defined as follicle-like structures, present on one or both ovaries, with a diameter of at least 2.5 cm during a minimum of ten days in the absence of luteal tissue (Kesler and Garverick, 1982;
Youngquist, 1986; Day 1991a; Woolums and Peter, 1994a). It has become clear though that this definition needs to be revised. First, the size limit is rather artificial as follicles might already become cystic at a smaller size, and dominant follicles ovulate on average at a size of 1.6 to 1.9 cm in dairy cows (Savio et al., 1990; Bleach et al., 2004; Lopez et al., 2004). Moreover, many researchers showed that COF are actually dynamic structures, which can regress and be replaced by new cysts (Kesler et al., 1980; Cook et al., 1990; Hamilton et al., 1995; Yoshioka et al., 1996). So the required individual persistency of ten days is questionable. In addition, in practice veterinarians generally do not have the opportunity to perform a second examination of an animal ten days after the initial diagnosis of COF to fulfill all the terms of the definition. The absence of a corpus luteum is another requirement, which is not always fulfilled (Al-Dahash and David, 1977). Non-steroidogenic cysts which are hormonally inactive do not influence the normal oestrous cycle, so they can occur together with a corpus luteum. Therefore, recent research articles define COF differently and perhaps more logically (Hamilton et al., 1995; Gümen et al., 2002; Zulu et al., 2002; Hatler et al., 2003), although a generally accepted definition is still lacking, which can also be attributed to the heterogeneity (type of cyst, time of occurrence, clinical signs) of the cysts.

Based on the current knowledge and recent literature, COF may be defined as follicles with a diameter of at least 2 cm that are present on one or both ovaries in the absence of any active luteal tissue and that clearly interfere with normal ovarian cyclicity.

Macroscopically, cysts can be subdivided into follicular and luteal cysts, which are considered to be different forms of the same disorder (Opsomer et al., 1997). The former probably evolve into the latter and consequently many intermediate forms exist with limited or extensive luteinization of the follicle wall. Determination of progesterone concentrations in blood plasma, milk or milk fat can help to make a distinction between the two types. Follicular cysts secrete little or no progesterone while luteal cysts clearly do (Garverick, 1997). However, the threshold values used in literature differ a lot (Leslie and Bosu, 1983; Nakao et al., 1983; Booth, 1988; Dinsmore et al., 1989; Douthwaite and Dobson, 2000), which makes it difficult to set a concentration threshold. And as mentioned, the many intermediate forms do not allow for a clear identification of cyst type. So classification is not easy and is subject to personal interpretation. Ultrasound can be useful in supplying extra information. Follicular cysts have a thin wall (≤ 3mm) and the follicular fluid is uniformly anechogenic, while luteal cysts have a thicker wall (> 3mm), which is visible as an echogenic
rim. Also, the latter often have echogenic spots and web-like structures in the follicular fluid (Edmondson et al., 1986; Jeffcoate and Ayliffe, 1995).

Follicular cysts initially continue to produce oestrogens in the absence of other follicles >5mm on ultrasound (Scott and Dobson, 1997). After a variable period of time oestrogen production may cease. The cyst becomes non-steroidogenic without luteinizing, thereby allowing a new follicular wave to emerge and follicles to grow beyond 5 mm (Dobson et al., 2000; Noble et al., 2000).

**Incidence and Symptoms**

Cystic ovarian follicles can occur at different times throughout lactation. The incidence varies between 6 and 30% (Erb and White, 1981; Kesler and Garverick, 1982; Refsdal, 1982; Bartlett et al., 1986; Youngquist, 1986; Day, 1991b; Lopez-Diaz and Bosu, 1992; Laporte et al., 1994, Opsomer et al., 1998). The diagnosis of COF is most often made during the first 60 days post partum (Kesler and Garverick, 1982; Bosu and Peter, 1987; Day 1991a,b), mainly because of the close monitoring of cow fertility during this period. The majority of all cysts occur throughout this stage (Refsdal, 1982; Bartlett et al., 1986; Laporte et al., 1994). The self-recovery percentage of these early cysts is 60-65% (Kesler and Garverick, 1982; Refsdal, 1982; Day 1991a,b). Despite this high self-recovery rate, the importance in dairy cow fertility is not negligible (López-Gatius et al., 2002). By delaying normal cyclicity, the time to first insemination increases and pregnancy rates after first insemination decrease (Shresta et al., 2004).

A genetic predisposition exists for COF (Kirk et al., 1982; Cole et al., 1986), but the heritability is rather low, being 0.07 to 0.12 (Lin et al., 1989; Uribe et al., 1995; Hooijer et al, 2001). However, the incidence in Dutch Holstein Friesian herds is actually increasing (Hooijer et al., 2001). Genetic selection to reduce the incidence of COF can be successful, despite the low heritability. By excluding bulls, which sired daughters with cysts, from breeding programs, cyst incidence was reduced by 50% over a 20-year period in a Swedish cattle population (Anonymous, 1978).

The clinical signs that accompany ovarian cysts are variable. Anoestrus often occurs, especially during the postpartum period and both with follicular and luteal cysts (Kesler and Garverick, 1982). Irregular oestrus-intervals, nymphomania, relaxation of the broad pelvic ligaments and development of masculine physical traits are other signs which may be present, especially later during lactation (Roberts, 1986; Youngquist, 1986). According to Youngquist
(1986) 80% of all cows suffering from COF are anoestrous while the remaining 20% are nymphomaniac, but this division seems too strict since many different signs may accompany cysts.

**Folliculogenesis and ovulation**

Before reviewing the existing literature on the pathogenesis of COF, normal follicle development and ovulation in cattle will be briefly discussed. Since COF are the result of an abnormality during follicle growth and/or ovulation, it is essential to know how normal follicle development and ovulation occur in cattle.

Follicle growth and development result from a complex interaction between the hypothalamic-pituitary axis and the ovaries. In addition, local factors in the ovary influence this process through paracrine/autocrine regulation. From the hypothalamus, Gonadotrophin Releasing Hormone (GnRH) is transported to the anterior pituitary where it stimulates the production and the release of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) (Roche et al., 1996). These latter two hormones stimulate follicular growth in the ovary.

Generally, the bovine oestrous cycle exhibits two or three waves of follicular growth (Savio et al., 1988). Each new follicle wave is preceded by an FSH surge (Ginther et al., 1999) since follicles require FSH support to grow beyond a size of 4 mm diameter. In a follicular wave, each follicle has the capacity to acquire dominancy, but the future dominant follicle starts to grow 6 h before the future subordinate ones and, therefore, has a small size advantage (Gibbons et al., 1997). Selection of the dominant follicle and deviation occur when the largest follicle reaches a size of \( \approx 8.5 \) mm (Ginther, 2000). At deviation, the largest follicle (dominant) continues to grow while the growth rate of the smaller follicles (subordinates) decreases. At this time the FSH-concentration has fallen below a critical level necessary to sustain follicle growth. However, the dominant follicle acquires even more LH-receptors in the granulosa cells, enabling the use of increased LH-concentrations for continued growth at the time of selection (Ginther et al., 1998). Also, the dominant follicle further reduces FSH release through a negative feedback by oestradiol and inhibin, thereby preventing the continued growth and development of any subordinate follicles (Ginther et al., 2000; Ginther et al., 2001).

In addition, the future dominant follicle exhibits changes in the Insulin-like Growth Factor (IGF)-System before the processes of selection and deviation. Decreased concentrations of binding proteins increase the bio-available fraction of IGF-1 and 2 in the follicular fluid of the
dominant follicle (Mihm et al., 2000; Austin et al., 2001; Beg et al., 2001). Both growth factors stimulate cell proliferation and oestradiol production and augment granulosa cell sensitivity for FSH (Spicer et al., 2001), thereby enabling the dominant follicle to further use the now very low FSH concentration and to continue growth. Other intrafollicular factors such as inhibin, activin and follistatin may (Stoebel and Moberg, 1982b; Martin et al., 1991; Austin et al., 2001) or may not (Lopez-Diaz and Bosu, 1992; Beg et al., 2001) play a role in the process of selection and deviation of the dominant follicle.

The dominant follicle will further grow and mature and will either ovulate, become atretic or develop into a cyst. To be able to ovulate, the dominant follicle must elicit a pre-ovulatory LH-surge through increased oestradiol production (Woolums and Peter, 1994a; reviewed by Mahesh and Brann, 1998).

**Pathogenesis of ovarian cysts**

Ovarian dysfunctions like cysts occur most often during the early postpartum period when there is a transition from the non-cyclic condition during pregnancy to the establishment of regular cyclicity. It is generally accepted that cystic follicles develop due to a dysfunction of the hypothalamic-pituitary-ovarian axis. This dysfunction has a multifactorial etiology, in which genetic, phenotypic and environmental factors are involved (Kesler and Garverick, 1982; Garverick, 1997; Peter, 2004). When discussing the pathogenesis of COF, a distinction may be made between a primary defect in the hypothalamus-pituitary and a primary defect at the level of the ovary in the follicle itself.

**Hypothalamic-pituitary dysfunction**

The most widely accepted hypothesis explaining the formation of a cyst is that LH release from the hypothalamus-pituitary is altered: the pre-ovulatory LH-surge is either absent, insufficient in magnitude or occurs at the wrong time during dominant follicle maturation, which leads to cyst formation (Day, 1991a; Lopez-Diaz and Bosu, 1992, Hamilton et al., 1995; Yoshioka et al., 1996) (Figure 1). This abberant LH release does not seem to be caused by a lower GnRH content of the hypothalamus, nor by reduced GnRH receptor numbers or LH content in the pituitary (Brown et al., 1986; Cook et al., 1991).

It is believed that an altered feedback mechanism of oestrogens on the hypothalamus-pituitary can result in an abberant GnRH and LH release and cyst formation. A GnRH/LH-
surge prematurely occurring during follicle growth, i.e. when no follicle capable of ovulation is present, can render the hypothalamus unresponsive to the feedback effect of oestradiol which results in the formation of ovarian cysts (Gümen et al., 2002; Gümen and Wiltbank, 2002). To restore the feedback mechanism, the hypothalamus needs to be exposed to progesterone (Ozturk et al., 1998; Gümen and Wiltbank, 2005a). Very recently, it was shown that a similar state of hypothalamic refractoriness to oestrogens and subsequent cyst formation can be achieved if the increase in progesterone after a spontaneous ovulation is prevented (Gümen and Wiltbank, 2005b). This physiological state of hypothalamic unresponsiveness to oestrogens seems to be present in the majority of cows with COF, as illustrated by the failure of an exogenous oestradiol treatment to elicit a timely LH-surge (Zaied et al., 1981; Refsal et al., 1987; Refsal et al., 1988; Dobson and Nanda, 1992). On the other hand, the refractoriness of the hypothalamus-pituitary for oestradiol in cows with COF seems to be a consequence rather than a cause of the disease. Removal of the cystic ovary by ovariectomy restores the feedback mechanism and the capacity of oestradiol to elicit an LH-surge, although the underlying mechanism is not known (De Silva and Reeves, 1988).

An altered feedback mechanism and GnRH/LH release may be attributed to factors interfering at the hypothalamic-pituitary level. Progesterone at suprabasal concentrations blocks the LH-surge, thereby inhibiting ovulation, but increases the LH pulse frequency (Stock and Fortune, 1993; Duchens et al., 1994). This results in an anovulatory, persistent follicle with a larger diameter and a longer lifespan than normal, and increased peripheral oestradiol concentrations (Stock and Fortune, 1993). These follicular and hormonal changes are very similar to observations made in cows with COF (Hamilton et al., 1995). Recently, Hatler et al. (2003) observed that at the time of diagnosis, most cysts are accompanied by suprabasal progesterone concentrations, which play a role in cyst turnover. These observations together with the similarities between persistent follicles, induced by suprabasal progesterone, and naturally occurring cysts, suggest a role for progesterone in the pathogenesis of COF.

Factors indirectly reducing GnRH/LH secretion like stress (Stoebel and Moberg, 1982a; 1982b; Dobson et al., 2000; Ribadu et al., 2000), intrauterine infections (Bosu and Peter, 1987; Peter et al., 1989) and seasonality (Emanuelson and Bendixen, 1991) are also considered to increase the risk of cyst formation.

In cystic cows, the formation of new cysts is accompanied by increased LH pulse frequencies and amplitudes (Cook et al., 1991; Hamilton et al., 1995). However hypersecretion of LH does not seem to be involved in cyst formation, but it may play a role in
cyst persistence (Hampton et al., 2003). Data obtained in sheep also dismiss an increased LH secretion as a primary cause of COF (Christman et al., 2000).

In conclusion, an aberrant LH-surge is likely the trigger for the development of COF. The abnormal LH release seems to be caused by an altered feedback mechanism of oestrogens on the hypothalamus-pituitary. The malfunctioning of the feedback mechanism can be caused by factors directly interfering at the hypothalamic-pituitary level or by an altered follicle growth and development disrupting the hypothalamic-pituitary-gonadal axis, as discussed below.

**Ovarian/Follicular dysfunction**

A primary dysfunction at the level of the follicle may disrupt the hypothalamic-pituitary-ovarian axis and cause the formation of COF (Figure 1). First of all, alterations in LH receptor expression and content may cause anovulation of the follicle. The LH-surge initiates a complex multi-gene, multi-step process in which timing is essential, finally leading to ovulation of the pre-ovulatory follicle (Robker et al., 2000). According to Kawate et al. (1990), FSH and LH receptor numbers in granulosa cells of cysts are decreased when compared to normal follicles, but this is contradicted by data from Odore et al. (1999) and Calder et al. (2001) who found similar receptor concentrations or higher levels of receptor mRNA expression, respectively. These discrepancies between studies may partly be explained by differences in methodology such as demonstration of the receptor itself or its mRNA, and the division of cysts into oestrogen-active and oestrogen-inactive. Still, such studies are incapable of clearly establishing a cause-effect relationship, since any detected changes may be primary or secondary to cyst formation. Therefore, in the same study Calder et al. (2001) studied developing “young cysts”. However, no significant differences in FSH/LH receptor mRNA were observed between these young cysts and dominant follicles, indicating that the increased LH mRNA expression in oestrogen-active cysts is a consequence rather than a cause of the cystic state. Young cysts were, however, studied in the presence of existing cysts, i.e. when the endocrine environment was already altered, and therefore the pathogenesis may differ from primary developing cysts.

Another receptor of interest is the oestradiol receptor β (ER-β). In rodents, the importance of this receptor in follicular growth and development has clearly been demonstrated (Wang and Greenwald, 1993; Robker and Richards, 1998) and its localisation in follicle cells throughout follicular development has been described in many mammals including cattle (Byers et al., 1997; Rosenfeld et al., 1999). More specifically, in rat ovarian follicles ER-β
mRNA expression precedes increased expressions of mRNAs for the LH receptor and specific steroidogenic enzymes (Bao et al., 2000). Therefore, alterations in expression of the ER-β might disrupt the local intra-ovarian paracrine/autocrine system, leading to an altered follicular development and steroidogenesis and finally formation of COF. However, this hypothesis is not supported by data from Calder et al. (2001) showing that ER-β mRNA expression was not altered in growing cysts. Odore et al. (1999) did, however, find decreased oestrogen receptor concentrations in follicular cysts, but the oestrogen receptor type was not defined, and once again this may be either cause or effect of the disorder.

Besides changes in receptor expression and content, alterations in steroidogenesis by the dominant follicle may also play a role in its cystic degeneration. After all, the dominant follicle has to elicit an LH-surge at the right time in its development by producing sufficient oestradiol. Oestrogen-active cysts show a higher expression of 3β-hydroxysteroid dehydrogenase mRNA, a steroidogenic enzyme (Calder et al., 2001), and cows developing a cyst have increased oestradiol concentrations during the early stages of follicular dominance (Beam, 1995). However, Calder et al. (2001) were unable to observe changes in mRNA expression of steroidogenic enzymes in the follicle wall of young growing cysts. They concluded that alterations of the endocrine system precede, and perhaps cause, the observed follicular alterations in cysts. In the study of Calder et al. (2001), young cysts did, however, develop in the presence of existing cysts, i.e. when the endocrine environment was already altered. As a consequence, the mechanism causing these “young cysts” to actually become cysts may differ from the mechanism(s) involved in primary cyst formation. The data from Beam (1995) suggest that steroidogenesis is enhanced during early development of future cysts, which may disrupt the hypothalamic-pituitary-gonadal axis. Through an increased positive feedback, LH release would be overstimulated and the final LH-surge would occur too early during follicle development. Due to immaturity, the follicle would not be able to respond with ovulation and may then become cystic.

Apart from changes in mRNA expression for certain receptors and steroidogenic enzymes, cell proliferation and apoptosis in the granulosa and theca interna cell layers also seem to be altered in cystic follicles. Early cystic follicles show an increase in apoptosis while cell proliferation is decreased (Isobe and Yoshimura, 2000a; 2000b). Although it is hard to establish a cause-effect relationship, alterations like these may disrupt normal follicle growth and steroidogenesis leading to cystic degeneration.

Recently, Imai et al. (2003) suggested that matrixmetalloproteinases (MMPs) could be involved in the formation of cysts: higher proMMP-2 and -9 levels were present in the
fOLLICULAR fluid of cysts than in the follicular fluid of normal dominant follicles. MMPs play a role in follicle wall remodelling and rupture at the time of ovulation (Smith et al., 1999, Robker et al., 2000) but hereto the inactive proMMP form needs to transformed to the active MMP form. This activation is triggered by the LH-surge (Robker et al., 2000). Since an aberrant LH-surge causes COF formation, the higher proMMP-2 and -9 levels in the follicular fluid of COF are most likely an indication of the lack of an LH-surge rather than a cause of COF formation.

Although studies focussing on differences between cystic and normal dominant follicles have greatly enhanced our knowledge, it is not possible to determine a cause-effect relationship. Therefore, future research should try to elucidate if, and what kind of, changes during follicle growth can interfere with normal follicle development and steroidogenesis finally leading to the formation of (a) cyst(s). However, due to the inability to predict the fate of a follicle (ovulation/atresia/COF), studying the follicular changes prior to natural cyst formation is almost impossible. Development of an accurate model for cyst induction which mimics the in vivo situation is therefore necessary, but extremely difficult. Perhaps an intensive monitoring of the growth and development of follicles which finally become cystic may reveal specific characteristics that allow for the classification of a follicle as a future cyst before it actually becomes one. This would offer the opportunity to identify follicular changes early on in cyst development.

Predisposing factors for COF

As mentioned before, COF are mainly observed in high yielding dairy cows during the first months post partum and milk yield is generally considered a risk factor (Emanuelson and Bendixen, 1991; Laporte et al., 1994; Beam, 1995; Rajala and Gröhn, 1998; Heuer et al., 1999; Hooijer et al., 2001; Lucy, 2001), although not all authors agree (Bartlett et al., 1986; Nanda et al., 1989). Moreover, besides the fact that COF are hereditary (see above), a genetic correlation between cysts and milk production traits was established, indicating that an ongoing selection for production parameters will increase the incidence of COF (Hooijer et al., 2001). What the genetic factor(s) is and how it promotes the formation of cysts is not known. However, the fact that cows do not develop a cyst during every lactation and during every ovarian cycle indicates that the gene(s) expression may be promoted by, or gains functional importance under, certain stressors, for example high milk yield and the associated negative energy balance (NEB) during the early postpartum period. At this time, energy
demands to sustain milk yield are higher than energy intake thus causing a NEB. This NEB is accompanied by several hormonal and metabolic adaptations, affecting ovarian function (Beam and Butler, 1999). Energy balance may be a more accurate parameter than milk yield to further elucidate the association between COF and production traits. Some animals can compensate for higher milk production through greater dry matter intake reducing the effect of milk yield on energy balance (Lucy, 2001). This could explain why not all authors (Bartlett et al., 1986; Nanda et al., 1989) observed a correlation between ovarian cysts and milk yield. However, when focusing on energy balance and the occurrence of COF, results still remain inconclusive. While Refsdal (1982), Sovani et al. (2000) and Zulu et al. (2002) observed a deeper NEB and increased mobilization of body reserves in cows developing cysts, Beam (1995) noticed that the nadir of the NEB occurred later post partum in cystic cows than in ovulatory cows. Moreover, cystic cows even mobilized less body reserves and derived a smaller percentage of their milk yield from body weight loss (Beam, 1995). Hooijer et al. (2003) were unable to find a more severe NEB, evaluated by the fat/protein ratio in milk, in cows with COF compared to ovulatory cows. However in an earlier study, Heuer et al. (1999) observed that a high fat/protein ratio, and, therefore, a more severe NEB, increased the risk of cyst occurrence. Data in sheep also suggest that an increased mobilization of body reserves, indicative for a deeper NEB, is linked with the occurrence of cystic follicles (Christman et al., 2000). Although a consensus is lacking, we conclude from literature that a link seems to exist between COF and the magnitude and/or duration of the NEB.

The possible underlying mechanism(s) is(are) also still unclear, but NEB may affect COF formation at both the level of the hypothalamus/pituitary and the ovary/follicle through associated hormonal and metabolic changes (Diskin et al., 2003; Lucy, 2003) (Figure 1). During NEB, circulating concentrations of IGF-1, insulin, glucose (Beam and Butler, 1999) and leptin (Block et al., 2001; Liefers et al., 2003) are reduced, while concentrations of metabolites such as non-esterified fatty acids (NEFA) (Rukkwamsuk et al., 2000) and β-hydroxybutyrate (BHB) are increased (Leroy et al., 2004). As mentioned earlier, the IGF-system plays an important role in follicle growth and development. Besides a direct effect, IGF-1 together with insulin indirectly stimulates follicular development through upregulation of the LH-receptor on granulosa cells (Davoren et al., 1986). Therefore, low systemic IGF-1 concentrations early post partum could contribute to anovulation and subsequent development of cystic follicles as shown by Zulu et al. (2002). However, data from Beam (1995) do not confirm this hypothesis. Also insulin itself is known to be a potent stimulator of follicle cell steroidogenesis and proliferation in vitro (Matamaros et al., 1990; Spicer and Echternkamp,
1995; Campbell et al., 1996; Gutierrez-Aguilar, 1997; Price and Silva, 1999) and *in vivo* (Simpson et al., 1994; Armstrong et al., 2002). As a result, reduced circulating insulin concentrations early post partum may play a role in ovarian dysfunction i.e. cyst formation. Besides low insulin concentrations, a general state of peripheral insulin resistance is present as well in high yielding dairy cows early post partum (Beck et al., 1983; Staufenbiel et al., 1992). Insulin resistance is regarded as an important factor in the pathogenesis of the PolyCystic Ovary Syndrome in women (Nestler, 1997; Nestler, 1998; Yenn, 1999) and COF have often been compared to this syndrome, justified or not. However, rather insulin insufficiency not insulin resistance has been observed in COF cows (Opsomer et al., 1999), indicating an altered interaction between glucose and insulin at the pancreatic level. In addition, in ewes it was not possible to induce cyst formation through establishment of a state of insulin resistance (Christman et al., 2000). Conclusively, IGF-1 and insulin are important stimulators of follicle growth and based on the limited number of publications on the subject, low concentrations of one or both of the hormones may contribute to the formation of COF. Further research should confirm whether or not this hypothesis is valid.

Leptin is a recently “new” hormone, produced by adipocytes, and is regarded as the ultimate factor linking metabolic status to reproduction (Barash et al., 1996). Depending on the metabolic state of the animal it has either a stimulatory effect or none at all on hypothalamic-pituitary function in cattle (Williams et al., 2002; Amstalden et al., 2005). Spicer (2001) hypothesized that above a certain threshold level, leptin acts as a trigger to initiate hypothalamo-pituitary gonadotropin secretion. Besides effects on the central nervous system, this hormone also seems capable of modulating ovarian function by acting directly on follicular cells. Both bovine granulosa and theca cells possess leptin receptors and *in vitro* leptin inhibits insulin stimulated steroidogenesis of granulosa and theca cells (Spicer et al., 1997; 1998). However, both basal, IGF-1 and LH-stimulated steroidogenesis and cell proliferation as well as insulin-stimulated cell growth are unaffected by lepoitin (Spicer et al., 1997; 1998; 2000). This indicates that in a low leptin environment (i.e poor body condition and poor nutrition), ovarian function is mainly regulated by gonadotropins and low insulin/IGF-1 concentrations. In a moderate to high leptin environment, as in obesity, leptin will limit ovarian steroidogenesis, stimulated by the high insulin/IGF-1 concentrations, to prevent overproduction (Spicer, 2001). In the postpartum dairy cow, a clear relationship between leptin profiles and first postpartum ovulation is lacking (Liefers et al., 2003), although a minimum permissive level of leptin seems required to induce the first postpartum
LH-surge (Huszenicza et al., 2001; Liefers et al., 2003). Therefore, leptin may play a role in cyst development.

According to Huszenicza et al. (1988) and Zulu et al. (2002), cows developing cysts have higher serum NEFA concentrations during the first week(s) post partum than ovulatory cows, although Beam (1995) was unable to observe this. Interestingly, in rats, elevated NEFA concentrations for 48 h can decrease insulin secretion by the β-cells of the pancreatic islets in response to a glucose challenge (Mason et al., 1999). Moreover, NEFA are cytotoxic for several cell types, including human granulosa cells (Shimabukuro et al., 1998; Cnop et al., 2001; Maedler et al., 2001; Mu et al., 2001; Lu et al., 2003; Ulloth et al., 2003). So (prolonged) exposure to high NEFA concentrations during periods of NEB may hamper follicle growth and development through these mechanisms, disrupting the complex endocrine system and promoting the formation of ovarian cysts. Besides NEFA, increased serum ketone concentrations also affect ovarian function (Huszenicza et al., 1988; Opsomer et al., 2000; Reist et al., 2000) indicating that these metabolites may be mediators of the negative effect of NEB on follicular development. High ketone concentrations increase the risk of cyst occurrence (Dohoo and Martin, 1984; Andersson et al., 1991) and consequently are likely candidates to be involved in the formation of COF.
Figure 1. Schematic representation of the pathogenesis of ovarian cysts and the possible pathways involved. An FSH surge stimulates the emergence of a new follicular wave, from which a single dominant follicle is selected at the time of deviation. Through a positive feedback loop oestradiol stimulates GnRH and LH pulsatility, which in turn support growth and development of the dominant follicle. Upon reaching preovulatory size, follicular steroidogenic activity reaches a peak and produces a preovulatory oestradiol surge. This surge
either fails to elicit a GnRH and subsequent LH-surge or the GnRH/LH-surge is delayed/mistimed. The dominant follicle, therefore, does not ovulate but, due to the ongoing LH pulsatility, continues to grow and becomes a cyst. The disruption of the hypothalamic-pituitary-gonadal axis can be caused by factors affecting the oestradiol feedback mechanism and GnRH/LH release at the hypothalamic-pituitary level (1) and/or by an aberrant follicle growth and development with alterations in receptor expression and steroidogenesis (2), leading to an altered oestradiol surge and feedback (3). Hypothalamic-pituitary function and follicular growth/development may be affected by NEB through metabolic/hormonal adaptations. In addition, in the situation of NEB, the expression of genetic hereditary factor(s) associated with COF may be promoted or the functional importance may increase, which in turn may affect follicle growth and hypothalamic-pituitary function.

**Conclusion**

Cystic ovarian follicles are one of the most frequent and important ovarian disorders in modern high yielding dairy cows that have been the subject of much research in recent decades. However, many aspects of the disease, and especially pathogenesis, remain unclear and inconclusive, as for example, illustrated by the lack of a clear definition. Especially the endocrine and follicular changes that precede cyst development are still unknown, mainly due to the heterogeneity and unpredictability of the disease. Studies aiming to elucidate the pathogenesis, have tried to do so by induction of cysts. This, however, may not mimic naturally-occurring cysts. Nevertheless such experiments have enhanced our knowledge about the endocrine and follicular changes that occur after cyst formation. Development of an accurate model mimicking the *in vivo* situation or identification of criteria to allow classification of a follicle as a future cyst before it actually becomes cystic, would be very valuable in studying the cellular and molecular changes that precede ovarian cyst formation.

Due to the genetic correlation with production traits and the high incidence of ovarian cysts during the period of NEB early post partum, future research should also focus on the effect of NEB-associated metabolic/hormonal changes and energy utilization on follicular development and steroidogenesis. Understanding how NEB affects cyst formation will help to optimize management and feeding practices in preventing the occurrence of COF.

Further research on cellular changes in follicular cysts may elucidate which genes show an altered expression pattern compared to normal dominant follicles and could therefore be involved in the primary development. Genetic knock-out models as well may help to determine which genes play a role in cyst formation. Identification of these genes would be an initial step in the process of identifying the hereditary factor(s), making it possible to
genetically screen bulls and cows for COF prior to their use in artificial insemination programs.
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Hormonal and metabolic profiles of high yielding dairy cows prior to ovarian cyst formation or first ovulation post partum

Modified from:

HORMONAL AND METABOLIC PROFILES OF HIGH YIELDING DAIRY COWS PRIOR TO OVARIAN CYST FORMATION OR FIRST OVULATION POST PARTUM
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Summary

The present study aimed to investigate the pathogenesis of cystic ovarian disease (COD) in high yielding dairy cows post partum (pp). Hormonal and metabolic profiles during the first 3 weeks pp as well as during the final week prior to ovulation/cyst formation, were compared between dairy cows that developed either an ovulatory follicle (OV) or a cyst (CYST) < day 60 pp. Thirty four lactations of 28 high yielding (9500 kg/305 days) Holstein-Friesian dairy cows were studied. Ovaries of cows were scanned twice a week from day 10 pp on, until ovulation/cyst formation. Milk yield data, body condition scores, and blood samples, for determination of oestradiol-17\(\beta\), insulin, \(\beta\)-OH-butyrate and non-esterified fatty acids, were collected simultaneously. Milk samples for progesterone analysis were collected daily. Four lactations were excluded from further analysis due to irregular pp ovarian cyclicity, excluding COD. Eight lactations (26.7%) developed a cyst, while 22 lactations ovulated < day 60 pp. Ovulation and cyst formation occurred at similar times pp. Metabolic and hormonal profiles did not differ between CYST and OV lactations during the first 3 weeks pp. In the final week prior to cyst formation/ovulation, insulin concentrations were lower in CYST than in OV lactations while no differences were observed for any of the other parameters tested. In two lactations, cyst formation was preceded by suprabasal progesterone and increased oestradiol-17\(\beta\) concentrations. These results suggest that cyst formation in high yielding dairy cows pp is associated with lower insulin levels but not with other distinct hormonal and metabolic alterations. However from this study, we can not exclude the involvement of subtle hormonal and metabolic changes in the pathogenesis of ovarian cysts. Suprabasal progesterone, and altered oestradiol-17\(\beta\) concentrations, seem to play a minor role in cyst formation.


Introduction

Cystic ovarian disease (COD) is an important ovarian dysfunction which alters and delays normal ovarian cyclicity in high yielding dairy cows. The pathogenesis of COD remains unclear, despite all the research that has been conducted. However, one constant in the pathogenesis is the lack of a pre-ovulatory LH-surge or the failure of this surge to occur at the right time during maturation of the dominant follicle (reviewed by Vanholder et al., 2002 and by Peter, 2004).

Most research focusing on the pathogenesis of COD has either been performed retrospectively in cows previously diagnosed with COD (De Silva and Reeves, 1988; Refsal et al., 1988; Yoshioka et al., 1996) or in studies in which cysts had been artificially induced (Refsal et al., 1987; Peter et al., 1989; Cook et al., 1990; Cook et al., 1991; Hamilton et al., 1995; Kaneko et al., 2002). Other articles mainly focused on the link between COD and milk yield (Gröhn et al., 1994; Laporte et al., 1994; Hooijer et al., 2001; López-Gatius et al., 2002; Hooijer et al., 2003). Although such kind of research has increased our knowledge tremendously, studying the period immediately prior to cyst formation in naturally occurring cases is more interesting to reveal possible hormonal and metabolic factors involved in its pathogenesis. Studies using this approach are however scarce (Beam, 1995; Zulu et al., 2002). Due to the inability to predict the occurrence of cysts, such studies are very labour intensive and the number of cystic animals obtained is usually limited.

Hormonal alterations during the final stages of follicle growth and maturation may trigger cyst formation. The dominant follicle’s fate, oestrogenic activity and period of dominance are controlled by LH: in the presence of a frequent LH-pulse pattern, the dominant follicle will produce sufficient oestradiol-17β (E$_2$) to induce a pre-ovulatory LH-surge and then ovulate (reviewed by Mihm et al., 2002). Progesterone (P$_4$) at so called suprabasal concentrations blocks the pre-ovulatory LH-surge but does not suppress LH-pulse frequency as normal luteal P$_4$ concentrations do. This results in an anovulatory, persistent follicle with a larger diameter and a longer lifespan than normal, accompanied by increased peripheral E$_2$ concentrations (Stock and Fortune, 1993). These follicular and hormonal changes are very similar to observations made in cows with COD (Hamilton et al., 1995). Moreover, 66% of COD cases were associated with suprabasal progesterone levels (P$_{4_{supra}}$) at the time of their diagnosis and a role for such levels in cyst turnover has been suggested (Hatler et al, 2003).
In the absence of a mature follicle, increased E$_2$ concentrations can trigger a pre-ovulatory LH-surge without subsequent ovulation and P$_4$ production (Gümen et al., 2002; Gümen and Wiltbank, 2002). Since P$_4$ exposure of the hypothalamus is necessary to reinitiate the E$_2$ feedback mechanism (Gümen and Wiltbank, 2002; 2005), a new dominant follicle then fails to trigger a LH-surge and becomes cystic (Gümen et al., 2002; Gümen and Wiltbank, 2002). The hypothalamic unresponsiveness is very similar to the situation in naturally occurring COD where an E$_2$ injection fails to elicit an LH-surge (De Silva and Reeves, 1981).

Besides hormonal imbalances, metabolic alterations are suggested to play a role in cyst formation (Zulu et al., 2002). This is supported by the high incidence of COD in dairy cows during the early postpartum (pp) period, when animals are in a negative energy balance, and by the association of COD with a high level of milk production (Gröhn et al., 1994; Laporte et al., 1994; Beam, 1995; Hooijer et al., 2001; López-Gatius et al., 2002; Hooijer et al., 2003). Several studies have shown that the energy balance pp affects follicular development and ovulation (Beam and Butler, 1997; 1999; Butler, 2003), but whether this can result in cyst formation still remains unclear.

Therefore the aims of this study were to investigate whether ovarian cysts, naturally occurring during the first 60 days pp in high yielding dairy cows, are preceded by 1) P$_4^{supra}$ and/or increased E$_2$ concentrations; and/or 2) altered metabolic profiles during the first weeks pp and during the final week prior to cyst formation.

**Materials and Methods**

*Animals*

Twenty eight multiparous high yielding Holstein-Friesian dairy cows that calved normally during fall/winter of 2002 and 2003 were used in this study. In total, data from 34 lactations were obtained. All experimental work was performed at the research dairy farm of the Ghent University (Biocentrum Agrivet, Melle, Belgium), following approval by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University. Animals were housed in a loose stable with cubicles and were milked on average 2.2 times per day by an automated voluntary milking system. The average 305-day milk yield of the herd was 9500 kg milk with 3.90% fat and 3.45% protein. Cows were fed according to their requirements for maintenance and milk production. The ration consisted of high quality roughages (maize silage, grass
silage and sugar beet pulp), soybean meal and concentrates. Each animal routinely received 500 ml propylene glycol daily as an oral drench during the first 3 days pp.

**Production parameters and diseases**

The 7 day-average daily milk yield was calculated by the automated milking system per cow. These values were recorded twice a week, concurrent with blood sampling and ultrasonography. Body condition scores (BCS) were recorded by the same experienced operator throughout the study at day 1 pp, day 10 pp, and then twice a week. Scoring was performed on a 5 point scale (1= thin, 5= fat), with 0.25 increments as described by Edmondson et al. (1989). The occurrence of retention of fetal membranes and/or endometritis, identified as risk factors for COD (Bosu and Peter, 1987; López-Gatius et al., 2002), were recorded per cow.

**Milk and blood sampling**

From day 1 pp, daily milk samples were collected and stored frozen (-20°C) until P₄ determination in milkfat. Blood samples were collected through coccygeal blood vessel puncture into Venoject® tubes (Terumo Europe N.V., Leuven, Belgium): one with EDTA as anticoagulant and one with a clotting activator (Autosep®: Gel + Clot. Act.). Samples were taken at day 1 pp, day 10 pp, and then twice a week. The clotting activated sample (serum) was allowed to clot and within 1.5 h after collection all samples were centrifuged (1400 × g, 30 min) and serum or plasma was stored frozen (-20°C) until determination of non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB), insulin and E₂ concentrations.

**Ultrasonography**

Ovaries of all cows were examined by linear array ultrasonography utilizing a 7.5 MHz rectal probe (PieMedical, Maastricht, The Netherlands) on day 10 pp. From then on ultrasonography was performed twice a week until ovulation or formation of a cystic follicle, or until day 60 pp. Ultrasound examinations were performed by the same operator throughout the study. At each session, the number of large (9-19 mm) follicles, the presence of a corpus luteum and/or ovarian cyst(s) on each ovary was recorded. The diameter of a follicle/cyst was the average of both the length and the width of the structure as determined by the built-in
software of the ultrasound. The cyst’s and the large follicles’ positions on the ovary were carefully recorded to make sure the same “cyst” was observed during the subsequent scanning sessions.

**Definition of ovarian cyst(s) and ovulation**

An ovarian cyst was defined as a follicular structure of 2.0 cm or more in diameter, that persists for at least 7 days (i.e. three or more scanning sessions) in the absence of a corpus luteum. Ovulation of a dominant follicle was defined by disappearance of the follicle, followed by formation of a corpus luteum and accompanied by an increase in P₄ in milkfat above 34 ng/ml, which according to our radio immuno assay (RIA) corresponds to 1 ng/ml in serum, as determined preliminary. The day of ovulation was determined as being the 5th day prior to a P₄ rise above the threshold value of 34 ng/ml, as done by Shresta et al. (2004).

**Classification of P₄ profiles during the final 7 days preceding ovulation/cyst formation**

Progesterone profiles were classified similarly to Hatler et al. (2003): P₄ was considered suprabasal when the levels were continuously between 14.4 and 63.6 ng/ml milkfat (corresponding serum values of 0.3 and 2 ng/ml, respectively). Progesterone was classified as low when the level dropped to less than 14.4 ng/ml milkfat for one or more days during the 7-day period. This classification is based on the fact that P₄ blocks the LH-surge and ovulation above serum concentrations of 0.3 ng/ml (Duchens et al., 1994; 1995), promoting prolonged growth and dominance of the follicle and this up to concentrations of 2 ng/ml in serum (Savio et al., 1993; Stock and Fortune, 1993).

**Hormone and biochemical analyses**

Progesterone concentrations in milkfat were analysed by RIA (Opsomer et al., 1999a). This assay had a detection limit of 25 pg and intra- and inter-assay coefficients of variation of 9.3% and 12.0% respectively. Oestradiol-17β concentrations in plasma were also analysed by RIA (Henry et al., 1987). The detection limit was 5 pg and the intra- and inter-assay coefficients of variation were 5.8% and 8.3%, respectively. Insulin concentrations in serum were analysed using a commercially available ELISA, validated for cattle (Immuno-Biological Laboratories, Hamburg, Germany), according to the instructions of the
manufacturer. Commercial kits were used for the measurements of BHB (Sigma Diagnostics Inc., St.Louis, USA) and NEFA (Wako Chemicals GmbH, Neuss, Germany) and these analyses were performed at the Department of Clinical Chemistry, University Hospital, Ghent, Belgium.

Statistical analyses

Parity, length of the dry period, BCS at the start of lactation, absolute BCS loss during the first 20 days pp and day pp of first ovulation/cyst formation were compared between groups by using an independent samples Student’s t-test.

Data of the first three weeks pp (milk yield, BCS, insulin, BHB and NEFA) as well as of the final week prior to ovulation/cyst formation (milk yield, BCS, insulin, BHB, NEFA and E2) (repeated measurements) were analysed using a linear mixed effects model, with follicle type (ovulatory or cystic follicle) and time pp or time to ovulation/cyst formation as fixed effects and lactation in cow as random effect (S-Plus 2000, Cambridge, USA). Data for BHB and NEFA were log transformed before statistical analysis to correct for departures from normality. Progesterone profiles were only analysed descriptively, based on their classification as mentioned before. Probability values of P<0.05 were considered significant. All data are presented as means ± SEM.

Results

Ovarian activity, ovulation, cyst formation and disease occurrence

In all lactations, ovarian activity and follicular growth, characterized by the appearance of a follicle > 8mm, occurred before day 21 pp. Ovarian cysts developed in 8 lactations (CYST) (26.7%), while 22 lactations ovulated (OV) before day 60 pp. More detailed information on each of the CYST lactations is presented in Table 1. In OV and CYST lactations, first ovulation or cyst formation occurred approximately at the same time pp (21.3 ± 2.5 vs 27.5 ± 4.7 days, respectively; P=0.23). Four lactations were excluded from further analysis: two in which the anovulatory period ≥ 60 days pp, one due to an irregular P4 profile after ovulation, making an accurate assessment of the day of ovulation impossible, and one due to irregular follicle development. The latter developed a follicle which crossed the 2 cm diameter limit but failed to persist for 3 consecutive ultrasound controls. At the third check up, the follicle
had diminished in size and seemed to luteinize, as indicated by thickening and irregular lining of the follicle wall and a simultaneous increase of P₄ in milkfat.

Five out of 22 OV lactations (22.7%) had an abnormal puerperium due to retention of the fetal membranes and/or endometritis, while only one (12.5%) of the CYST lactations suffered from both of these puerperal disorders.

Table 1. Detailed information on each of the ovarian cysts observed: cow identity (ID) and parity, day post partum of initial detection, number of cysts, size of the cyst(s) at the three consecutive ultrasound (US) sessions and days of observed presence from 1st to 3rd US.

<table>
<thead>
<tr>
<th>Cow ID</th>
<th>Parity</th>
<th>Day pp of detection</th>
<th># of cysts</th>
<th>1st US (mm)</th>
<th>2nd US (mm)</th>
<th>3rd US (mm)</th>
<th>Days 1st-3rd US</th>
</tr>
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<tr>
<td>936</td>
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* 1st US = day of detection.
§ At 2nd and 3rd US, two additional cysts were observed with a diameter of 22 and 21 mm at the 2nd US and of 23 and 23 mm at the 3rd US.
a,b Size of the largest cyst.

Parity, length of the dry period and BCS at start of lactation

Parity and length of the dry period were similar between OV and CYST lactations (3.1 ± 0.3 versus 2.9 ± 0.4 lactations and 62.5 ± 8.9 versus 64.1 ± 10.3 days, respectively). The BCS at the start of lactation was also similar between the two groups (P=0.67).
Progesterone profiles and \(E_2\) concentrations prior to cyst formation and ovulation

All OV and 6 of the CYST lactations had low \(P_4\) profiles prior to ovulation and cyst formation, respectively. A \(P_4\) profile of an OV lactation is shown in Figure 1A. Two CYST lactations (CYSTsupra) did however have \(P_4\)supra concentrations. The concentrations in these two lactations were continuously between 14.4 and 46 ng/ml milkfat during the final 7 days preceding cyst formation (Figure 1B).

Three other CYST lactations developed suprabasal \(P_4\) concentrations between 1-17 days after formation. These hormonal changes were often associated with thickening and irregular lining of the cyst wall on ultrasound, indicating luteinization of the cyst.

Oestradiol-17\(\beta\) concentrations during the final week prior to ovulation or cyst formation were significantly increased in CYST compared to OV lactations (\(P<0.05\)). Due to the fact that \(P_4\)supra itself actually increases \(E_2\) levels, the \(E_2\) concentrations of the CYSTsupra lactations and the other CYST lactations were compared separately with those of the OV lactations. Oestradiol-17\(\beta\) concentrations in the CYSTsupra lactations were higher than in OV lactations (\(P<0.05\)), while the other CYST lactations had similar levels than the OV lactations.

**Figure 1.** Milkfat progesterone profiles of an ovulatory lactation (OV) with low levels prior to ovulation (A) and of a cystic lactation (CYST) (cow 1016) with suprabasal levels prior to cyst formation (B). Ovulation occurred on day 12 pp in the OV lactation (arrow), while in the CYST lactation the dominant follicle passed the 2 cm diameter limit on day 37 pp (arrow) and persisted for at least 8 days (3\(^{rd}\) control by ultrasound on day 44). The white bar indicates the suprabasal progesterone range (from 14.4 ng/ml to 63.6 ng/ml milkfat).
Milk yield, metabolic parameters and body condition score loss

The average daily milk yield steadily increased while the BCS decreased with time pp (P<0.05) and these changes over time were similar between OV and CYST lactations (Figure 2A-B). Also the absolute loss of body condition during the first 20 days pp was not significantly different between groups (0.48 ± 0.7 for OV vs 0.34 ± 0.10 for CYST; P=0.34). Metabolic profiles (NEFA, BHB and insulin concentrations) as well did not differ between OV and CYST lactations during the first 20 days pp (Figure 3A-C). In both groups, NEFA and insulin concentrations decreased with time pp (P<0.05).

![Figure 2](image.png)

**Figure 2.** Profiles of mean daily milk yield (A) and body condition score (BCS) (B) of cystic (CYST, n= 8) and ovulatory (OV, n= 22) lactations during the first 3 weeks pp.
Figure 3 A-C. Profiles of mean non-esterified fatty acids (NEFA) (A), β-hydroxybutyric acid (BHB) (B), and insulin (C) serum concentrations of cystic (CYST, n= 8) and ovulatory (OV, n= 22) lactations during the first 3 weeks pp.

When focussing on the final week prior to ovulation and cyst formation, BCS, milk yield and NEFA and BHB concentrations were similar between OV and CYST lactations. However, insulin concentrations were significantly lower in the final week prior to cyst formation than prior to ovulation of the dominant follicle (P<0.05) (Table 2).
Table 2. Mean daily milk yield (kg), body condition score (BCS), insulin (µIU/ml), non-esterified fatty acids (NEFA) (mEq/L) and β-hydroxybutyric acid (BHB) (mM) concentrations during the final week prior to ovulation (OV) or cyst formation (CYST). Data are mean ± SEM.

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<tr>
<th>Parameter</th>
<th>OV</th>
<th>CYST</th>
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<tr>
<td>Milk yield</td>
<td>38.8 ± 1.1</td>
<td>41.5 ± 2.4</td>
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<tr>
<td>BCS</td>
<td>2.83 ± 0.06</td>
<td>2.69 ± 0.13</td>
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<tr>
<td>insulin</td>
<td>8.92 ± 0.74 (^{a})</td>
<td>5.61 ± 0.73 (^{b})</td>
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<tr>
<td>NEFA</td>
<td>0.52 ± 0.04</td>
<td>0.46 ± 0.05</td>
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<tr>
<td>BHB</td>
<td>1.00 ± 0.10</td>
<td>1.07 ± 0.26</td>
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\(^{a,b}\) Means with different superscripts within the same row differ significantly (P<0.05).

Discussion

In this study, we have investigated the relationship between hormonal or metabolic parameters and the formation of ovarian cysts during the first 60 days pp in high yielding dairy cows. Compared to most research on COD, this longitudinal study allowed us to investigate the period prior to natural cyst formation which is rather unique and has only been done before by Beam (1995) and Zulu et al. (2002). To define an ovarian cyst, a size limit of 2 cm rather than 2.5 cm was chosen based on the fact that dominant follicles ovulate on average between 1.6 and 1.9 cm diameter in dairy cows (Savio et al., 1990; Bleach et al., 2004; Lopez et al., 2004). A smaller size limit than the traditional 2.5 cm has also been used in other recent studies on ovarian cysts (Hamilton et al., 1995; Gümen et al., 2002; Zulu et al., 2002; Hatler et al., 2003).

Despite the fact that in the study of Hatler et al. (2003) the majority of COD cases (66%) were associated with P\(_{4}\)\(^{ supra}\) levels at the time of diagnosis, our results show that at the time of formation, only a minority of cysts (25%) are associated with P\(_{4}\)\(^{ supra}\) levels. Besides different definitions for ovarian cysts (follicle >1.7 cm) and P\(_{4}\)\(^{ supra}\) (0.1-1 ng/ml in serum), the results of Hatler et al. (2003) may be explained by luteinization of the cysts. Since they performed rectal examinations every 9 to 14 days to detect cysts, the actual “age” of the cyst was not known and it could already have luteinized. This is supported by the fact that in our study 5 of the CYST lactations (62.5%) had suprabasal P\(_{4}\) concentrations between 0-17 days after
formation. In an earlier study of Bosu and Peter (1987), cyst formation was not associated with increased P₄ concentrations. However, their study did not specifically focus on an association between COD and P₄supra profiles.

Whether in our study, the two cysts associated with P₄supra actually formed due to inhibition of the pre-ovulatory LH-surge by the P₄supra concentrations (Sirois and Fortune, 1990; Stock and Fortune, 1993), could not be determined since no LH analyses were performed. Such P₄supra levels fail to suppress LH pulse frequency, thereby promoting growth and steroidogenesis of the dominant follicle (Savio et al., 1993; Bergfeld et al., 1996). The increased E₂ concentrations in the two CYSTsupra lactations were most likely an illustration of this.

The source of P₄supra concentrations in our study is not known. Incomplete luteolysis of the pregnancy CL is highly unlikely, since P₄ dropped to basal levels immediately after parturition. Neither of the lactations ovulated/formed a CL before the period of P₄supra. In one of the CYSTsupra lactations a dominant follicle from the previous follicular wave seemed to luteinize on ultrasound, which may have produced enough P₄ to reach suprabasal levels. Another possible source of P₄ in cattle is the adrenal gland. In situations of stress adrenal P₄ production is stimulated by an increased release of ACTH from the pituitary (Watson and Munro, 1984). Quantifying stress is however difficult and not being the aim of the study, this was not investigated. In literature it is also mentioned that body fat may act as passive storage for P₄ since this is a lipophilic molecule. Progesterone diffuses in and out of lipid tissue according to changes in plasma concentrations. However, some would remain in the body fat, only being released when the tissue is mobilized (Hamudikuwanda et al., 1996). This implies that cows losing more body fat, have higher P₄ levels in serum (in the absence of any luteal tissue). However, the two CYSTsupra lactations did not clearly lose more in body condition prior to or during the periods of P₄supra concentrations than the others with low P₄ levels.

Our study indicates that the majority of ovarian cysts early pp are formed by other mechanisms than P₄supra. Recent research has shown that a prematurely (i.e. when no ovulatable follicle is present) induced pre-ovulatory LH-surge, by treatment with exogenous estrogens, which is not followed by a rise in P₄ renders the hypothalamus insensitive to E₂. New follicles developing under these conditions do not ovulate and become cystic (Gümen et al., 2002; Gümen and Wiltbank, 2002). This refractoriness of the hypothalamus is similar to the situation in naturally occurring cysts where injection of E₂ does not elicit an LH-surge (Zaied et al., 1981), unless the cystic ovary is removed (De Silva and Reeves, 1988). In our study, E₂ concentrations prior to cyst formation were elevated. Due to the small number of
Chapter 4.

CYST lactations, this was the result of increased E2 concentrations, associated with $P_{4\supra}$, in the two CYST$_{\supra}$ lactations alone. Beam (1995) observed increased E2 levels in cows prior to development of cysts, but $P_4$ was not determined. An association between development and persistence of cysts and increased E2 concentrations has also been reported by Hamilton et al. (1995). However, that study focussed on the subsequent follicular wave forming (a) new cyst(s) in cows with induced and natural follicular cysts present i.e. when the endocrine system was already disrupted. The elevated E2 concentrations are therefore most likely the result of an increased production by the cysts, due to overstimulation by the elevated LH-pulsatility.

When focussing on productive and metabolic aspects of OV and CYST lactations, no differences could be observed during the first 3 weeks pp for any of the parameters tested, neither when looking more specifically at the final week prior to ovulation or cyst formation, with the exception of insulin. Lactations developing cysts had significantly lower insulin concentrations than OV lactations during the final period preceding cyst formation/ovulation. A previous study already revealed that insulin secretion after an intravenous glucose challenge is impaired in COD cows (Opsomer et al., 1999b). Insulin acts on the ovary and exerts direct and indirect stimulatory effects on granulosa and theca cell growth and steroidogenesis, both in vitro (Spicer and Echternkamp, 1995; Gutiérrez et al., 1997; Spicer et al., 2002) and in vivo (Matamaros et al., 1990; Simpson et al., 1994). Moreover, insulin seems to be important for follicular maturation (Landau et al., 2000) and normal ovarian function pp (Miyoshi et al., 2001), as it increases the steroidogenic capacity of the dominant follicle (Butler et al., 2004). Insulin also stimulates LH-receptor expression in granulosa cells, mainly indirectly through IGF-1 receptor upregulation (Davoren et al., 1986). Therefore, altered insulin concentrations may affect follicular development, maturity and responsiveness to LH stimulation, which could lead to anovulation and cyst formation. Based on this, the lower insulin levels seem to contradict the higher E2 concentrations observed in the CYST lactations. However, as shown before only the CYST$_{\supra}$ lactations actually had elevated E2 levels, which can be attributed to the action of $P_{4\supra}$.

Although the results of Beam’s study (1995) are more or less in accordance with our data, he did not observe any difference in insulin concentrations between cystic and ovulatory lactations. Moreover, he did record a higher milk yield and less mobilization of body reserves in proportion to milk production in cystic cows. His observations indicate that differences in energy utilization/partitioning between cystic and ovulatory cows may be one of the key factors (Beam, 1995).
Contrary to our and Beam’s (1995) study, Huszenicza et al. (1988) and Zulu et al. (2002) did observe increased NEFA levels pp during one or more weeks in cows developing ovarian cysts. Besides differences in the definition of COD, cystic cows in the study of Zulu et al. (2002) had a higher BCS during the dry period and at the time of parturition than ovulatory animals, which may result in increased mobilization of body fat and higher NEFA levels pp. Increased NEFA concentrations have been shown to be cytotoxic for bovine granulosa cells in vitro (Vanholder et al., in press), which could hamper follicle growth.

An increased milk yield during early lactation is considered a risk factor for the occurrence of COD by several authors (Gröhn et al., 1994; Laporte et al., 1994; López-Gatius et al., 2002; Hooijer et al., 2003) and production traits seem to be genetically correlated to COD (Hooijer et al., 2001). Although these studies link milk yield to cyst formation, data from our and Beam’s (1995) study indicate that this is not due to significant differences in mean energy balance and hormonal/metabolic parameters prior to ovulation or cyst formation. Hooijer et al. (2003) as well could not detect a more severe NEB, determined by the fat/protein ratio in milk, in COD cows. Beam (1995) however suggests that changes in energy balance may be more relevant than absolute values. He noticed that in cystic cows the nadir of the energy balance occurred later than in ovulatory cows. On the other hand, differences in energy balance between cows that ovulate and those that form a cyst may be very subtle, if present. This is indicated by the fact that when negative energy balance is severe, ovarian activity is delayed and anovulation occurs (Opsomer et al., 2000; Butler, 2003), due to cessation of follicle growth at a size of ~9mm (Wiltbank et al., 2002). Since this is clearly not the case in cystic follicles, which easily reach ovulatory size, only subtle alterations of certain metabolic and hormonal parameters may be involved.

In conclusion, the results of the present study suggest that ovarian cyst formation is associated with lower insulin levels but not with other distinct hormonal and metabolic alterations. However, from this study we can not exclude the involvement of subtle hormonal and metabolic changes in the pathogenesis of ovarian cysts. Suprabasal progesterone, and altered E₂ concentrations, seem to be involved in the formation of only a minor percentage of ovarian cysts.

Acknowledgements

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References


CHAPTER 5

Effect of non-esterified fatty acids on follicular cell function and viability in vitro
Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro

Modified from:

EFFECT OF NON-ESTERIFIED FATTY ACIDS ON BOVINE GRANULOSA CELL STEROIDOGENESIS AND PROLIFERATION IN VITRO
Vanholder T, Leroy Jlmr, Van Soom A, Opsomer G, Maes D, Coryn M, de Kruif A
Summary

In high yielding dairy cows, the negative energy balance (NEB) during the first weeks post partum may influence dominant follicle growth and steroidogenesis. Since non-esterified fatty acid (NEFA) concentrations are elevated during NEB and are shown to be toxic for several cell types, we investigated the individual and combined effects of the three main NEFAs on granulosa cell proliferation and steroidogenesis in vitro. Granulosa cells from large follicles were cultured for two days in serum free medium in the presence of palmitic (C16:0) (PA), stearic (C18:0) (SA) and/or oleic acid (C18:1) (OA). Addition of 150, 300 or 500 µM of PA and SA inhibited cell proliferation (P<0.05) while OA only elicited such an effect at 500 µM (P<0.01). In the combination treatment (150 µM of each fatty acid), cell numbers were also reduced (P<0.01). These inhibitory effects on cell number are partly due to the induction of apoptosis by these NEFAs, as was demonstrated by annexin V-FITC/propidium iodide staining of the granulosa cells. Oestradiol-17β production was stimulated by all doses of PA, by 300 and 500 µM of SA and by 500 µM of OA (P<0.05). Combined treatment with 150 µM of each fatty acid also stimulated oestradiol-17β production per 10⁴ cells (P<0.05). We can conclude that PA, SA and to a lesser degree OA modulate granulosa cell proliferation and steroidogenesis in vitro. These effects may be involved in the occurrence of ovarian dysfunction during the postpartum period in high yielding dairy cows.
**Introduction**

Reproductive performance in high yielding dairy cows has declined worldwide over the past few decades (Lucy, 2001). Almost half (49%) of modern dairy cows suffer from an ovarian dysfunction during the postpartum period, leading to a prolonged interval to first ovulation (Opsomer et al., 1998).

Negative energy balance (NEB) is a risk factor for the occurrence of delayed cyclicity (Beam and Butler, 1999; Opsomer et al., 2000). Especially the interval from parturition to the energy balance nadir influences follicular competence early post partum. Follicles emerging before the NEB nadir have a reduced growth and produce less oestradiol, and therefore require more time and a larger size to achieve a sufficient oestradiol production, capable of triggering ovulation (Beam and Butler, 1997). The inhibitory effect of NEB on follicular growth and development can be exerted through different mechanisms, like reduced LH-pulsatility and lower IGF-1 concentrations (Beam and Butler, 1999).

During periods of NEB, NEFA concentrations in circulation are elevated and total serum concentrations of palmitic (C16:0) (PA), stearic (C18:0) (SA) and oleic acid (C18:1) (OA) are increased (Rukkwamsuk et al., 2000). Although there does not seem to be a significant active uptake of NEFA by the ovary (Rabiee et al., 1997), alterations in serum concentrations induced through fasting, are closely reflected in the follicular fluid of dominant follicles (Comin et al., 2002; Jorritsma et al., 2003). This has been confirmed by Leroy et al. (2004a) who showed that in cattle the NEFA concentration in follicular fluid of large follicles is highly correlated with the concentration in serum. Moreover, in high yielding dairy cows post partum, NEB associated changes in serum NEFA concentrations are well reflected in the follicular fluid of the dominant follicle (Leroy et al., 2004b).

Recent research has indicated that especially PA and SA have a negative effect on human granulosa cell viability in vitro (Mu et al., 2001), already at concentrations frequently encountered in the serum of high yielding dairy cows (Beam and Butler, 1997; Reist et al., 2000; Rukkwamsuk et al., 2000). Consequently, NEB may also affect follicular growth and development through increased NEFA concentrations acting on ovarian cells. Therefore the aim of this study was to examine the individual and combined effects of PA, SA and OA on bovine granulosa cell steroidogenesis and proliferation in vitro.
Materials and Methods

Reagents and hormones

Dulbecco’s modified Eagle’s medium (DMEM)/ nutrient mixture F-12 Ham (1:1 mixture) with glutamine, sodium bicarbonate and Hepes, fatty acid free bovine serum albumin (BSA), apotransferrin, sodium selenite, insulin (bovine, 10mg/mL 25mM Hepes), androstenedione, kanamycin, trypan blue 0.04% solution, PA, SA and OA were all purchased from Sigma (Bornem, Belgium). Gentamycin and trypsin were obtained from Gibco/InVitroGen (Merelbeke, Belgium) and porcine FSH was kindly provided by Prof. Beckers (University of Liège, Belgium). Percoll™ was purchased from Amersham Biosciences (Uppsala, Sweden) and heparin was obtained from Leo Pharma (Zaventem, Belgium). Ethanol was purchased from Vel/Merck Eurolab (Zaventem, Belgium).

Granulosa cell collection

Ovaries were collected at a local abattoir and immediately transported (30°C) to the laboratory. After washing of the ovaries in saline with kanamycin at room temperature (20°C), large (>8mm) follicles were dissected free of ovarian stroma and follicle health was assessed according to the criteria described by Kruip and Dieleman (1982). Granulosa cells were collected by follicle puncture, as described by others (Spicer and Stewart, 1996; Bosc and Nicolle, 1997; Basini et al., 2002). Healthy follicles were punctured with a 19-gauge needle and a sterile syringe flushed with heparin. Follicular fluid was repeatedly aspirated to disrupt the granulosa cell layer, subsequently pooled and centrifuged (800 x g, 10min). The resulting granulosa cell pellet was loaded on a Percoll™ 45% gradient and centrifuged (720 x g, 30 min) to discard any red blood cells. Granulosa cells were then washed in culture medium and after centrifugation (800 x g, 10 min) resuspended. Viability of the cell suspension was determined by use of trypan blue exclusion dye. Cell viability averaged 44.1 ± 3.0 % at the time of plating.
Granulosa cell culture

The serum free culture system used was an adaptation of the system described by Gutiérrez et al. (1997). Briefly, granulosa cells (≈7 \times 10^4 cells per well) were cultured in a total volume of 250 µL DMEM/Nutrient mixture F-12 Ham with 2.5mM glutamine, 14.3mM sodium bicarbonate and 15mM Hepes supplemented with BSA 0.1%, apotransferrin (2.5 µg/mL), sodium selenite (4 ng/mL), gentamycin (20 µg/mL), insulin (50 ng/mL), porcine FSH (1ng/mL), and androstenedione (1 µg/mL) in 96-well tissue culture plates (Nunc, Roskilde, Denmark). Palmitic acid, SA and OA were dissolved in pure ethanol at a concentration of 50 mg/mL. According to treatment PA, SA or OA in ethanol were added in volumes to obtain final test concentrations of 150, 300 and 500 µM of the fatty acid concerned. For the combination treatment, fatty acids in ethanol were added in volumes to obtain a concentration of 150 µM of each fatty acid. Control wells received equal volumes of ethanol as the test groups. Plates were incubated for 48 h in a humidified incubator at 38.5°C in 5% CO₂:95% air. At the end of the culture period, medium was collected (125µL) and the number of granulosa cells was determined in each well.

The system was validated to see whether granulosa cells retained their specific follicular properties in the culture system. After 48 h of culture, cell numbers and E₂ and P₄ concentrations in culture medium were determined. Granulosa cells produced 1,907 ± 274 pg/10⁴ cells oestradiol and 1,926 ± 264 pg/10⁴ cells progesterone, yielding an E₂/P₄ ratio of 0.99. This ratio is higher than the one determined by Gutiérrez et al. (1997) and implies that the granulosa cells do not show extensive luteinization after 48 h of culture. This is supported by the cellular morphology we observed at the end of culture: granulosa cells form tight clumps of spherical cells, as described by Gutiérrez et al. (1997). We also tested whether the concentration of the substrate could be a limiting factor for the E₂ production. Therefore cells were cultured for 48 h with 1 and 10 µg/ml androstenedione and E₂ concentrations in medium were measured. No difference was recorded between treatments (1,976 ± 364 pg E₂/10⁴ cells and 1,950 ± 522 pg E₂/10⁴ cells respectively), indicating that 1 µg/ml androstenedione is sufficient as substrate to support E₂ production.
Chapter 5.1

**Determination of cell number**

At the end of the culture period, the number of granulosa cells in each well was determined with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Benelux, Leiden, The Netherlands). This assay is based on the ability of living cells to reduce the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), to a soluble formazan product. The quantity of formazan product is directly proportional to the number of living cells in culture. Briefly, at the end of the culture period, after removal of 125 µL of medium for hormone analysis, 25 µL of the MTS solution was added to each well and incubated for 2h in a humidified incubator at 38.5°C in 5% CO₂:95% air. After incubation, the assay plates were read using a multiwell plate reader (Spectrafluor Plus Microplate Reader, Tecan®) at a wavelength of 492 nm. A standard curve relating absorbance directly to the number of cells was constructed by incubating known numbers of cells with the MTS solution under the same culture conditions. These cells were first cultured for 48 h under the same culture conditions and with 0, 150, 300 and 500 µM of the fatty acid concerned. Cells were rinsed with PBS (without Ca²⁺ and Mg²⁺) and treated with trypsin (0.25%, 10 min at 37°C). Cell aggregates were disrupted by repeated pipetting of the cell suspension and they were finally resuspended in culture medium. After determination of concentration, cells were resuspended in a total volume of 125 µL medium at different numbers ranging from 1x10⁴ to 1.5x10⁵ cells. For each treatment, a standard curve was constructed for each replicate. Corrections were made for differences in absorbance between test media by subtracting the absorbance values of the test medium without cells incubated with the CellTiter 96® AQueous One Solution Cell Proliferation Assay from the absorbance values of the test wells. There was a linear relationship between the number of cells and the absorbance values (r>0.95). The number of cells in each well of the culture plates was estimated from the resulting regression equation.

**Hormone Analysis**

At the end of the culture period, media were collected and stored frozen (-20°C) until determination of oestradiol-17β (E₂) and progesterone (P₄) concentrations through radio immuno assay (RIA), as described earlier (Henry et al., 1987). The detection limit for E₂ was 5 pg and the intra- and inter-assay coefficients of variation were 5.75% and 8.30%,
respectively. For P₄ the detection limit was also 5 pg and the intra- and inter-assay coefficients of variation were 7.05 % and 8.75 % respectively.

Detection of apoptosis

Granulosa cells (≈6 x 10⁵ cells per well) were cultured on round cover slips in 4-well plates under serum free conditions as described above for 48 h in the presence of 300 μM OA, SA or PA or no fatty acid at all. After 40 h, apoptosis was induced in the positive control by addition of staurosporine (20 μM in ethanol stock solution) to a final concentration of 1 μM. Early and late apoptotic changes were detected using an Annexin V-FITC/Propidium Iodide Vybrant Apoptosis Kit (Molecular Probes, Leiden, The Netherlands). After 48 h, cells were rinsed with PBS and then incubated with 100 μL 1X Annexin Binding Buffer containing 20 μL annexinV-FITC and 2 μL propidium iodide (PI) at room temperature, for 15 minutes, in the dark. To visualize nuclear changes, cells were also stained with Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). After incubation, cells were washed with 100μL 1X Annexin Binding Buffer, and the cover slips with the adherent cells were removed from the wells and mounted upside down on a glass slide. Fluorescence microscopic analysis was performed using a Leica DMR microscope (Van Hopplyns NV, Brussels, Belgium). Healthy cells only stain Hoechst positive, while early apoptotic cells also stain positive for annexin. Late apoptotic or necrotic cells stain positive for both Hoechst, annexin and propidium iodide.

Statistical Analysis

Each culture experiment was performed three times and within each replicate quadruplicate culture wells were used per treatment. For the apoptosis staining, the experiment was repeated two times. Statistical analysis was performed after conversion of the data to percentages of respective controls (ethanol treated wells were taken as 100%), in order to minimize variation between replicate experiments. Data were tested for normality and homogeneity of variance and logarithmic transformation was performed when these requirements were not fulfilled. The results are expressed as the mean ± SEM. For all experiments, E₂ production was calculated per 10⁴ viable cells.

The effects of PA, SA and OA on the amount of proliferation of bovine granulosa cells and the production of E₂ by these granulosa cells were analysed using analysis of variance,
with the type of fatty acid as fixed factor, replicate as random factor and the amount of cell proliferation or E₂ production per 10⁴ viable cells as dependent variables in the model. Pairwise comparisons between the groups were made using post hoc Scheffé’s test. The number of granulosa cells showing apoptosis in the different treatment groups, as determined by the annexin-FITC/PI staining method, was analysed using logistic regression models. Differences were considered to be significant if P-values were lower than 0.05. The statistical analyses were performed using SPSS 11.0 (SPSS 11.0 for Windows, Chigaco, IL).

Results

Effect of NEFA on cell proliferation

The saturated fatty acids PA and SA had a significant inhibitory effect on cell proliferation at the three concentrations tested (P<0.05) (Figure 1). This effect was not dose-dependent for PA since all three concentrations reduced cell numbers to the same extent (P>0.05) (Figure 1A). Stearic acid however had a more severe negative effect on cell proliferation at 300 µM and 500 µM than at 150 µM (P<0.01) (Figure 1B).

The mono-unsaturated OA only inhibited cell proliferation significantly at the highest concentration of 500µM (P<0.01) (Figure 1C). This concentration reduced cell numbers by 76 % compared to controls.

The combination treatment with 150 µM of each of the fatty acids also reduced cell numbers significantly compared to controls (P<0.01) (Figure 1D). Cell numbers were reduced by 35 %.
Figure 1 A-D. Effects of different concentrations of palmitic (A), stearic (B) and oleic (C) acid alone or combined (D) on granulosa cell proliferation (cells per well; mean ± SEM) after 48 h of culture. The NEFA-combination (D) contains 150µM of each of the fatty acids. Data are expressed as percentage of controls. Means with different superscripts differ significantly (P<0.05).
Induction of apoptosis by NEFA

Compared to controls, significantly more early apoptotic cells were present in the OA and PA treatments and significantly more late apoptotic/necrotic cells were observed in all three treatment groups (Figure 2). Hence the percentage of healthy cells, showing no apoptosis/necrosis, was significantly reduced in all treatment groups compared to controls.

Figure 2. Effect of different NEFA treatments on granulosa cell health after 48 h of culture. Cell health was assessed through fluorescent microscopic analysis after staining with Hoechst and annexin-FITC/propidium iodide. Within each experimental group, significant differences with the corresponding controls are indicated by different superscripts (P<0.05).

Effect of NEFA on E2 production

Treatment of granulosa cells with different concentrations of PA had a significant stimulatory effect on E2 production (P<0.01). This effect was not dose-dependent. For SA, a stimulatory effect was only significant at the two highest concentrations (P<0.01) (Figure 3).

When cells were treated with 500 µM OA, E2 production was significantly stimulated (P<0.05). Lower OA concentrations had no effect (P>0.05) (Figure 3C).

The combination treatment with 150 µM of each fatty acid also stimulated E2 production significantly (P<0.05) (Figure 3D).
Figure 3 A-D. Effects of different concentrations of palmitic (A), stearic (B) and oleic (C) acid alone or combined (D) on E₂ production (pg/10⁴ cells; mean ± SEM) after 48 h of culture. The NEFA-combination (D) contains 150µM of each of the fatty acids. Data are expressed as percentage of controls. Means with different superscripts differ significantly (P<0.05).

Discussion

In this study we examined the effect of different NEFAs on bovine granulosa cell proliferation and health, and steroidogenesis. We used a serum free culture system to postpone luteinization of the granulosa cells, which occurs more rapidly in the presence of serum (Gutiérrez et al., 1997). In a previous experiment, a negative effect of albumin bound oleic acid on granulosa cell E₂ production was seen (Vanholder et al., 2003). In the controls however albumin had a negative effect on cell number and a positive effect on steroidogenesis (data not published). The commercial formulation we used contained oleic acid and albumin in a 2:1 molar ratio, but it was shown by Melsert et al. (1991) that at this or a higher ratio oleic acid can counteract albumin effects. To eliminate albumin effects and any interactions between albumin and fatty acids, we used unbound fatty acids, dissolved in ethanol, as described by the manufacturer. The use of dissolved fatty acids (in NaOH) is documented by Mu et al. (2001). Although this does not mimic the in vivo situation where fatty acids are mainly bound to albumin, cellular uptake and metabolisation of unbound fatty acids also occurs (McArthur et al., 1999).

We observed an inhibitory effect of PA, SA and to a lesser degree OA on granulosa cell proliferation, both individually and in combination. These results are partially in agreement
with the study performed on human granulosa cells by Mu et al. (2001). In addition they found the effect of each of the fatty acids to be dose-dependent and OA to be already inhibitory at a concentration of 300µM. These discrepancies may be due to differences in species (human versus bovine), previous hormonal stimulation of granulosa cells (IVF in women), culture system (serum containing versus serum free), cell type (more luteinization due to presence of serum), days in culture (3 days versus 2 days) and concentrations of fatty acids that were tested. In luteinized granulosa cells, a negative effect of OA on cell proliferation was also noticed by Jorritsma et al. (2004). The concentration used however was supraphysiological (1mM) and the incubation period was substantially longer (5 days). Moreover, they used medium with serum and albumin-bound OA.

The observed inhibitory effects of fatty acids on cell survival and proliferation are most likely due to the induction of apoptosis, since more early apoptotic cells were present when granulosa cells were treated with 300 µM of either OA or PA. In the SA treated group, a higher percentage of cells was late apoptotic/necrotic. This indicates that SA is more toxic and that it induces apoptosis or even necrosis more quickly. The induction of apoptosis by these fatty acids was shown to occur in several cell types like human granulosa cells (Mu et al., 2001), rat Leydig cells (Lu et al., 2003), in vitro nerve growth factor differentiated cells (Uolloth et al., 2003) and rat pancreatic β cells (Shimabukuro et al., 1998), although in the latter also necrosis was induced (Cnop et al., 2001).

Although negative effects of fatty acids on cell survival and proliferation were observed, E2 production was stimulated when PA and SA were present in the culture medium. A relative shortage of substrate (androstenedione) per cell is very unlikely to be the cause, since increasing the dose of substrate did not influence the outcome of the experiments (data not shown). Moreover, the androstenedione concentration we used is equal to or exceeds the ones used by other authors (Gutiérrez et al., 1997; Spicer et al., 2002). One mechanism that could be involved is that apoptotic granulosa cells can maintain steroidogenesis as long as the steroidogenic organelles remain intact (Amsterdam et al., 1997). Granulosa cells even show enhanced and more efficient steroidogenesis during apoptosis, due to clustering of the steroidogenic organelles (Keren-Tal et al., 1995). Although fatty acid treated cells were mainly late apoptotic/necrotic (Figure 2), this mechanism may still apply. Since our aim was to compare the percentages of early apoptotic cells between treated and untreated groups, we used a single staining technique to detect early apoptotic cells. This single technique does not allow making a distinction between late apoptotic and necrotic cells (Van Cruchten and Van den Broeck, 2002). Therefore a certain percentage of the cells deemed late apoptotic/necrotic
could still be steroidogenically active and contribute to the E\textsubscript{2} production. This hypothesis is supported by the results of the cell proliferation assay, which measures metabolically active, living cells. Relative to the respective controls, the percentages of living cells in the fatty acid treated groups determined by the proliferation assay are higher than the percentages of healthy and early apoptotic cells in the same fatty acid treated groups determined by staining of the cells.

Another action of fatty acids is that they affect membrane stability. The ability of a fatty acid to penetrate cell membranes is dependent on its saturation and the stereometric position of double bond(s) (MacDonald and MacDonald, 1988). Saturated fatty acids increase and unsaturated ones decrease membrane stability (MacDonald and MacDonald, 1988; Stryer, 1995). This characteristic could be important since synthesis of oestrogens from androgens is catalyzed by the aromatase enzyme complex consisting of the aromatase cytochrome P450 and the NADPH-cytochrome P450 reductase (Conley and Hinshelwood, 2001) which are both membrane anchored proteins (Stryer, 1995). This mechanism could explain the stimulatory effect on steroidogenesis of the saturated fatty acids. In addition free fatty acids can stimulate ovarian NADPH dependent enzymes by reducing NADP\textsuperscript{+} to NADPH (Stevenson et al., 1973). The aromatase enzyme complex, which converts androgens to oestrogens, is such an enzyme (Stryer, 1995). The stimulatory effect OA at the highest concentration may be exerted through this mechanism.

The observed effects of NEFAs on granulosa cell proliferation and steroidogenesis in vitro could be involved in ovarian dysfunction early post partum in vivo. As stated earlier, dominant follicles emerging before the energy nadir have a slower growth rate and therefore require more time to achieve ovulatory size (Beam and Butler, 1997). Since our results indicate that free fatty acids inhibit cell proliferation in vitro, already at concentrations frequently found in serum (Beam and Butler, 1997; Reist et al., 2000; Rukkwamsuk et al., 2000) as well as in follicular fluid (Leroy et al., 2004a, 2004b) of early postpartum cows, this mechanism may play a role in the reduced growth of early postpartum dominant follicles in vivo. These follicles also show a diminished E\textsubscript{2} production (Beam and Butler, 1997). Our results indicate that the effect on steroidogenesis is dependent on the type of fatty acid, the concentration and the presence of more than one fatty acid. Moreover, oestrogen synthesis is dependent on the provision of substrate (androgens), produced by the theca cells. An excess of substrate was however present in our in vitro system. The effect of NEFA on theca cell steroidogenesis is currently under investigation and may be a limiting factor. It has been demonstrated in rat Leydig cells that NEFA are capable of inhibiting LH induced testosterone
production (Meikle et al., 1989) by reducing the amount of free cholesterol available for steroidogenesis (Meikle et al., 1996). A similar effect in theca cells would limit androgen production and therefore substrate availability for granulosa cell steroidogenesis. The net in vivo effect would then be an inhibitory one on both granulosa cell steroidogenesis and proliferation. Further research is however needed on the effect of NEFA on granulosa and theca cell steroidogenesis.

Dominant follicles that become cystic have an increased overall and peak E2 production compared to ovulatory dominant follicles of the first postpartum wave (Beam, 1995). Mistimed elevated E2 concentrations can elicit a GnRH/LH surge which, in the absence of a dominant follicle, is not followed by ovulation and/or luteinization. The lack of an increase in progesterone concentration after the GnRH/LH surge renders the hypothalamus insensitive to further oestradiol stimulation. In that way, cyst-like follicles can develop (Gümen and Wiltbank, 2002). Although mean energy balance and homeorhetic fluctuations of metabolic hormones did not differ between cows having either ovulatory follicles or cysts, the NEB nadir occurred later in the latter group (Beam, 1995). As observed by Mu et al. (2001), the in vitro effect of NEFAs was also time dependent. Therefore, in addition to the concentration of NEFAs, also the duration of exposure to these NEFAs could play a role in modulation of the effect on granulosa cells. This could explain how follicular competence increases after the NEB nadir (Beam and Butler, 1997). However other mechanisms modulating the effect of NEB on follicular development and competence are probably also involved.

**Conclusion**

We have demonstrated that NEFAs, especially saturated fatty acids, inhibit bovine granulosa cell survival and proliferation. Effects on E2 production were mainly stimulatory or without effect, depending on the concentration tested, the saturation of the fatty acid and the presence of other fatty acids. These effects of NEFAs on granulosa cell proliferation may therefore be a possible mechanism through which the NEB influences folliculogenesis during the early postpartum period in high yielding dairy cows. Further research is needed on the effect of NEFA on granulosa and theca cell steroidogenesis.
Acknowledgements

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References


Effect of non-esterified fatty acids on bovine theca cell steroidogenesis and proliferation in vitro

Modified from:

EFFECT OF NON-ESTERIFIED FATTY ACIDS ON BOVINE THECA CELL STEROIDOGENESIS AND PROLIFERATION IN VITRO
Vanholder T, Leroy JLMR, Van Soom A, Maes D, Coryn M, Fiers T, de KruijF A, Opsomer G
Animal Reproduction Science, in press
Summary

Elevated serum non-esterified fatty acid (NEFA) levels associated with a negative energy balance (NEB) may affect ovarian function and hence reproductive performance in high yielding dairy cows. We have investigated the individual and combined effects of the three major NEFAs on bovine theca cell proliferation and steroidogenesis in vitro. Theca cells from healthy large follicles (>8mm) obtained from slaughterhouse ovaries were cultured in serum free medium in the presence of 0, 50, 150 and 200 µM of palmitic acid (PA; C16:0); 0, 50, 150 and 250 µM of stearic acid (SA; C18:0); and/or 0, 50, 150 and 250 µM of oleic acid (OA; C18:1). Progesterone and androstenedione concentrations were measured in spent medium after 48 h of culture and cell numbers were determined spectrophotometrically per culture well. Cell viability was assessed by annexin-V FITC/propidium iodide staining. Only the treatment with 200 µM of PA inhibited cell proliferation (P<0.001) when tested individually, both of the mixtures tested (M1= 100 µM of PA, 130 µM of SA and 140 µM of OA; M2= 200 µM PA, 260 µM of SA and 280 µM of OA) reduced cell numbers (P<0.001). Progesterone and androstenedione production, both per well and per 10⁴ cells, were not affected by any of the treatments, with the exception of M2. This mixture reduced progesterone production per well and per 10⁴ cells (P<0.05). The effects observed were most likely caused by the cytotoxic action of the NEFAs, as demonstrated by the increased percentage of early apoptotic (M1) and late apoptotic/necrotic cells (M1 and M2) in the combination treatments (P<0.05). When combined, elevated physiological concentrations of PA, SA and OA can modulate theca cell proliferation and steroidogenesis in vitro by reducing theca cell viability. These NEFAs may be one of the mediators through which NEB compromises ovarian functioning and thus fertility in high yielding dairy cows.
Introduction

Around 50% of modern high yielding dairy cows suffer from an ovarian dysfunction during the early postpartum period, leading to delayed resumption of cyclicity (Opsomer et al., 1998; Shrestha et al., 2004a) and prolonged calving intervals (Shrestha et al., 2004b). Many factors can delay resumption of ovarian cyclicity postpartum. Negative energy balance (NEB) seems to be a major risk factor (Beam and Butler, 1999; Opsomer et al., 2000). These effects of NEB on ovarian function may be exerted through decreased LH-pulsatility and reduced circulating IGF-1, insulin and glucose concentrations (Beam and Butler, 1999). However, metabolic alterations associated with NEB may also affect ovarian function by acting directly on the ovary. Increased lipolysis of body fat also occurs during periods of NEB, causing an elevation in serum concentrations of non-esterified fatty acids (NEFAs) (Rukkwamsuk et al., 2000). These NEFAs may affect follicular growth and fertility by acting directly on the oocyte and on other cell types within the growing follicle. Recent research has shown that, in vitro NEFAs have a negative effect on oocyte maturation (Leroy et al., 2004a) and granulosa cell viability (Vanholder et al., 2005), but a positive effect on granulosa cell production of oestradiol (Vanholder et al., 2005). These effects are already noticeable at concentrations frequently encountered in the follicular fluid of the dominant follicle in high yielding dairy cows (Leroy et al., 2004a, 2004b).

A close interaction exists between granulosa and theca cells in the growing follicle. In vitro, theca cells stimulate granulosa cell viability (Allegrucci et al., 2003) and suppress granulosa cell apoptosis (Tajima et al., 2002). In addition, theca cells provide the substrate (androgens) for granulosa cell steroidogenesis. These close interactions indicate that effects on granulosa cells may be indirectly modulated by effects on theca cells. A major difference is that theca cells are surrounded by a tight capillary network, while granulosa cells are embedded in follicular fluid. Since serum concentrations of NEFA are approximately 40% higher than those in the follicular fluid of the dominant follicle in high yielding dairy cows early postpartum (Leroy et al., 2004a), theca cells are generally exposed to higher NEFA concentrations than granulosa cells. Our aim was to investigate the effects of the three most important NEFAs at concentrations encountered in the serum of high yielding dairy cows in the postpartum period, both individually and in combination on theca cell proliferation and steroidogenesis in vitro.
Materials and Methods

Reagents and hormones

Dulbecco’s modified Eagle’s medium (DMEM)/ nutrient mixture F-12 Ham (1:1 mixture) with glutamine, sodium bicarbonate and Hepes, fatty acid free bovine serum albumin (BSA), apotransferrin, sodium selenite, insulin (human, 10mg/mL, 25mM Hepes), ovine LH, kanamycin, trypan blue 0.04% solution, palmitic acid (PA), stearic acid (SA) and oleic acid (OA) were all purchased from Sigma (Bornem, Belgium). Gentamycin and trypsin were obtained from Gibco/InVitroGen (Merelbeke, Belgium). Percoll™ was purchased from Amersham Biosciences (Uppsala, Sweden) and heparin was obtained from Leo Pharma (Zaventem, Belgium). Ethanol was purchased from Vel/Merck Eurolab (Zaventem, Belgium).

Theca cell collection

Ovaries were collected at a local abattoir and immediately transported (30°C) to the laboratory. Large (>8mm) follicles were dissected free of ovarian stroma and follicle health was assessed according to the criteria described by Kruip and Dieleman (1982) after first washing the ovaries in saline with kanamycin at room temperature (20°C). Collection of theca cells was done as described by Meidan et al. (1990), with small adaptations. Briefly, healthy follicles were punctured with a 19g needle and a sterile syringe while follicular fluid was repeatedly aspirated to disrupt the granulosa cell layer. Follicles were hemisected in physiological saline after puncture and the follicle wall gently scraped and flushed to remove remaining granulosa cells. The theca interna was peeled away from the theca externa under a stereo microscope. The theca interna was then incubated in PBS (without Ca²⁺ and Mg²⁺) with 0.25% trypsin and 0.02% EDTA for 15 min at 37°C to further remove any adherent granulosa cells. The pieces of theca interna were cut up and incubated for 45 min at 37°C in culture medium containing collagenase I (3mg/ml) and DNAse I (5 U/ml). The suspension was filtered through a steel mesh filter with 140 µm pores after repeated pipetting. The cell suspension was washed with culture medium and centrifuged (800 x G, 10 min). After decanting and resuspension in culture medium, the resulting theca cell pellet was loaded on a Percoll™ 45% gradient and centrifuged (720 x G, 30 min) to remove any red blood cells. Theca cells were then washed twice in culture medium and resuspended after centrifugation.
Viability of the cell suspension was determined by use of trypan blue exclusion dye. Cell viability averaged 78.6 ± 3.6 % at the time of plating.

Theca cell culture

Theca cells were cultured in a serum free culture system. The cells (≈7 x 10⁴ cells per well) were cultured in a total volume of 250 µL DMEM/Nutrient mixture F-12 Ham with 2.5mM glutamine, 14.3mM sodium bicarbonate and 15mM Hepes supplemented with BSA 0.1%, apotransferrin (2.5 µg/mL), sodium selenite (4 ng/mL), gentamycin (20 µg/mL), insulin (10 ng/mL) and ovine LH (0.1ng/mL) in 96-well tissue culture plates (Nunc, Roskilde, Denmark). Palmitic acid, SA and OA were dissolved in pure ethanol (Hinckley et al., 1996; Hirabara et al., 2003), at concentrations of 25, 12.5 or 50 mg/mL, respectively. For the individual fatty acid treatments, PA, SA or OA in ethanol were added in volumes to obtain final test concentrations of 50, 150 or 200µM for PA and of 50, 150 or 250µM for SA and OA. Combination treatments were either a mix of 100 µM of PA, 130 µM of SA and 140 µM of OA (M1) or a mix of 200 µM PA, 260 µM of SA and 280 µM of OA (M2). The composition of the mixtures was based on in vivo experiments which had given us a clear indication of the concentrations and ratios of the individual fatty acids in the serum of high yielding dairy cows in early lactation (Leroy et al., 2004a). The concentrations of PA, SA, and OA in the combination treatments corresponded to the concentrations of these fatty acids present when the total serum NEFA concentration was 500 or 1000 µM, respectively (Leroy et al., 2004a). Control wells received the same volume of ethanol as the test groups with the highest NEFA concentration. Plates were incubated for 48 h in a humidified incubator at 38.5°C in 5% CO₂:95% air. Medium was collected (150µL) at the end of the culture period for hormone analysis and the number of theca cells was measured in each well.

Determination of cell number

The number of theca cells in each well was determined with the CellTiter 96® AQ_{bous} One Solution Cell Proliferation Assay (Promega Benelux, Leiden, The Netherlands). This assay is based on the ability of living cells to reduce the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to a soluble formazan product. The quantity of this product is directly proportional to the number of living cells in culture. Briefly, 20 µL of the MTS solution was added to each well
and incubated for 2h in a humidified incubator at 38.5°C in 5% CO₂:95% air at the end of the culture period and after removal of 150 µL of medium for hormone analysis. The assay plates were read using a multiwell plate reader (Spectrafluor Plus Microplate Reader, Tecan®) at a wavelength of 492 nm after incubation. The relationship between absorbance and cell number was established by incubating 2 x 10⁵ cells with the MTS solution under the same culture conditions. These cells were first cultured for 48 h under the same culture conditions, then rinsed with PBS (without Ca²⁺ and Mg²⁺) and treated with trypsin (0.25%, 10 min at 37°C). Cell aggregates were disrupted by repeated pipetting of the cell suspension and were finally resuspended in culture medium. After determination of the concentration, 2 x 10⁵ cells were resuspended in a total volume of 800 µL medium, and then 160 µL of the MTS solution was added and also incubated for 2h in a humidified incubator at 38.5°C in 5% CO₂:95% air. The solution was serially diluted in culture medium after incubation; triplicate 120 µL aliquots were placed in a 96-well plate and the absorbance was measured at 492 nm. A regression equation was fitted to determine the relationship between absorbance and cell number. For each replicate, this was linear with r >0.99. A standard curve per replicate was constructed for each treatment. Corrections were made for differences in absorbance between test media by subtracting the absorbance values of the test medium without cells incubated with the CellTiter 96® AQueous One Solution Cell Proliferation Assay from the absorbance values of the test wells. The number of cells in each well was estimated from the resulting regression equation.

**Hormone Analysis**

At the end of the culture period, 150 µL medium per well was collected, pooled per treatment and stored frozen (-20°C) until determination of progesterone (P₄) and androstenedione (A₄) concentrations. Progesterone was measured using a RIA as described earlier (Henry et al., 1987). The detection limit was 5 pg and the intra- and inter-assay coefficients of variation were 7.05 % and 8.75 %, respectively. Androstenedione was measured using a commercial RIA (Diagnostic Systems Laboratories Inc., Webster, Texas, USA). This assay had a detection limit of 51 pg/ml with intra- and inter-assay coefficients of variation of 4.7% and 5 %, respectively.
Evaluation of morphology and viability of theca cells

Theca cells (≈6 x 10^5 cells per well) were cultured on round cover slips in 4-well plates under serum free conditions as described above for 48 h and in the absence (controls) or presence of the three fatty acids at different concentrations (M1 and M2 treatment). Apoptosis was induced in the positive control after 36 h by a 12h incubation in the presence of staurosporine (20 µM in ethanol stock solution) at a final concentration of 1 µM. Early and late apoptotic changes were detected using an Annexin V-FITC/Propidium Iodide Vybrant Apoptosis Kit (Molecular Probes, Leiden, The Netherlands). Cells were rinsed with PBS after 48 h and then incubated with 100 µL 1X Annexin Binding Buffer containing 20 µL annexinV-FITC and 2 µL propidium iodide (PI) at room temperature, for 15 min, in darkness. Cells were also stained with Hoechst 33342 (Molecular Probes, Leiden, The Netherlands) to visualize nuclear changes. After incubation, cells were washed with 100µL 1X Annexin Binding Buffer, and the cover slips with the adherent cells were removed from the wells to be mounted upside down on a glass slide. Fluorescence microscopic analysis was performed using a Leica DMR microscope (Van Hopplynus NV, Brussels, Belgium). Healthy cells only stain Hoechst positive, while early apoptotic cells also stain positive for annexin V. Late apoptotic or necrotic cells stain positive for both Hoechst, annexin V and propidium iodide.

Statistical analysis

Each culture experiment was performed four times and triplicate culture wells were used per treatment within each replicate. The experiment was repeated two times for the evaluation of cell morphology and viability by staining. Data were tested for normality and homogeneity of variance prior to statistical analysis and logarithmic transformations were performed when these requirements were not fulfilled.

The effects of PA, SA and OA on proliferation of bovine theca cells were analysed using analyses of variance, with the type of fatty acid as a fixed factor, replicate as a random factor and the number of cells per well as a dependent variable in the model (mixed model). The effects of different concentrations of PA, SA and OA on theca cell P₄ and A₄ production, expressed both per well and per 10^4 cell, were analysed by one-way ANOVA. Pairwise comparisons between the groups were made using the post hoc Scheffé’s test. The viability of the theca cells in the different treatment groups, as determined by the annexin V-FITC/PI
staining method, was analysed using logistic regression models. Differences were considered to be significant if P-values were lower than 0.05. The statistical analyses were performed using SPSS 11.0 (SPSS 11.0 for Windows, Chicago, IL).

Results

Effect of NEFAs on cell proliferation

The number of cells in control wells had fallen a bit below the original plating density of \(\approx 70,000\) cells to \(63,516 \pm 7,010\) cells (mean \(\pm\) SEM) at the end of culture. In the individual fatty acid treatments, no effect on cell proliferation was observed and cell numbers at the end of culture were similar to those in the corresponding controls, with the exception of the 200µM PA treatment (Figure 1A-C). At this concentration, PA had an inhibitory effect on cell proliferation (\(P<0.001\)). It reduced cell numbers by 33.8% compared to controls, and by 37.3% and 32.2% compared to the 50µM and 150µM PA treatments (Figure 1A). Theca cell numbers were lower in the 150 µM SA group than in the 250µM group (\(P<0.05\)) (Figure 1B).

When looking at the effects of both combination treatments, both M1 and M2 reduced cell numbers (\(P<0.001\)) and this effect was not dose dependent (\(P=0.4\)). The number of cells compared to controls was reduced in M1 and M2 by 34.5% and 40.7%, respectively (Figure 1D).
Figure 1 A-D. Effects of different concentrations of palmitic (A), stearic (B) and oleic (C) acid alone or combined (D) on theca cell proliferation after 48 h of culture. The NEFA-combination M1 contains 100 µM of PA, 130 µM of SA and 140 µM of OA and M2 contains 200 µM PA, 260 µM of SA and 280 µM of OA. Data are expressed as mean percentages (± SEM) relative to controls (control wells with ethanol were taken as 100%). Means with different superscripts differ significantly (P<0.05).
Effects of NEFAs on theca cell morphology and viability

After 48 h, theca cells in control wells formed tight clumps of round cells. In NEFA-treated groups and especially in the combination treatments (M1 and M2), theca cells were more dispersed and less aggregated. Cells did maintain their rounded appearance.

In terms of cell viability, more cells were apoptotic (M1) and late apoptotic/necrotic (M1 and M2) in the combination treatments than in the negative controls after 48 h of culture ($P<0.05$) (Figure 2). Also, M2 was more cytotoxic than M1, as indicated by the higher percentage of cells in the late apoptotic/necrotic stage and the lower percentage of healthy and early apoptotic cells ($P<0.05$) (Figure 2). Late apoptotic/necrotic cells were characterized by highly condensed, small pycnotic nuclei, visualised by Hoechst staining. Healthy cells (i.e. no staining by annexin nor propidium iodide) on the other hand had large nuclei with nucleolus, showing no condensation of nuclear material.

![Figure 2](image-url)

**Figure 2.** Effect of different NEFA treatments on theca cell viability after 48 h of culture. Cell viability was assessed through fluorescent microscopic analysis after staining with Hoechst and annexin V-FITC/propidium iodide. The M1 treatment contains 100 µM of PA, 130 µM of SA and 140 µM of OA and M2 contains 200 µM PA, 260 µM of SA and 280 µM of OA. Within each cell viability status, significant differences between treatments are indicated by different superscripts ($P<0.05$).

Effect of NEFAs on progesterone and androstenedione production

Results are shown in Table 1. None of the individual fatty acid treatments had an effect on $P_4$ production by the theca cells. Only M2 inhibited $P_4$ production in the combination...
treatments, both per well (P<0.01) and per 10^4 cells (P<0.05). The M2 decreased P_4 per well by 54.1% and per 10^4 cells by 66.1% compared to controls. A negative trend was noticed on P_4 production per well for M1 (P=0.074). Neither the individual fatty acids, nor any of the combination treatments influenced A_4 production.

**Table 1.** Effect of different concentrations of palmitic, stearic and oleic acid alone or in combination on theca cell progesterone and androstenedione production per well and per 10^4 cells after 48 h of culture.

<table>
<thead>
<tr>
<th>Fatty acid µM</th>
<th>Progesterone ng/well</th>
<th>Progesterone pg/10^4 cells</th>
<th>Androstenedione pg/well</th>
<th>Androstenedione pg/10^4 cells</th>
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<td>203 ± 34^a</td>
<td>698 ± 440</td>
<td>28 ± 16</td>
</tr>
<tr>
<td>M1</td>
<td>2.1 ± 0.5^ab</td>
<td>149 ± 30^ab</td>
<td>636 ± 358</td>
<td>58 ± 42</td>
</tr>
<tr>
<td>M2</td>
<td>0.9 ± 0.1^b</td>
<td>69 ± 17^b</td>
<td>185 ± 115</td>
<td>13 ± 8</td>
</tr>
</tbody>
</table>

Means ± SEM. M1 contains 100 µM of PA, 130 µM of SA and 140 µM of OA and M2 contains 200 µM PA, 260 µM of SA and 280 µM of OA. ^a^b Means with different superscripts within the same column and fatty acid treatment differ significantly (P<0.05).
Discussion

The NEB during the early postpartum period has a negative effect on ovarian activity and fertility of high yielding dairy cows (Butler, 2003). To determine whether this effect may also be mediated through elevated serum concentrations of NEFAs, the in vitro effect of the three major NEFAs on bovine theca cell proliferation and steroidogenesis was tested. Concentrations and ratios of NEFAs used in this study were based on in vivo observations (Leroy et al., 2004a). Although in vivo NEFAs are mainly bound to albumin, dissolved unbound fatty acids were used (Hinckley et al., 1996; Mu et al., 2001; Hirabara et al., 2003; Lu et al., 2003) to avoid counteracting effects between albumin and the fatty acids (Melsert et al., 1991). It has been shown that both forms of fatty acids are taken up by the cells (McArthur et al., 1999).

The results from the present study show that a combination of different NEFAs at concentrations observed during periods of NEB in vivo, negatively affects theca cell proliferation in vitro. This is in agreement with data from a previous experiment that we conducted on bovine granulosa cells (Vanholder et al., 2005). However, in that study as well as in others using luteinized bovine granulosa cells (Jorritsma et al., 2004) and human granulosa cells (Mu et al., 2001) inhibitory effects of the individual fatty acid(s), especially the saturated ones (Vanholder et al., 2005; Mu et al., 2001), on cell proliferation were also observed. In the present experiment on theca cells, only PA at a concentration of 200µM elicited a comparable effect. With exception of the study of Jorritsma et al. (2004) in which a supraphysiological dose of OA was tested, comparable NEFA concentrations were used in the other studies. The fact that we failed to show any effect of the individual fatty acids on theca cells, at concentrations harmful to granulosa cells, is most likely due to differences in cell type and environment. As mentioned previously, granulosa cells are surrounded by the follicular fluid and the basal membrane, being an avascular compartment. The theca interna on the other hand, is rich in blood capillaries. Since NEFA concentrations are approximately 40% lower in the follicular fluid of a dominant follicle than in blood serum (Leroy et al., 2004a), granulosa cells are usually exposed to lower NEFA concentrations than theca cells during the period of NEB. Whether NEFA metabolism differs between granulosa and theca cells is not known. The in vivo situation may relate to the higher in vitro sensitivity of granulosa cells to NEFAs, when compared to theca cells. Also, methodological differences
such as species used, days in culture, and the presence of serum in the medium could account for the differences between our study and the one of Mu et al. (2001).

The reduced cell proliferation observed in the M1 and M2 treatments is probably due to cytotoxic effects of NEFAs, reducing theca cell viability. This is illustrated by the higher percentage of early apoptotic (M1) and late apoptotic/necrotic cells (M1 and M2) in the combination treatments. In the M2 group more cells were late apoptotic/necrotic than in the M1 treatment, indicating that NEFA cell toxicity was concentration dependent. The induction of apoptosis and/or necrosis by NEFAs, individually or combined and at different concentrations, has also been described for other cell types like rat Leydig cells (Lu et al., 2003), nerve growth factor differentiated PC12 cells (Ulloth et al., 2003) and rat pancreatic β cells (Shimabukuro et al., 1998; Cnop et al., 2001; Maedler et al., 2001).

Despite the negative effects of the highest PA, the M1 and the M2 treatments on theca cell proliferation and viability, steroidogenesis was hardly affected. Only the M2 treatment reduced P₄ production, while none of the other treatments had any effect. Androstenedione production was not significantly altered.

Although Stevenson and Robinson (1973) initially demonstrated a stimulatory effect of free fatty acids on ovarian steroidogenesis, more recent research suggests an inhibitory effect. In mouse Leydig cells, NEFAs reduce steroid production by limiting cholesterol availability (Meikle et al., 1989). The effect of NEFAs on testosterone synthesis in mouse Leydig cells is highly dependent on the type, the concentration and the combination of the fatty acid(s) tested, with OA being more inhibitory than SA, and with PA actually being stimulatory. Oleic acid exerts its inhibitory effect through blocking of the LH-stimulated hydrolysis of cholesterol esters used for steroidogenesis (Meikle et al., 1996). Many steroidogenic tissues (Liscum and Dahl, 1992), like human (Azhar et al., 1998) and rodent (Reaven et al., 1995) granulosa cells, use cholesterol esters for steroidogenesis. Therefore, the inhibitory effect of the M2 NEFA mixture on P₄ production in our study could be due to the mechanism described by Meikle et al. (1996). A lack of effect of M1 on P₄ production, despite the reduced cell viability, may be due to the following mechanism. Apoptotic follicle cells maintain their steroidogenesis as long as the steroidogenic organelles remain intact (Amsterdam et al., 1997) and this until complete cell destruction (Amsterdam et al., 2003). During the initial stages of apoptosis (up to 24h after the onset), steroidogenesis is even enhanced due to clustering of the steroidogenic organelles, which increases the efficiency of this process (Keren-Tal et al., 1995; Amsterdam et al., 2003). An initial increase in steroidogenesis in apoptotic cells may mask a reduction in steroidogenesis in the remaining
living cells. At the end of culture, this would result in an apparently similar production per living cell in the treatment and control groups. Being more toxic than M1 (Figure 2), the M2 treatment would cause a faster progression to the final phase of total cell collapse and hence shorten the phase of increased steroidogenesis. In that way, an inhibitory effect of NEFAs on steroidogenesis in living cells could not be masked by the apoptotic cells in the M2 treatment.

Absence of an effect on steroidogenesis of the individual fatty acid and M1 treatments may also be attributed to counteracting effects of the different NEFAs in the mixture, biphasic effects of the fatty acids as described for linoleic acid (Meikle et al., 1989) and the presence of a certain threshold concentration, specific for each NEFA, as shown before by Meikle et al. (1989) and by Vanholder et al. (2005). While the M2 treatment reduced P₄, the A₄ production was unaffected. This may seem contradictory to what Meikle et al. (1989, 1996) observed in Leydig cells. Besides the involvement of other unidentified mechanisms, this may be explained by differences in cell type. The studies of Meikle et al. (1989, 1996) were done using Leydig cells which continued to secrete high amounts of androgens in vitro when stimulated by LH. Theca cells on the other hand, luteinize readily in vitro and this will cause a shift from androgen to progesterone production. Hence, A₄ concentrations at the end of culture were low and variable, while progesterone levels were higher and showed less variation, as illustrated by the smaller SEM in Table 1. Therefore, when culturing theca cells in vitro, it is more realistic to demonstrate an effect of NEFA on P₄ production than on A₄ production. The latter is supported by data from Meikle et al. (1989): no effect of NEFA on testosterone synthesis could be demonstrated when Leydig cells were cultured in the absence of LH because testosterone synthesis was low.

The net result of NEFAs on follicular steroidogenesis remains unclear. The lack of effect on thecal A₄ production in the present study together with previous data on bovine granulosa cells (Vanholder et al., 2005) suggests a slightly positive action. In vivo data on the other hand, have demonstrated a negative relationship between follicular NEFA and oestradiol concentrations in cows (Comin et al., 2002; Jorritsma et al., 2003). In these in vivo studies an energy deficit was elicited by feed restriction. Such a dietary state also altered other parameters, besides NEFAs, that can affect/modulate ovarian steroidogenesis. The latter may help to explain the in vitro versus in vivo results. Further in vitro studies on co-cultures of granulosa and theca cells are indicated to elucidate the overall effect of NEFAs on follicular steroidogenesis.

The negative effect of high NEFA concentrations on theca cell proliferation in vitro, together with the obtained results on granulosa cells (Vanholder et al., 2005), indicate that
NEFAs may play a role in ovarian dysfunction early postpartum. In combination with the low LH-pulsatility and low concentrations of insulin, glucose and IGF-1, NEFAs may be responsible for the reduced growth rate of dominant follicles developing prior to the NEB nadir, as observed by Beam and Butler (1997). Also, NEFAs may be involved in the formation of cystic follicles. Interference with the regulation of cell growth, differentiation and apoptosis may lead to the development of such follicles (Peter, 2004). Namely in the granulosa cell layer and the theca interna of early cystic follicles, cell proliferation was decreased and apoptosis frequency increased (Isobe and Yoshimura, 2000a; 2000b). Our results on theca and granulosa cells (Vanholder et al., 2005) show that NEFAs were capable of causing similar changes in vitro, which may hint their involvement in the pathogenesis of cystic follicles. The latter may also explain the high percentage of cystic follicles diagnosed during the early postpartum period, i.e. when NEFA levels are high.

**Conclusion**

The present study demonstrates that a mixture of the three major NEFAs OA, SA and PA, at physiological concentrations and molar ratios, inhibited bovine theca cell proliferation and decreased cell viability as well as progesterone production. Effects on androstenedione production were however absent. Individual fatty acids, with the exception of PA, did not alter any of the parameters investigated.

An inhibitory effect of NEFAs on theca cell proliferation, together with their detrimental effects on granulosa cells, may therefore be one of the mechanisms through which the NEB may negatively influence folliculogenesis during the early postpartum period in high yielding dairy cows.

**Acknowledgements**

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References


CHAPTER 6

Effects of β-OH-butyrate on bovine granulosa and theca cell function in vitro

Modified from:

EFFECTS OF β-OH-BUTYRATE ON BOVINE GRANULOSA AND THECA CELL FUNCTION IN VITRO
Vanholder T, Leroy JL, Coryn M, Fiers T, de Kruijff A, Opsomer G
REPRODUCTION IN DOMESTIC ANIMALS, IN PRESS
**Summary**

In this study, the effects of \( \beta \)-OH butyrate (BHB) levels, associated with a negative energy balance, on bovine granulosa and theca cell function were investigated *in vitro*. Granulosa and theca cells of healthy large follicles (>8mm), obtained from slaughterhouse ovaries, were cultured in serum free medium containing 0, 0.5, 1 or 1.5 mM BHB and 3 mM glucose, to mimic the situation in the early postparum dairy cow. Hormone concentrations (progesterone, oestradiol-17\( \beta \) and/or androstenedione) in spent medium and cell numbers were measured after 48 h of culture. No effects of BHB on theca cell numbers or steroid production were observed. In granulosa cells, all BHB treatments evenly increased cell numbers (*P*<0.05), while they reduced progesterone and oestradiol-17\( \beta \) production per cell (*P*<0.05). These effects may be attributed to the use of BHB as energy source which is however differently metabolised than glucose. Conclusively, in the presence of physiological glucose concentrations BHB can modulate granulosa but not theca cell function *in vitro*.

**Introduction**

Ovarian activity in the postpartum (pp) dairy cow is strongly influenced by the depth and duration of the negative energy balance (NEB) (Butler, 2003). This effect is mediated through altered hormone and metabolite levels such as non-esterified fatty acids (NEFA) and ketones, which may act directly on ovarian cells (Lucy et al., 2003). *In vitro* high NEFA levels affect follicular cell function (Vanholder et al., 2005) and oocyte maturation (Leroy et al., 2004a) but information on an effect of high ketone levels on ovarian cells is lacking. The aim of the present study was therefore to investigate the effects of \( \beta \)-OH butyrate (BHB) on bovine granulosa and theca cell function *in vitro*. To mimic the situation in the early postparum dairy cow when ketones serve as an alternative energy source, the glucose concentration in culture medium was 3 mM (Leroy et al., 2004b).

**Materials and Methods**

All products were purchased from Sigma (Bornem, Belgium) and Gibco/InVitroGen (Merelbeke, Belgium), unless stated otherwise. Granulosa cells were collected as previously
described (Vanholder et al., 2005) while theca cells were collected according to Meidan et al. (1990). The theca cell suspension was additionally filtered (140 µm pores) and loaded on a Percoll™(Amersham Biosciences, Uppsala, Sweden) 45% gradient to remove red blood cells. Viability of granulosa and theca cell suspensions was determined by use of trypan blue and averaged 57.1 ± 7.9 % and 67.8 ± 9.2 %, respectively, at the time of plating. Granulosa and theca cells (~7 x 10⁴ cells per well) were cultured separately in a total volume of 250 µL DMEM /Nutrient Mixture Ham’s F-10 (1:1 mixture) with 3 mM glucose, 2.5mM glutamine, 14.3mM sodium bicarbonate and 15mM Hepes supplemented with BSA 0.1%, apotransferrin (2.5 µg/mL), sodium selenite (4 ng/mL), gentamycin (20 µg/mL), insulin (10 ng/mL) and porcine FSH (1ng/ml) or ovine LH (0.1ng/mL). Medium also contained 0 (control), 0.5, 1 or 1.5 mM of BHB (monosodium salt). After 48 h in a humidified incubator at 38.5°C in 5% CO₂:95% air, medium was collected and stored frozen (-20°C) until hormone analyses. The number of cells per well was measured with the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Benelux, Leiden, The Netherlands) (Vanholder et al., 2005). Progesterone (P₄) and oestradiol-17β (E₂) concentrations were measured using a RIA (Henry et al., 1987). Androstenedione (A₄) was measured using a commercial RIA (Diagnostic Systems Laboratories Inc., Webster, Texas, USA). Each culture experiment was performed three times and triplicate culture wells were used per treatment within each replicate. Data were analysed using analyses of variance (mixed model with concentration of BHB as a fixed factor, replicate as a random factor). Pairwise comparisons between the groups were made using post hoc Scheffé’s test (SPSS 11.0 for Windows, Chigaco, IL). The level of significance was P<0.05. The results are expressed as the mean ± SEM.

Results

The number of granulosa cells at the end of culture was higher in all three BHB treatments compared to controls (P<0.05), although no dose dependency was observed (Figure 1).

When looking at steroidogenesis, E₂ and P₄ production per well were not affected by treatment. However, E₂ and P₄ production per 10⁴ cells were reduced by all three BHB treatments (P<0.05) and once again this effect was not dose dependent (Table 1). Contrary to the observations in granulosa cells, none of the BHB concentrations had any effect on theca cell proliferation/survival. Steroidogenesis was not affected either by any of the treatments.
Figure 1. Effect of different concentrations of β-hydroxybutyrate (BHB) on granulosa cell numbers (cells per well; mean ± SEM) after 48 h of culture. Means with different superscripts differ significantly ($P<0.05$).

Table 1. Effect of different concentrations of β-hydroxybutyrate (BHB) on granulosa cell oestradiol-17β and progesterone production per well and per 10⁴ cells after 48 h of culture.

<table>
<thead>
<tr>
<th>β-hydroxybutyrate</th>
<th>Oestradiol-17β ng/well</th>
<th>Oestradiol-17β ng/10⁴ cells</th>
<th>Progesterone ng/well</th>
<th>Progesterone ng/10⁴ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>15.3 ± 3.0</td>
<td>5.1 ± 1.0</td>
<td>20.6 ± 4.4</td>
<td>6.1 ± 1.0</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>16.0 ± 3.1</td>
<td>3.7 ± 0.8</td>
<td>16.2 ± 3.2</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td>1 mM</td>
<td>15.0 ± 3.5</td>
<td>3.2 ± 0.9</td>
<td>17.2 ± 3.7</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>1.5 mM</td>
<td>14.2 ± 2.3</td>
<td>3.5 ± 0.7</td>
<td>16.3 ± 3.7</td>
<td>3.3 ± 0.5</td>
</tr>
</tbody>
</table>

Means ± SEM. a,b Means with different superscripts within the same column differ significantly ($P<0.05$).

Discussion

In this study, the effect of BHB on bovine granulosa and theca cell function in vitro was investigated. Treatment with BHB resulted in a higher number of granulosa cells which can be attributed to the use of ketones as an alternative energy source at low glucose levels (Veech, 2004). In vivo, ovarian cells do use ketones as energy substrate (Rabiee et al., 1997). This was further confirmed by repeating the experiment using standard medium with 17.5 mM glucose, where no effect was observed (data not shown). Theca cell numbers were not
increased by BHB supplementation, indicating that they are less affected by low glucose concentrations, as has been observed in lymphocytes as well (Franklin et al., 1991). Granulosa cell steroidogenesis was however reduced by BHB treatments. Although ketones generate energy through metabolism in the Krebs’ cycle, they can not, like glucose, be used in the pentose phosphate pathway (Nehlig, 2004). This pathway yields NADPH, which is required for steroid production (Stryer, 1995). Therefore, it is likely that, due to the higher cell numbers in BHB groups, per cell a shortage of glucose for generation of NADPH occurred, limiting steroid production. Opposite effects on granulosa cell numbers and steroidogenesis, due to diversions in energy metabolism, have also been observed by Rooke et al. (2004).

Conclusively, in the presence of physiological glucose concentrations BHB can modulate granulosa but not theca cell function in vitro. This may be attributed to the use of BHB as an alternative energy source.

Acknowledgements

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References


mRNA transcription levels of insulin receptor isoforms A and B, insulin-like growth factor receptors 1 and 2, and luteinizing hormone receptor in follicular cysts and dominant follicles in the bovine

Modified from:

mRNA TRANSCRIPTION LEVELS OF INSULIN RECEPTOR ISOFORMS A AND B, INSULIN-LIKE GROWTH FACTOR RECEPTORS 1 AND 2, AND LUTEINIZING HORMONE RECEPTOR IN FOLLICULAR CYSTS AND DOMINANT FOLLICLES IN THE BOVINE

Vanholder T, Goosens K, Peelman LJ, Leroy JLMR, Coryn M, de Kruijff A, Opsomer G Reproduction, Submitted
Summary

The objective of the study was to compare transcription levels of mRNA encoding for the insulin receptor isoforms A (IR-A) and B (IR-B), the insulin-like growth factor receptors 1 (IGFR-1) and 2 (IGFR-2) and the LH receptor (LHR) in granulosa and theca cells, between follicular cysts (COF) and dominant follicles (DF). Paired ovaries were collected in the slaughterhouse and theca and granulosa cells were collected per follicle or follicular cyst. Oestradiol and progesterone concentrations were determined in follicular fluid for classification of the follicular cysts as oestrogen-active (COF-E2) or as oestrogen-inactive (COF-P4) and exclusion of atretic follicles. Real-Time RT-PCR was applied to determine and compare mRNA transcription levels. Both for granulosa and theca cells, three stable reference genes were selected to serve as internal controls. No differences in mRNA transcription levels for LHR, IGFR-1, IGFR-2, IR-A and IR-B could be observed between DF and COF-E2. The latter, however, tended to have a higher relative percentage of IR-A in granulosa cells than the dominant follicles (P= 0.059). Oestrogen-inactive cysts had lower thecal LHR mRNA transcription levels and lower IR-A/IGFR2 and IGFR1/IGFR2 mRNA ratios than DF (P<0.05). The relative percentage of IR-A was higher in granulosa cells of COF-P4 than in DF (P<0.01). Compared to COF-E2, COF-P4 had a lower IGFR1/IGFR2 mRNA ratio (P<0.05) and tended to have lower LHR mRNA levels (P=0.054) and a lower IR-A/IGFR2 mRNA ratio (P=0.059) in theca cells. Granulosa cells of COF-P4 had a lower IR-A/IGFR2 mRNA ratio than granulosa cells of COF-E2 (P<0.05). These results show that transcription levels of mRNA encoding for IR’s, IGFR’s or LHR do not differ between DF and COF-E2. Transition from an oestrogen-active to an oestrogen-inactive follicular cyst seems to be associated with changes in transcription levels of mRNA encoding for IR’s, IGFR’s or LHR in granulosa and/or theca cells.
Cystic ovarian follicles (COF) are a common ovarian disorder in high-yielding dairy cows in the early postpartum period. Despite being the subject of much research, the exact etiology and pathogenesis of cyst formation remain unclear. Most research has focussed on the hypothalamus-pituitary and the GnRH/LH release after stimulation by estrogens (reviewed by Garverick, 1997 and by Peter, 2004), although an aberrant follicle growth and differentiation may be involved in cyst formation as well (Peter, 2004). Due to the impossibility to predict a follicle’s fate, it is however extremely difficult to study any follicular changes that precede natural cyst formation. Studies have therefore focussed on differences between cystic ovarian follicles and normal dominant follicles regarding gonadotropin and steroid receptors, steroidogenic enzymes or cell proliferation and apoptosis (Kawate et al., 1990; Odore et al., 1999; Isobe and Yoshimura, 2000a; 2000b; Calder et al., 2001). Although this approach does not prove a cause-effect relationship, it may indicate which follicular functions are altered and are therefore likely candidates to be involved in the pathogenesis of cysts. In women, this approach has helped to elucidate which genes are linked to the PolyCystic Ovary Syndrome (PCOS) (Franks et al., 1997; Urbanek et al., 1999), a disorder which has similarities with COF in cows.

Recently, it was suggested that both insulin (Opsomer et al., 1999; Vanholder et al., 2005) and IGF-1 (Zulu et al., 2002) may play a role in cyst formation in the early postpartum cow. Insulin (Landau et al., 2000; Miyoshi et al., 2001; Butler et al., 2004) and the IGFs (Spicer and Echternkamp, 1995; Schams et al., 2002) are important pro-gonadotrophic factors for follicular growth and development. Besides a direct stimulatory effect, IGF-1 and insulin indirectly stimulate follicular development through upregulation of the LH-receptor on granulosa cells (Davoren et al., 1986). Therefore, alterations in receptor expression for these hormones may lead to an abberant follicle growth and the formation of COF. In addition, two different isoforms of the insulin receptor (IR) have recently been identified in the cow (Neuvians et al., 2003) and the same isoforms were previously demonstrated in man (Seino and Bell, 1989), rat (Goldstein and Dudley, 1990) and sheep (McGrattan et al., 1998). Both isoforms bind insulin and IGF-1 (Mosthaf et al., 1990; Yamaguchi et al., 1991), but IR-A can also bind IGF-2 with a similar affinity than insulin, which IR-B can not. As a result, IR-A can also mediate mitogenic effects of IGF-2, besides metabolic effects of insulin, while IR-B can only do the latter (Frasca et al., 1999; Kalli et al., 2002). Consequently, differences in
expression of IR receptor isoforms may influence the effects of insulin and the IGFs through altered receptor ratios.

However, up to now no information on the transcription levels of mRNAs for these receptors in COF in comparison to normal dominant follicles is available. The objective of this study was therefore to determine whether transcription levels of mRNA for IR isoforms A and B, IGFR1, IGFR2 and LHR differed between normal dominant follicles and follicular cysts and as such could be an indication of their involvement in the pathogenesis of COF.

Materials and Methods

Collection of ovaries and isolation of theca and granulosa cells

Paired ovaries were collected at a local abattoir and immediately transported on ice (4°C) to the laboratory. After washing in saline, ovary pairs were classified as being cystic or non-cystic. Cystic ovaries were defined as having one or more follicles >25 mm in the absence of any luteal tissue. Non-cystic ovaries had follicles <20 mm in the presence of luteal tissue. Healthy large (>10mm) dominant follicles according to the criteria of Kruip and Dieleman (1982) were selected from the non-cystic ovaries. From the cystic ovaries, only follicular cysts with no or just limited luteinisation were selected. Granulosa and theca cells were collected per follicle/cyst. Granulosa cells were collected by follicle puncture and repeated aspiration of the follicular fluid to disrupt the granulosa cell layer. Follicles were then hemisected and gently scraped and flushed with PBS (without Ca^{2+} and Mg^{2+}) to remove remaining granulosa cells. Per follicle, follicular fluid and PBS flushings were collected separately in Venoject® tubes with EDTA as anticoagulant (Terumo Europe N.V., Leuven, Belgium) to prevent clotting. After centrifugation (800 x g, 10 min), a sample of the follicular fluid was collected and stored frozen (-20°C) until hormone analyses to determine follicular health or type of cyst. The granulosa cell pellet was washed with PBS (without Ca^{2+} and Mg^{2+}) and snap frozen in liquid N₂ (-196°C) after centrifugation (660 x g, 10 min). The theca interna was peeled away from the remaining follicle wall under a stereo microscope, washed in PBS (without Ca^{2+} and Mg^{2+}) and homogenized in liquid N₂ (-196°C) with mortar and pestle.

Both granulosa and theca interna samples were stored at -80°C until total RNA extraction.
**Hormone analysis**

Oestradiol-17β (E$_2$) and progesterone (P$_4$) concentrations in follicular fluid were measured to determine follicle health or the steroidogenic status of the follicular cyst. A ratio E$_2$/P$_4$ < 1 in follicular fluid of normal follicles is indicative for atresia (Badinga et al., 1992; Landau et al., 2000) and samples from such follicles were excluded from further analyses. Follicular cysts were classified as oestrogen-active cysts (COF/E$_2$) (E$_2$/P$_4$ > 1 and P$_4$ < 100 ng/ml follicular fluid) or as oestrogen-inactive, progesterone-producing cysts (COF- P$_4$) (E$_2$/P$_4$ < 1 and P$_4$ > 100 ng/ml follicular fluid) (Boryczko et al., 1995). Steroid concentrations were measured by using a RIA, as described earlier (Henry et al., 1987). The detection limit for E$_2$ was 5 pg and the intra- and inter-assay coefficients of variation were 5.75% and 8.30%, respectively. For P$_4$ the detection limit was also 5 pg and the intra- and inter-assay coefficients of variation were 7.05 % and 8.75 % respectively.

**Total RNA extraction and cDNA synthesis**

Total RNA was isolated from theca and granulosa cells using Total RNA Isolation Reagent (TRIR, ABgene, Epsom, United Kingdom) according to the manufacturer’s instructions. After extraction, RNA was dissolved in 10 mM Tris HCl pH 8.0 and quantified at a wavelength of 260 nm by spectrophotometry. The integrity of RNA was verified by optical density (OD) absorption ratio OD$_{260nm}$/OD$_{280nm}$ between 1.8 and 2.0.

For genomic DNA removal an in-solution DNase digestion was carried out by treating 1µg of RNA with 2 units of RQ1 DNase (Promega, Leiden, The Netherlands) followed by a spin-column purification (Microcon YM-100, Millipore, Brussels, Belgium). A minus Reverse Transcription (RT) control was performed with primers for GAPDH to check the removal of all the contaminating genomic DNA.

First-strand cDNA was synthesized from 1µg of RNA using the iScript cDNA synthesis kit (Bio-Rad, Nazareth, Belgium), following the manufacturer’s instructions. The iScript Reverse Transcriptase is a modified MMLV-derived reverse transcriptase and the iScript Reaction Mix contains both oligo(dT) and random primers. After the RT reaction and RT control with primers for GAPDH, the cDNA was diluted fourfold in 10 mM Tris HCl pH 8.0.
Reference gene selection and determination of expression stability

Reference gene selection and primer design

To be able to compare results between samples, internal reference genes have to be selected to normalize results. Eight reference genes (GAPDH, Histone H2A, HPRT1, SDHA, YWHAZ, 18S rRNA, ACTB, TBP) that belong to different functional classes to reduce the chance that the genes might be co-regulated, were selected. Primers used for these reference genes were previously designed and optimised (K Goossens unpublished observations).

Real-Time quantitative PCR and reference gene expression stability

All PCR reactions were performed in a 15 µl reaction volume on the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Nazareth, Belgium) using the Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen, Merelbeke, Belgium) and 200 nM of each specific primer. Per cell type, both cDNA samples from cystic and normal dominant follicles were randomly used. The PCR program consisted of an initial step at 50°C for 2 min, a denaturation step at 95°C for 2 min to activate the Taq DNA polymerase, followed by 45 cycles of denaturation at 95°C for 20 seconds and a combined primer annealing/extension at the specific annealing temperature for 30 seconds during which fluorescence was measured. A melt curve was produced to confirm a single gene-specific peak and to detect primer/dimer formation by heating the samples from 70 to 95°C in 0.5°C increments with a dwell time of 10 seconds at each temperature while continuously monitoring the fluorescence. PCR efficiencies were calculated using a relative standard curve derived from a cDNA mixture (a four-fold dilution series with five measuring points). This cDNA mixture was obtained by pooling cDNA from the individual samples. Each reaction was run in duplicate, including a no-template control.

Reference gene expression stability was determined by using the geNorm Visual Basic application for Microsoft Excel (Vandesompele et al., 2002). By calculation of a relative gene expression stability $M$ and step-wise exclusion of the gene with the highest $M$-value the three most stable reference genes were selected. Due to a low PCR efficiency, the expression stability of ACTB and TBP in granulosa cells, and of 18SrRNA and TBP in theca cells could not be determined.
**Determination of target and selected reference gene transcription levels**

**Target gene primer design**

Primers for target genes were designed with the Primer3 program (URL: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) using the EMBL data base or used according to literature (Table 1) and were commercially synthesised (Sigma-Genosys, Bornem, Belgium). The reported bovine sequences were used and the specificity of the primers was tested using a BLAST analysis (URL: http://www.ncbi.nlm.nih.gov/blast/) against the genomic NCBI database. To check for the formation of secondary structures and hair-pins of the amplified segment at the annealing temperature, M-fold analysis was performed (URL: http://www.bioinfo.rpi.edu/applications/mfold). For the IR isoforms, one primer couple amplified both the IR-B and IR-A isoforms, while the other primer couple only amplified the IR-A isoform by using a boundary spanning (exon 10-12) reverse primer. The relative quantity of IR-B was calculated by subtracting Ct (Cycle threshold) values of IR-A from the respective Ct-values of IR. The PCR products were cloned (pCR 2.1 vector, Invitrogen, Merelbeke, Belgium) and sequenced for verification (Thermo Sequenase Primer Cycle Sequencing Kit, Amersham Bioscience, Roosendaal, The Netherlands) with an ALF Express sequencer (Amersham Bioscience, Roosendaal, The Netherlands). All products showed ≥ 99% homology with the original bovine sequences.

**Table 1.** Target gene transcripts, primer sequences and resulting fragment size.

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequence of nucleotide</th>
<th>Fragment size (bp)</th>
<th>EMBL/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGFR-1</td>
<td>for 5’-AGCGCCTCCAACCTTTGCTCCTCTCCACTTTCT</td>
<td>232</td>
<td>X54890</td>
</tr>
<tr>
<td>IGFR-2</td>
<td>for 5’-CTGCCCTGGTACCGAAGACA</td>
<td>133</td>
<td>NM174352</td>
</tr>
<tr>
<td>IR-A</td>
<td>for 5’-GGAGTCTGCTGGCAGGATG</td>
<td>135</td>
<td>AY574999/Neuvians et al. (2003)</td>
</tr>
<tr>
<td>IR-B</td>
<td>for 5’-GGAGTCTGCTGGCAGGATG</td>
<td>217</td>
<td>AY574999</td>
</tr>
<tr>
<td>LHR</td>
<td>for 5’-CTGGAGAAGATGCCACACGAG</td>
<td>209</td>
<td>U41413</td>
</tr>
</tbody>
</table>
Real-Time quantitative PCR

Real-time quantitative PCR was performed for the target and the three selected reference genes as described above. All samples were assayed for the same gene in the same run, in duplicate, including a no-template control. Per follicle type and per tissue, 5 samples were analysed. As mentioned before, PCR efficiencies were calculated using a relative standard curve derived from a four-fold dilution series of cDNA with five measuring points. This cDNA was synthesized after total RNA isolation from one additional theca or granulosa cell sample.

Calculation of a normalization factor and data transformation

After Real-time PCR, a normalization factor was calculated based on the geometric mean of the three internal reference genes. Per target gene, the Ct values of the samples were transformed to raw quantities according to the comparative Ct method. The normalized expression level of each gene in each sample was calculated by dividing the raw quantity by the appropriate normalization factor as described by the geNorm Visual Basic application for Microsoft Excel (Vandesompele et al., 2002). If the threshold value was not crossed (no Ct value), a Ct value of 45 (maximum value) was assigned to the sample to allow further processing of the data.

Statistical Analysis

All data are presented as mean ± SEM. The normalized gene expression levels were analysed using analyses of variance (SPSS 11.0 for Windows, Chigaco, IL) after logarithmic or square root transformations to correct for departures from normality and homogeneity of variance. Probability values of P<0.05 were considered significant.
Chapter 7

Results

Reference gene expression stability

In granulosa cells of normal dominant follicles and cystic ovarian follicles, the three most stable reference genes of the ones tested were, in order of increasing stability, GAPDH, YWHAZ and SDHA (Figure 1). In theca cells on the other hand, GAPDH was the least stable while SDHA, YWHAZ and HPRT1 were the most stable (Figure 2). Consequently, GAPDH, YWHAZ and SDHA for granulosa cells and SDHA, YWHAZ and HPRT1 for theca cells were selected as internal reference genes for experiment 2.

Figure 1. Average expression stability values (M) of the remaining reference genes during the stepwise exclusion of the least stable one, in bovine granulosa cells.

Figure 2. Average expression stability values (M) of the remaining reference genes during the stepwise exclusion of the least stable one, in bovine theca cells.
Steroid hormone concentrations in follicular fluid

The E\textsubscript{2} and P\textsubscript{4} concentrations in the follicular fluid of the dominant follicles, COF- E\textsubscript{2} and COF- P\textsubscript{4} are summarized in Table 2.

Table 2. Hormone concentrations in the follicular fluid of dominant follicles (DF) (n=5), oestrogen-active cysts (COF-E\textsubscript{2}) (n=5) and oestrogen-inactive, progesterone-producing cysts (COF- P\textsubscript{4}) (n=5).

<table>
<thead>
<tr>
<th>Follicle type</th>
<th>Oestradiol-17\beta (ng/ml)</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DF</td>
<td>290 ± 118</td>
<td>94 ± 39</td>
</tr>
<tr>
<td>COF-E\textsubscript{2}</td>
<td>250 ± 189</td>
<td>44 ± 14</td>
</tr>
<tr>
<td>COF- P\textsubscript{4}</td>
<td>5 ± 1</td>
<td>153 ± 11</td>
</tr>
</tbody>
</table>

Means ± SEM.

Target genes transcription levels

Transcription levels of mRNA for IGFR1, IGFR2, IR-A, IR-B and LHR in granulosa cells

In all granulosa cell samples, all five receptor mRNAs could be demonstrated. For none of the receptors, differences in relative quantities of mRNA could be observed between the DF, the COF-E\textsubscript{2} and the COF-P\textsubscript{4} groups (Figure 3). However, relative percentages of the two IR isoforms did differ between groups: in DF, IR-B made up for 45.9 ± 10.2 % of the total quantity of IR, which tended to be higher than in COF-E\textsubscript{2} (12.4 ± 9.8 %; P=0.059) and was significantly higher than in COF-P\textsubscript{4} (5.5 ± 3.2 %; P<0.05). When looking at ratios of the other receptors that bind the same hormones, there were no clear differences between groups either, except for the ratio IR-A/IGFR2. In granulosa cells of COF-E\textsubscript{2} this ratio was significantly higher than in granulosa cells of COF-P\textsubscript{4} (P<0.05) (Figure 4).

Within each follicle type, transcription levels of mRNA for a certain receptor exhibited a large variation, as illustrated in table 3.
Figure 3. Normalized expression levels of mRNAs for different receptors in granulosa cells of dominant follicles (DF), oestrogen-active cysts (COF-E₂) (n=5) and oestrogen-inactive, progesterone-producing cysts (COF-P₄) (n=5). Data are means ± SEM.

Figure 4. Ratios of normalized receptor mRNA expression levels in granulosa cells of dominant follicles (DF), oestrogen-active cysts (COF-E₂) (n=5) and oestrogen-inactive, progesterone-producing cysts (COF-P₄) (n=5). Data are means ± SEM. Within receptor ratio, bars with different superscripts differ significantly (P<0.05).
Table 3. Normalized expression levels of mRNAs for different receptors in granulosa cells of dominant follicles (DF) (n=5), oestrogen-active cysts (COF-E2) (n=5) and oestrogen-inactive, progesterone-producing cysts (COF-P4) (n=5).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Group</th>
<th>IGFR1</th>
<th>IGFR2</th>
<th>IR-B</th>
<th>IR-A</th>
<th>LHR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF</td>
<td>20 (1.2-27.2)</td>
<td>4.9 (1-10.5)</td>
<td>10.5 (2.3-38.6)</td>
<td>12.6 (2.4-17.7)</td>
<td>90.9 (11.8-172.3)</td>
</tr>
<tr>
<td></td>
<td>COF-E2</td>
<td>14.4 (6.4-26.6)</td>
<td>4.9 (1-12.8)</td>
<td>1.4 (0-4.4)</td>
<td>15.9 (5.4-24.4)</td>
<td>31.5 (4.7-75.2)</td>
</tr>
<tr>
<td></td>
<td>COF-P4</td>
<td>74.4 (1-288)</td>
<td>14.3 (1.9-27)</td>
<td>0.5 (0-1.1)</td>
<td>10.6 (1-14.8)</td>
<td>26.2 (1-47.5)</td>
</tr>
</tbody>
</table>

Means and range.

Transcription levels of mRNA for IGFR1, IGFR2, IR-A, IR-B and LHR in theca cells

Contrary to the granulosa cells, in two theca cell samples of the COF-P4 group no mRNA encoding for the LHR could be demonstrated. All other mRNAs were present in all theca cell samples of the different follicle types.

Transcription levels of mRNA encoding for IGFR-1 were similar in DF and COF-E2 and tended to be higher in DF than in COF-P4 (P=0.079) while no differences were observed between COF-E2 and COF-P4. When looking at IGFR-2, IR-B and IR-A mRNA levels, these were similar in all three follicle types. The LHR mRNA levels on the other hand were significantly higher in DF than in COF-P4 (P<0.05) and tended to be higher in COF-E2 than in COF-P4 as well (P=0.054). No differences in LHR mRNA levels were present between DF and COF-E2 (Figure 5). When looking at receptor ratios, they clearly differed between groups. The IR-A/IGFR2 ratio in COF-P4 was significantly lower than in DF (P<0.01) and tended to be lower than in COF-E2 (P=0.059). No difference between DF and COF-E2 was observed. The IGFR1/IGFR2 ratio in COF-P4 was also significantly lower than in DF (P<0.01) and than in COF-E2 (P<0.05), while once again ratios in DF and COF-E2 were not different (Figure 6). The relative percentages of the two IR isoforms did not differ between groups and overall averaged 75% for IR-A and 25% for IR-B.

Also in theca cells, transcription levels of mRNA for a certain receptor showed a large variation within each follicle type.
Figure 5. Normalized expression levels of mRNAs for different receptors in theca cells of dominant follicles (DF), oestrogen-active cysts (COF-E2) (n=5) and oestrogen-inactive, progesterone-producing cysts (COF-P4) (n=5). Data are means ± SEM. Within receptor type, bars with different superscripts differ significantly (P<0.05).

Figure 6. Ratios of normalized receptor mRNA expression levels in theca cells of dominant follicles (DF), oestrogen-active cysts (COF-E2) (n=5) and oestrogen-inactive, progesterone-producing cysts (COF-P4) (n=5). Data are means ± SEM. Within receptor ratio, bars with different superscripts differ significantly (P<0.05).

Discussion

In the present study we have quantitatively compared mRNA transcription levels of several receptors, important for follicle growth and development, between dominant follicles
and COF by using Real-Time PCR. To be able to determine transcription levels in both granulosa and theca cells, dominant follicles and cystic follicles were obtained from slaughterhouse ovaries. The only drawback of this set up is that the previous history of the animal, and more specifically the “age” and possible treatment of the cyst are unknown.

When performing Real-Time quantitative PCR, selection of appropriate internal control genes (endogeneous genes) is necessary to be able to correct for differences in amount of starting material, enzymatic efficiencies and overall transcriptional activity in different cell types. These internal control genes should not vary in the investigated cells or tissues (Vandesompele et al. 2002). Many studies use only one control gene, without proper validation of its expression stability. Endogeneous gene expression can however vary considerably, as has been shown for GAPDH (reviewed by Bustin 2000). Moreover, at least three proper control genes should be used for calculation of a normalization factor. We have therefore determined the expression stability of 6 internal control genes in theca and granulosa cells of dominant follicles and COF in a first experiment by using the geNorm Visual Basic application for Microsoft Excel (Vandesompele et al. 2002). Expression stability of these internal control genes did differ between the two tissues, although SDHA and YWHAZ were the most stable genes in both cell types. Consequently, these two reference genes together with GAPDH for granulosa cells and HPRT1 for theca cells were used as internal control genes when determining trancription levels of the target genes.

All 5 receptors’ mRNA was observed in both granulosa and theca cell samples of dominant follicles and COF. However, in two theca cell samples of COF-P4 no mRNA for the LHR could be demonstrated. The expression of LHR mRNA (Calder et al., 2001), IGFR-1 mRNA (Armstrong et al., 2000; Schams et al., 2002) and IGFR-2 mRNA (Spicer et al., 2004) in bovine follicles has previously been demonstrated, as well as the presence of mRNA for both IR isoforms in corpus luteum (Neuvians et al., 2003). However, the expression of IR-A and IR-B mRNA in bovine granulosa and theca cells has, to our knowledge, never been reported before. Overall, the relative percentages of the two IR isoforms were similar to those reported in granulosa cells of normal and cystic human follicles (Phy et al., 2004), with approximately 75-80 % of the total IR mRNA being IR-A mRNA.

In granulosa cells, no differences in mRNA transcription levels for any of the receptors could be detected between DF and COF. In theca cells, mRNA transcription levels of receptors were similar between groups as well, except for IGFR-1 and LHR mRNA levels which were higher in DF than in COF-P4. Our results on the LHR disagree with certain
previous publications, but agree with others. Kawate et al. (1990) concluded that fewer LH receptors were present in granulosa cells of follicular and luteinized cysts and in theca cells of follicular cysts when compared to normal follicles, but this is contradicted by data from Odore et al. (1999). They observed similar receptor concentrations in follicular cysts and dominant follicles and actually lower concentrations in dominant follicles in comparison to luteinized cysts. The major difference with our study is that both Kawate et al. (1990) and Odore et al. (1999) demonstrated actual receptor presence, while we have monitored receptor mRNA transcription. The presence of mRNA does not guarantee actual receptor expression, since posttranscriptional modulations may occur. On the other hand, oestrogen and progesterone concentrations in the cysts we classified as COF-E2 were a lot higher, respectively lower than in the cysts classified as follicular by Kawate et al. (1990) and Odore et al. (1999). In addition, we only selected DF with an E2/P4 >1 in follicular fluid, to prevent inclusion of atretic follicles (Badinga et al., 1992; Landau et al., 2000), which was not done by Odore et al. (1999). Consequently, differences in steroidogenic activity in cysts and in follicular health of DF may account for some of the contradictions between studies.

Calder et al. (2001), who demonstrated mRNA transcripts by in situ hybridization, did observe increased LHR mRNA levels in granulosa cells of E2-producing cyst. However, they obtained COF from live animals shortly after cyst formation while in our study the “age” of the cysts was unknown. Consequently, receptor mRNA levels may have been down regulated by the continuously high LH concentrations in cystic animals (Hamilton et al., 1995), resulting in steroidogenically less active cysts as indicated by the lower oestrogen concentrations in the follicular fluid in the COF-E2 in our study. On the other hand, Calder et al. (2001) also observed lower or even absent LHR mRNA transcripts in theca and granulosa cells of COF-P4.

Currently we are aware of only one publication (abstract) on IGF receptors in COF of cattle. Greenaway et al. (2004) observed reduced IGFR-1 expression in granulosa cells of cystic follicles, while we saw no differences in mRNA levels. In our study, only theca cells of COF-P4 tended to have lower levels than DF. Due to the limited information in the abstract, no explanation for the differences with our study can be given. In women with PCOS, no alterations in follicular IGFR-1 and -2 expression could be observed (el-Roeiy et al., 1994; Voutilainen et al., 1996). Although COF and PCOS have as many differences as similarities, these reports in women support our findings in cows.

As mentioned before, no publications on IR-isoform expression in bovine follicles and cysts are available. In women with PCOS, overall IR mRNA transcription in follicle cells is
increased, while transcription levels of the individual isoforms were not different (Phy et al., 2004). The latter results support our findings in the present study, although extrapolation of results between PCOS and COF has to be done with caution due to endocrinological differences (i.e. insulin resistance) which may affect receptor expression. In our study, the relative abundance of the IR-isoforms in granulosa cells, but not in theca cells, did differ between the groups with the percentage of IR-A being higher in COF than in DF. Based on the functional differences between the IR-A and IR-B isoforms (Frasca et al., 1999), this may indicate that granulosa cells of COF are more mitogenically stimulated than granulosa cells of DF. The latter may require more metabolic stimulation by insulin for the production of the oestrogen surge to elicit the pre-ovulatory LH surge.

The lower ratios of IR-A/IGFR2 and of IGFR-1/IGFR-2 in granulosa and/or theca cells of COF-P4 compared to COF-E2 and/or DF are most likely an indication of a reduced proliferative activity in COF-P4. The mitogenic effects of IGF-2 are mediated through IR-A and IGFR-1 (Frasca et al., 1999; Neuvians et al., 2003). The IGFR-2 on the other hand acts as a regulator of circulating IGF-2 levels: after binding of IGF-2 to the receptor the complex is internalized and degraded (Jones and Clemmons, 1995). Consequently, increased expression of IGFR-2 in comparison to IR-A and IGFR-1 will result in more internalisation and degradation of IGF-2. As a result, less IGF-2 will be available to bind on IR-A and IGFR-1 to stimulate cell mitogenesis. This hypothesis is supported by data from Isobe and Yoshimura (2000b) who observed less mitogenic activity in COF.

In conclusion, no obvious differences could be observed between DF and COF-E2, although the latter tended to have a higher relative percentage of IR-A in granulosa cells than dominant follicles. Progesterone producing cysts had lower mRNA transcription levels and ratios for certain receptors in comparison to DF and COF-E2. Our results suggest that COF formation is not associated with altered transcription patterns of mRNA encoding for IR’s, IGFR’s or LHR. Transition from an oestrogen-active to an oestrogen-inactive follicular cyst seems to be associated with changes in transcription levels of mRNA encoding for IR’s, IGFR’s or LHR in granulosa and/or theca cells.

Acknowledgements

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References


CHAPTER 8

General Discussion
The scope of the present thesis was to gain further insight in the aetiology of cystic ovarian follicles (COF) in high yielding dairy cows. More specifically the first aim was to elucidate the role of the negative energy balance (NEB) in cyst formation, especially focussing on how altered metabolite and hormone concentrations may affect follicular growth and development at the ovarian level. The second aim was to determine whether gene transcription levels of receptors for hormones important for follicular growth and development, differ between cystic follicles and normal dominant follicles, and could therefore be likely candidates to be involved in the pathogenesis of COF.

Despite the abundance of scientific articles on COF, it was still unclear if and how metabolic adaptations, associated with a NEB through a high milk yield, do influence cyst formation. Therefore in the first study, we compared hormonal and metabolic profiles between animals developing a cyst and animals ovulating a dominant follicle, in the first 60 days post partum (pp) (Chapter 4). By monitoring the cows in this early postpartum period, we were able to compare hormone and metabolite serum concentrations prior to cyst formation or ovulation i.e. during the final stages of follicle growth.

Our results show that no clear metabolic and hormonal alterations precede cyst formation, except for insulin. Serum insulin concentrations were significantly lower in the period immediately prior to cyst formation than prior to ovulation of a dominant follicle. These findings suggest that insulin may be an important metabolic factor in the pathogenesis of COF, as was already hinted in a previous study demonstrating an impaired insulin secretion after an intravenous glucose challenge in COF cows (Opsomer et al., 1999). This hypothesis is further supported by in vivo data demonstrating an important role for insulin in postpartum ovarian function (Miyoshi et al., 2001; Balogh et al., 2003; Butler et al., 2004). Based on the known stimulatory effects of insulin on follicle cells, both in vitro (Davoren et al., 1986; Spicer and Echternkamp, 1995; Gutiérrez et al., 1997; Spicer et al., 2002) and in vivo (Matamaros et al., 1990; Simpson et al., 1994), and its importance in follicular maturation (Landau et al., 2000), low insulin concentrations may trigger cyst formation through insufficient stimulation of follicle cell proliferation and steroidogenesis. The latter may cause a retarded maturation/growth of the dominant follicle and insufficient oestradiol-17β production. The altered follicular growth pattern and oestradiol-17β production will disrupt the hypothalamo-pituitary-gonadal axis through insufficient positive feedback. This would
finally result in an aberrant LH-surge and the subsequent development of a cystic follicle. However, we did not observe lower oestradiol-17β concentrations in cystic cows, but this may be attributed to the study design. Due to practical restrictions, the number of COF cows and the sampling frequency (2x/week) in our study were limited, which may have masked such subtle differences.

Besides a direct effect on ovarian cells, low insulin concentrations may also indirectly affect follicle growth by reducing GnRH and LH pulse secretion from the hypothalamus-pituitary (Miller et al., 1995; Miller et al., 1998; Tanaka et al., 2000) (Figure 1).

Lower insulin concentrations may be the phenotypic link between production and COF. Insulin concentrations are lower in cows selected for milk yield (Bonczek et al., 1988), and insulin responsiveness to a glucose challenge is also lower in lactating than in nonlactating animals (Sano et al., 1993). Low insulin concentrations reduce glucose uptake by the peripheral tissues, thereby increasing glucose availability for milk production by the udder. Since insulin is not required for glucose uptake by the udder, the mammary gland is guaranteed sufficient glucose for milk synthesis (Collier, 1985). Further selection for milk yield would imply animals with lower insulin concentrations and, based on our results, an increased prevalence of COF.
Figure 1. Schematic model of how low insulin concentrations may cause cyst formation. Low insulin concentrations insufficiently (+) stimulate follicle cell proliferation and oestradiol-17β production. The reduced oestradiol-17β feedback, together with the low insulin concentrations result in a reduced gonadotropin release. Dominant follicle growth is retarded and the altered follicular growth pattern and oestradiol-17β production disrupt the hypothalamo-pituitary-gonadal axis. This finally results in an aberrant LH-surge and the subsequent development of a cystic follicle.

The lactations in which COF developed in our study, were not characterized by a higher milk production i.e. more energy expenditure. Milk yield was however not corrected for fat and protein content and together with the limited number of animals, this may have masked any differences in actual energy expenditure through milk yield. On the other hand, yield on its own is perhaps not a reliable parameter to assess the metabolic stress on an animal, since a higher production (energy loss), is usually compensated by an increased feed intake (energy intake) (Lucy, 2001).

The absence of metabolic alterations other than insulin prior to cyst formation does not exclude their involvement in the pathogenesis of cysts. Due to practical restrictions in the
study design, subtle alterations may have gone unnoticed. Up to ovulatory size the development of COF and normal follicles seems very similar (Beam, 1995; Calder et al., 2001). Consequently, any metabolic differences, if present, are expected to be subtle, since severe metabolic alterations will result in cessation of follicle growth, instead of development of COF (Butler, 2003). Hence, other metabolic parameters, besides insulin, may be involved as well. In addition, not only absolute concentrations are important, also the duration of the period during which these parameters are altered is known to play a decisive role in the development of COF (Beam, 1995). Other studies do, however, report increased NEFA concentrations during one or more weeks pp in cows developing ovarian cysts (Huszenicza et al., 1988; Zulu et al., 2002). Discrepancies between studies may be attributed to differences in study design, frequency of sampling and definition of COF.

The results of our first study do not indicate the involvement of reproductive hormones in the pathogenesis of ovarian cysts, although a role for slightly elevated, so called suprabasal, progesterone concentrations can not be confirmed nor dismissed. The number of cyst cases which developed in the presence of such progesterone profiles was limited and due to the restrictions of the study design other parameters like LH, which are affected by progesterone and play a role in cyst formation, were not determined. While suprabasal progesterone concentrations are likely to be associated with cyst turn-over (Hatler et al., 2003), their involvement in primary cyst formation, if at all, seems to be limited to a minor percentage of the cases.

Although we were not able to demonstrate this, metabolites such as NEFAs may be involved in cyst formation either through a longer period with elevated concentrations (Beam, 1995) and/or through actually higher concentrations (Huszenicza et al., 1988; Zulu et al., 2002). However, the mechanism through which increased NEFA concentrations can promote cyst development is not known. It has been shown that they can act as metabolic messengers, affecting gonadotropin secretion at the hypothalamic-pituitary level (Canfield and Butler, 1990). However in human medicine, several studies have demonstrated that elevated NEFA concentrations affect the functioning and viability of different cell types (Shimabukuro et al., 1998; Cnop et al., 2001; Lu et al., 2003; Ulloth et al., 2003), such as follicle cells, even after a limited period of time (Mu et al., 2001). Since high yielding dairy cows experience a prolonged pp period of NEB with elevated NEFA concentrations (Butler, 2003), NEFAs may affect ovarian function by a direct action on follicle cells as well. No information on this subject was, however, available. Therefore we studied the effect of physiological NEFA
concentrations on bovine granulosa and theca cell proliferation, viability and steroidogenesis \textit{in vitro} (Chapter 5). \textit{In vivo}, a close interaction exists between granulosa and theca cells within the follicle. Theca cells produce androgens, which diffuse through the basal membrane to the granulosa cells where they are converted to oestrogens. In addition, theca and granulosa cells interact and secrete substances which promote each other’s growth and differentiation (Yada et al., 1999; Tajima et al., 2002). Despite their close interaction \textit{in vivo}, the effect of NEFAs on bovine granulosa and theca cells was examined in two separate studies. We based this experimental set-up on \textit{in vivo} data demonstrating that the ratios and concentrations of the individual NEFAs differ between physiological compartments within the follicle. Granulosa cells are surrounded by the follicular fluid and the basal membrane, being an avascular compartment. The theca interna cells on the other hand are embedded in a dense network of blood capillaries. Leroy et al. (2005) demonstrated that both the overall NEFA concentration and the ratios of the individual fatty acids differ between follicular fluid and blood. To be able to subject granulosa and theca cells to different NEFA concentrations and ratios, separate cultures had to be used.

Our results revealed that treatment with physiological NEFA concentrations reduced both granulosa (Chapter 5.1) and theca cell numbers (Chapter 5.2) after an incubation of 48h. Saturated fatty acids, and especially palmitic acid, were more potent in reducing cell numbers with granulosa cells seeming to be more sensitive to the effect of NEFAs than theca cells. The latter may be attributed to the \textit{in vivo} situation where granulosa cells are exposed to lower NEFA concentrations than theca cells. Concentrations in follicular fluid are on average 40% lower than in blood serum (Leroy et al., 2005) which could explain the higher sensitivity of granulosa cells \textit{in vitro}. To further elucidate how NEFAs exerted their inhibitory effects on cell numbers, granulosa and theca cells were stained with annexin/propidium iodide to reveal whether NEFA treatment affects cell viability and induces apoptosis. The induction of apoptosis, alone or in combination with the induction of necrosis, seems to be the mechanism through which NEFAs exert their effects on several different cell types (Shimabukuro et al., 1998; Cnop et al., 2001; Lu et al., 2003; Ulloth et al., 2003). This was also the case in our studies as incubation of granulosa and theca cells with a combination of the different fatty acids for 48h resulted in reduced numbers of healthy, viable cells. Simultaneously, early stages of apoptosis and late apoptotic/necrotic cells were present, clearly illustrating the cytotoxic effect of elevated physiological NEFA concentrations.

Despite the cytotoxic effect on granulosa cells, oestradiol-17\(\beta\) production per metabolically active cell was increased. Although this may seem contradictory, it has been
shown that apoptotic granulosa cells maintain their steroidogenesis as long as the steroidogenic organelles remain intact (Amsterdam et al., 1997) and this until complete cell destruction (Amsterdam et al., 2003). Up to 24h after the onset of apoptosis, granulosa cells actually show an enhanced steroidogenesis due to clustering of the steroidogenic organelles, which increases the efficiency of this process (Keren-Tal et al., 1995; Amsterdam et al., 2003). However, in our culture system, granulosa cells had an excess of androstenedione for oestrogen production at their disposal, which may not be the case in vivo. In vivo, granulosa cells are dependent on androgens from theca cells for oestradiol-17β production. Androstenedione production by theca cells was, however, not affected by NEFA treatments: only progesterone production was reduced by the highest NEFA combination treatment.

Although extrapolation of in vitro results to the in vivo situation should be done with great care, these in vitro results indicate that NEFAs may affect ovarian functioning and possibly cyst formation through an additional direct effect on follicle cells. By decreasing cell viability and induction of apoptosis, follicular growth and development may be retarded. In addition, if the in vitro increased oestradiol-17β production also occurred in vivo, an immature follicle would produce too much oestrogen too soon. Higher oestrogen concentrations prior to cyst development have been observed by Beam (1995), although we were not able to clearly show this in our first study. Together, reduced follicle growth and/or increased steroidogenesis may disrupt the hypothalamic-pituitary-gonadal axis, possibly resulting in the formation of a cystic follicle. Peter (2004) also suggested that interference with the regulation of cell growth, differentiation and apoptosis may lead to the development of cystic follicles.

**Figure 2.** Hypothetical model of how increased NEFA concentrations may cause cyst formation. By decreasing cell viability and induction of apoptosis, follicular growth and development are retarded. If oestradiol-17β production is increased, an LH-surge will be elicited prematurely during follicle growth i.e. the follicle is not capable of responding with ovulation and may become cystic. If oestradiol-17β production is not or just slightly increased, the follicle will insufficiently exert a positive feedback on the hypothalamus-
pituitary for maximal LH release. As a result, the final LH-surge does not occur or its magnitude is insufficient to trigger ovulation. The follicle then becomes cystic.

Besides a direct effect on ovarian cells, NEFAs may also indirectly influence follicular growth and development by reducing receptor expression and interfering with receptor-ligand interactions at the cell surface. By affecting the structure and fluidity of cell membranes (MacDonald and MacDonald, 1988) and by altering the membrane surface charge in rat ovarian luteal cells (Kolena et al., 1999), NEFAs are capable of destabilizing the ovarian LH receptor, and reducing its binding capacity of LH (Scsukova et al., 2000; Kolena et al., 2002). In addition, NEFAs induce insulin resistance in peripheral tissues in humans (Van Epps-Fung et al., 1997; Boden, 2001). These additional effects of NEFAs may also affect follicular growth and development, since especially LH and insulin are important for the final maturation and ovulation of the dominant follicle.

In addition to the interference of NEFAs on insulin signalling in target tissues, free fatty acids are also capable of modulating insulin secretion by the pancreas (Mason et al., 1999; Maedler et al., 2001; Gravena et al., 2002). Prolonged exposure of rat islets of Langherhans to physiologically elevated NEFA concentrations reduced insulin secretion in response to stimulation by glucose (Mason et al., 1999). Whether a similar effect is exerted in cattle remains to be determined.

Conclusively, our in vitro data together with the in vivo observations of Beam (1995), Huszenicza et al. (1988) and Zulu et al. (2002) indicate that NEFAs are likely to be involved in the pathogenesis of COF. Several pathways may be involved, but at the level of the ovary a negative effect may already be exerted. Further research is needed to clarify the role of NEFAs in cyst formation. Especially the interaction with insulin in the pp dairy cow needs further clarification.

Besides increased NEFA concentrations, the early pp period in high yielding dairy cows is also characterized by elevated ketone concentrations in serum. Ketones have been identified as a risk factor for delayed cyclicity (Reist et al., 2000) and it has been shown that they can modulate functioning of cells of the immune system (Franklin et al., 1991; Nonnecke et al., 1992; Hoeben et al., 1997; Sartorelli et al., 1999; Sartorelli et al., 2000; Lacetera 2002). To reveal whether they are capable of influencing follicular cell function, we studied the effect of increased β-OH-butyric acid (BHB) concentrations, the main ketone body in the pp dairy cow, on bovine granulosa and theca cell function in vitro (Chapter 6). At physiological
glucose concentrations, BHB actually enhanced granulosa cell survival, probably by serving as an alternative energy source. This hypothesis is supported by the lack of BHB effect when cells were cultured in a high glucose medium, i.e. when energy availability was sufficient. Theca cells were hardly affected by BHB, indicating that they may be less sensitive to low glucose concentrations than granulosa cells. Steroidogenesis of theca cells was not affected while in granulosa cells production per cell was reduced. This can be attributed to differences in metabolism of ketones compared to glucose. Although ketones generate energy through the Krebs’ cycle, they can not, like glucose, be used in the pentose phosphate pathway (Nehlig, 2004). This pathway yields NADPH, which is required for steroid production (Stryer, 1995). Therefore, it is likely that, due to the higher cell numbers in BHB groups, a shortage of glucose for generation of NADPH occurred, limiting steroid production per cell. The lack of a negative effect of ketones on cell function is also reported by Lacetera et al. (2002). They showed that in sheep, immune cell function was altered by high NEFA concentrations, but not by high BHB concentrations.

In conclusion, our in vitro data imply that despite the association of increased ketone concentrations with delayed ovarian cyclicity, as shown by others, ketones themselves do not exert any negative effects on ovarian cells. Consequently, ketone concentrations in the pp dairy cow seem to be an indicator of the severity of NEB, but not a mediator of the negative effects of the NEB on reproduction.

Considering the overall picture in the early pp high yielding dairy cow when glucose and insulin concentrations are reduced, and ketone and NEFA concentrations are increased, the following hypothesis may apply: follicle cells will try to compensate for the low glucose by using ketones as alternative energy substrate. Due to restrictions in the metabolism of ketones, follicular steroidogenesis may be reduced. Low insulin concentrations and the possible induction of insulin resistance by high NEFA concentrations may contribute to the reduced follicle growth and steroidogenesis by insufficient stimulation of glucose uptake and cell proliferation. In addition, the elevated NEFA concentrations may exert a toxic effect on the follicle cells and may further reduce insulin release from the pancreas, worsening the situation. Consequent reduced follicle growth and steroidogenesis may insufficiently stimulate GnRH/LH release from the hypothalamus-pituitary. Depending on the severity of the metabolic alterations, the dominant follicle may undergo atresia or develop into a cyst due to the inability to elicit an LH-surge or to elicit one of sufficient magnitude.
Although a genetic background for COF has been demonstrated (Kirk et al., 1982; Cole et al., 1986; Hooijer et al., 2001), it is not clear which genes are involved. The difficulties in studying the underlying genetics of COF are the lower heritability and the unpredictability of the disorder. A cow which is genetically predisposed to develop COF, does not do so in every lactation, nor in every ovarian cycle. Consequently, accurately classifying an animal as a “COF-cow” or as a “normal” cow is almost impossible. Therefore, it is not feasible to study differences in gene expression at cow level. However, studying gene expression patterns in COF in comparison with normal dominant follicles can be valuable. Despite the inability to demonstrate a cause-effect relationship, this approach may indicate which genes have an altered expression profile and are therefore likely candidates to play a role in the pathogenesis of COF. In women, this approach has helped to elucidate which genes are linked to PCOS (Franks et al., 1997; Urbanek et al., 1999), a disorder with some similarities with COF.

When comparing gene expression patterns between populations (COF versus normal follicles), two different approaches are possible. A first approach is to look at which genes are expressed in both populations using techniques like cDNA micro–arrays and differential display. By subsequent cloning and sequencing, the genes that are differentially expressed, can be identified. This approach has a high-throughput (simultaneous quantification of thousands of genes), but is labour intensive, rather expensive and only allows for the detection of large differences in gene expression (Ding and Cantor, 2004). Changes in gene expression between COF and normal dominant follicles can, however, be expected to be subtle since the development of future COF and normal follicles up to the ovulatory size seems to be similar (Beam, 1995; Calder et al., 2001).

The second approach determines whether the expression patterns of certain selected genes differ between two populations by using fine-scale, more accurate and specific RNA quantification methods like Real-time PCR (Ding and Cantor, 2004). This method allows for the detection of relatively small expressional changes, but does require the selection of a certain number of candidate genes. Based on the importance of insulin and the IGF’s for follicular growth and development during the early pp period (Butler, 2003; Butler et al., 2004), and the association of insulin (Chapter 4) and IGF-1 (Zulu et al., 2002) with cyst formation, the genes encoding for their receptors were selected as candidate genes, together with the LH receptor gene. The transcription levels of the different mRNAs encoding for these receptors were determined in COF and dominant follicles by using real-time PCR (Chapter 7). This novel PCR technique enables a quantitative comparison of results between populations, which is not possible when using classical RT-PCR. Before performing real-time
PCR, accurate selection of internal control genes per tissue/cell type is however necessary to correct for differences in amount of starting material, reaction efficiency etc. between the different groups. Consequently, six commonly used reference genes were selected from literature and by using the geNorm Visual Basic application for Microsoft Excel (Vandesompele et al., 2002), the three most stable genes in theca interna and granulosa cells were selected.

For the final experiment, cystic follicles and dominant follicles were obtained from slaughterhouse ovaries. Although in this situation, the previous history of the animal, and more specifically the “age” of the cyst are unknown, it enables the determination of mRNA transcription levels in both granulosa and theca cells. Hormone concentrations in follicular fluid were determined to accurately classify follicles as healthy and to classify COF as oestrogen- or progesterone-producing.

Our results revealed that transcription levels of mRNA encoding for the insulin receptors type A (IR-A) and B (IR-B), IGF receptors type 1 (IGFR-1) and 2 (IGFR-2) and LH receptor (LHR) did not differ between dominant follicles and oestrogen-producing cysts. The relative percentages of IR-A and IR-B did tend to differ between dominant follicles and oestrogen-producing cysts, with IR-A being higher and IR-B being lower in oestrogen-producing cysts. Progesterone-producing cysts had reduced LHR mRNA quantities and lower IR-A/IGFR2 and IGFR1/IGFR2 mRNA ratios compared to dominant follicles and oestrogen-producing cysts. These alterations were most likely indicators of follicular degeneration. The reduced expression of the LHR in progesterone-producing cysts is in accordance with previous studies (Brown et al., 1986; Calder et al., 2001) and is an indication of the loss of oestrogenic capacity and the chronic nature of the cyst.

Publications on receptor expression or receptor mRNA expression in COF and dominant follicles are scarce. Only the expression of the LH receptor or its mRNA has been studied in a few other papers (Brown et al., 1986; Kawate et al., 1990; Odore et al., 1999; Calder et al., 2001), which either contradict or agree with our results. The contradictions may be attributed to differences in experimental design such as the hormonal classification of the cysts/follicles, demonstration of the receptor or just its mRNA, and how cysts were obtained. Only one publication (abstract) on expression of IGF receptor type 1 in COF exists (Greenaway et al., 2004), but due to the limited information in this abstract a comparison of results between studies is difficult. To our knowledge, no publications on insulin receptor-isoform expressions in bovine follicles and/or cysts are currently available.
In human medicine, IGFR-1 and -2 (el-Roeiy et al., 1994; Voutilainen et al., 1996) and IR-A and IR-B expressions (Phy et al., 2004) have been studied in cystic follicles from PCOS women, with IR-A and IR-B showing an equally increased expression. Although the latter contradicts our findings in cattle, extrapolation of results between PCOS and COF has to be done with great caution due to the many, especially endocrinological differences (i.e. insulin resistance) which may affect receptor expression. Therefore, more research is needed to confirm our findings on the IGF and insulin receptors in COF in cattle. Especially the relative abundance of the two insulin receptor isoforms needs further investigation, since this tended to differ between dominant follicles and COF. Based on the functional differences between the IR-A and IR-B isoforms (Frasca et al., 1999), an altered ratio of the two isoforms may affect follicle development and function. An interesting approach for future research would be to collect granulosa cells from COF early pp through transvaginal puncture and aspiration. Although this method does not allow for the study of expression patterns in theca cells, information on the endocrinological and metabolic status of the animal would be available through analyses of blood and follicular fluid samples.

In summary, we were unable to show that alterations in transcription levels of mRNAs encoding for the insulin receptor A and B, the IGF receptors 1 and 2 and the LH receptor are likely to play a role in primary cyst formation. The transition from an oestrogen-producing cyst to a progesterone-producing cyst does however seem to be associated with changes in transcription levels of mRNA encoding for the LH receptor and altered receptor mRNA ratios in granulosa and/or theca cells.

**Overall conclusion and future research**

In conclusion, in this thesis it has been demonstrated that COF formation during the early pp period is associated with lower insulin concentrations during the final phase of dominant follicle growth. In addition, NEB may also promote cyst formation through increased NEFA concentrations that exert a direct negative effect on follicle cells. Increased BHB concentrations do not adversely affect follicle cell function and in a low glucose environment may promote follicle cell survival. No obvious alterations in mRNA expression for the insulin, IGF and LH receptors seem to be associated with COF formation, although the ratio of the two insulin receptor isoforms tended to differ between cysts and dominant follicles.
Several issues described and discussed in this thesis need further investigation and clarification to increase understanding of the aetiology of COF, which is still rather limited.

The association of low insulin concentrations with COF, demonstrated in Chapter 4, needs to be confirmed by others. Also, the reason why cows developing COF had reduced insulin concentrations is not clear and therefore needs to be studied more in detail. Several possibilities exist. The first one is that COF cows are more genetically selected for a high milk yield and consequently secrete less insulin. As mentioned before, selection for milk yield is linked to lower insulin concentrations (Bonczek et al., 1988). A second possibility is that, based on the inheritance of COF, a genetic defect, not linked to milk yield, causes a reduced pancreatic insulin release in these animals. And finally, it is also possible that other metabolic adaptations associated with a high milk yield, like elevated NEFA-concentrations, interfere/influence pancreatic insulin release.

Future studies should therefore look at pancreatic insulin release in COF and “normal” cows, perhaps through a combination of in vivo studies and in vitro experiments with pancreatic cell cultures. To determine whether a genetic defect causes the reduced insulin secretion, it may be interesting to genetically compare bulls that are known to sire daughters with a high incidence of COF and bulls that do not. Also the effect of prolonged exposure of pancreatic islets of Langerhans to high NEFA concentrations on insulin secretion needs to be studied in cows.

Considering our in vitro effects of NEFAs on follicle cells in Chapter 5, the role of increased NEFA concentrations on COF development also needs further clarification. Perhaps by using an appropriate animal model, it may be possible to determine whether increased NEFA concentrations during follicle growth and development can result in the formation of COF.

Understanding what causes the low insulin concentrations and the exact role of NEFAs in cyst development, may offer opportunities to optimize feeding and management practices aimed to increase insulin secretion and reduce NEFA concentrations in dairy cows early post partum. It may also indicate whether further selection for milk yield can be achieved without increasing the incidence of COF.

Future research should also continue to focus on differences in gene expression between COF and normal dominant follicles. More specifically, the expression of genes encoding for steroidogenic enzymes and receptors for insulin and growth factors needs to be studied.
further. Our results have to be confirmed and more information is needed on the importance
of the ratio of the insulin receptor isoforms in COF formation.

Collection of cells from COF early pp through transvaginal follicle puncture may be
useful since additional information on the endocrinological and metabolic status of the animal
and the cyst would be available through analyses of blood and follicular fluid samples.
Identification of genes with an altered expression pattern may lead to the development of
genetic knock-out models to determine the gene’s role in cyst formation.
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Chapter 8. General Discussion


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Cystic ovarian follicles (COF) are an important ovarian dysfunction in high yielding dairy cows during the early postpartum period. Cystic follicles are generally defined as large (> 2.5 cm) anovulatory follicles that persist on the ovary for at least 10 days in the absence of a corpus luteum. It has become clear though that the definition and terminology need to be revised, but due to the heterogeneity of the disease this is not easy to achieve. Despite the large number of scientific papers on the subject the exact etiology and pathogenesis of COF remain unclear (Chapter 3). The most widely accepted hypothesis is that there is a dysfunction of the hypothalamic-pituitary-ovarian axis. More specifically, the pre-ovulatory LH-surge is either absent, insufficient in magnitude or occurs at the wrong time during dominant follicle maturation. However, the cause of this defective LH secretion is unknown. Primary alterations at both the level of the hypothalamus-pituitary and at the level of the ovary may disrupt the reproductive axis. Several publications indicate that a phenotypic link exists between COF and milk production. Generally, a high milk yield is associated with a negative energy balance (NEB) and, based on the detrimental effect of the NEB on reproduction, may therefore be the link between production and COF. In addition, due to the hereditariness (a) genetic factors seem to be involved as well. In order to prevent the occurrence of COF, a better understanding of how and why ovarian cysts develop is warranted.

The general aims of the present thesis were to increase our knowledge on the role of the NEB in cyst formation (Chapter 4), and more specifically how elevated metabolite concentrations may affect follicular growth and development by acting directly on follicle cells (Chapter 5 and 6). In addition, we aimed to determine whether gene transcription levels of receptors for certain hormones, important for follicular growth and development, differ between cystic follicles and normal dominant follicles, and could therefore be likely candidates to be involved in the pathogenesis of COF (Chapter 7).

In Chapter 4, we investigated whether cyst formation was preceded by altered hormonal and metabolic profiles, indicative for a NEB. One or more lactations of high yielding dairy cows were closely monitored during the postpartum period: daily milk samples were collected for progesterone analysis, blood sampling for hormone and metabolite analyses and ultrasound examination of the genital tract to monitor follicular growth and cyst formation were performed twice a week. Eight of the 30 lactations (26.7%) developed a cyst, while 22 lactations (73.3%) ovulated before day 60 post partum. First ovulation and cyst formation
occurred at similar times post partum. Metabolic and hormonal profiles did not differ between cystic and ovulatory lactations during the first 3 weeks post partum. In the final week prior to cyst formation/ovulation, insulin concentrations were lower in cystic than in ovulatory lactations while no differences were observed for any of the other parameters tested. In two lactations, cyst formation was preceded by suprabasal progesterone and increased oestradiol-17β concentrations. These results suggest that cyst formation in high yielding dairy cows post partum is associated with lower insulin concentrations but not with other distinct hormonal and metabolic alterations. However from this study, we can not exclude the involvement of subtle hormonal and metabolic changes in the pathogenesis of ovarian cysts. Suprabasal progesterone, and altered oestradiol-17β concentrations, seem to play a minor role in cyst formation.

Although we were not able to demonstrate a link between metabolic alterations, other than insulin, and cyst formation, subtle alterations may have gone unnoticed in the first study. Other reports suggest that increased NEFA concentrations may play a role in cyst development. In addition, metabolic alterations typically associated with a NEB may affect ovarian function by acting directly on follicle cells. Therefore, the effect of increased NEFA concentrations on follicular cell function was investigated in vitro (Chapter 5). Granulosa (Chapter 5.1) and theca cells (Chapter 5.2) were cultured during 48 h in the presence of different physiological concentrations of the three most abundant fatty acids (palmitic, stearic and oleic acid) in follicular fluid and blood serum, respectively. In vitro concentrations and ratios of the different fatty acids in culture medium were based on in vivo data.

In granulosa cells, mainly the saturated fatty acids reduced cell numbers while the mono-unsaturated oleic acid only exerted such an effect at the highest concentration tested. A combination of the three fatty acids clearly reduced cell numbers as well. Theca cells on the other hand seemed less sensitive to the effect of the fatty acids, since only the highest concentration of palmitic acid reduced cell numbers. However when combined, the three fatty acids clearly exerted a negative effect on theca cell numbers. These inhibitory effects were due to induction of apoptosis/necrosis in both granulosa and theca cells. When looking at steroidogenesis, oestradiol-17β production by the granulosa cells was stimulated by palmitic, stearic and to a lesser extent by oleic acid. When combined, the fatty acids also stimulated oestradiol-17β production. Theca cell steroidogenesis was hardly affected. Based on the in vitro results, NEFAs are likely to affect ovarian functioning during periods of NEB through a direct toxic effect on follicular cells and modulation of steroidogenesis.
Besides increased NEFA concentrations, a NEB is also characterised by elevated ketone concentrations, β-hydroxybutyrate being the most important one. Due to the association of increased ketone concentrations with a delayed postpartum ovarian activity, the effect of physiological β-hydroxybutyrate concentrations on follicular cell function was investigated \textit{in vitro} (Chapter 6). No detrimental effects on granulosa and theca cell viability could be observed. At low glucose concentrations, β-hydroxybutyrate actually supported granulosa cell survival, probably by acting as an alternative energy source. Granulosa cell steroidogenesis was reduced by β-hydroxybutyrate treatments, which, due to the higher cell numbers, may be attributed to a relative shortage per cell of molecules involved in steroid production. Conclusively, physiological β-hydroxybutyrate concentrations do not adversely affect follicular cell function \textit{in vitro}.

In Chapter 7, transcription levels of mRNA encoding for the insulin receptor isoforms A (IR-A) and B (IR-B), the insulin-like growth factor receptors 1 (IGFR-1) and 2 (IGFR-2) and the LH receptor (LHR) in granulosa and theca cells were compared between COF and dominant follicles (DF). Paired ovaries were collected in the slaughterhouse and theca and granulosa cells were collected per follicle or cyst. Oestradiol and progesterone concentrations were determined in follicular fluid for classification of the cysts as oestrogen-producing (COF-E2) or as progesterone-producing (COF-P4) and exclusion of atretic follicles. Real-Time RT-PCR was applied to determine and compare mRNA transcription levels. Both for granulosa and theca cells, three stable reference genes were selected to serve as internal controls. No differences for any of the receptors could be observed between DF and COF-E2. The latter, however, tended to have a higher relative percentage of IR-A in granulosa cells than dominant follicles. Theca cells of COF-P4 on the other hand, had lower LHR mRNA levels and lower IR-A/IGFR2 and IGFR1/IGFR2 mRNA ratios than DF and COF-E2. In addition, granulosa cells of COF-P4 had a lower IR-A/IGFR2 mRNA ratio than granulosa cells of COF-E2 and a higher relative percentage of IR-A than granulosa cells of DF. These results show that transcription levels of mRNA encoding for IR’s, IGFR’s or LHR do not differ between DF and COF-E2, with exception of the IR-A/IR-B ratio. Transition from an oestrogen-producing cyst to a progesterone-producing cyst seems to be associated with changes in transcription levels of mRNA encoding for IR’s, IGFR’s or LHR in granulosa and/or theca cells. In conclusion, our results do not indicate a role for altered transcription levels
of mRNA encoding for the IGFR’s or LHR in the pathogenesis of COF. The observation of differences in IR isoform abundance needs further clarification.

Finally, in the general discussion (Chapter 8) the main results are summarized and discussed in view of the current knowledge on the subject.

From the results described in the present thesis, the following conclusions can be drawn:

1. Lower insulin concentrations during the final stages of dominant follicle development and maturation are associated with cyst formation in high yielding dairy cows post partum. No other distinct hormonal and metabolic alterations seem to precede cyst formation.

2. Non-esterified fatty acids are toxic for both granulosa and theca cells in vitro, at concentrations present in the follicular fluid and serum of dairy cows during a negative energy balance.

3. Ketones, and more specifically β-hydroxybutyrate, are not detrimental for follicle cells in vitro and are therefore more an indicator for the NEB than a mediator of its effects.

4. Transcription levels of mRNA encoding for the insulin receptors A and B, the insulin-like growth factor receptors 1 and 2, and the LH receptor, are not altered in oestrogen-producing cysts in comparison to normal dominant follicles. In progesterone-producing cysts, the transcription levels of certain mRNAs are altered, which are likely an indication of a loss of oestrogenic capacity and follicular degeneration.
Een belangrijke ovariële afwijking bij hoogproductieve melkkoeien kort na de partus is het optreden van Cysteuze Ovariële Follikel(s) (COF). Cysteuze ovariële follikels worden gedefinieerd als grote (> 2.5 cm) anovulatoire follikels die gedurende minstens 10 dagen op het ovarium persisten, in afwezigheid van een corpus luteum. Het is echter duidelijk dat zowel deze definitie als de terminologie moeten herzien worden, maar dit is niet evident gezien de heterogeniteit van de aandoening. Ook al is er reeds veel onderzoek naar verricht, de etiologie en de pathogenese van COF blijven onduidelijk (Hoofdstuk 3). Een algemeen aanvaarde hypothese is dat de hypothalamus-hypofyse-ovarium as ontregeld is. Meer bepaald blijkt de pre-ovulatoire LH-piek afwezig of te laag te zijn of niet op het juiste moment tijdens de groeifase van de dominante follikel op te treden. De oorzaak van deze afwijkende LH-secretie is echter niet bekend. Primaire afwijkingen zowel ter hoogte van de hypothalamus-hypofyse als ter hoogte van het ovarium-follikel kunnen het endocriene systeem ontregelen. Verschillende publicaties hebben aangetoond dat er een fenotypische link bestaat tussen het optreden van COF en melkproductie. Het onderliggende mechanisme is tot op heden onbekend. Een hoge melkproductie gaat echter meestal gepaard met een negatieve energiebalans (NEB). Gezien de nadelige invloed op de fertiliteit, zou aldus de NEB de link kunnen zijn tussen productie en COF. Daarnaast toont de erfelijkheid van de aandoening aan dat er eveneens genetische factoren bij betrokken zijn. Om het optreden van COF te kunnen voorkomen, is een betere kennis van de pathogenese noodzakelijk.

Dit proefschrift heeft als belangrijkste doelstellingen de algemene kennis omtrent de rol van de NEB in het ontstaan van COF te vergroten (Hoofdstuk 4), en meer bepaald na te gaan hoe verhoogde metaboliectenconcentraties, eigen aan deze NEB, de folliculaire groei en ontwikkeling rechtstreeks kunnen beïnvloeden (Hoofdstukken 5 en 6). Daarnaast is onderzocht of er verschillen bestaan in de transcriptie van genen van bepaalde hormoonreceptoren tussen cysteuze en normale dominante follikels. De overeenkomstige hormonen zijn belangrijk voor de finale follikelgroei en –ontwikkeling, en verschillen in receptortranscriptie kunnen een indicatie zijn voor hun betrokkenheid in de pathogenese van COF (Hoofdstuk 7).

In Hoofdstuk 4 wordt beschreven in hoeverre cystenvorming wordt voorafgegaan door afwijkende hormonale en metabole profielen, indicatief voor een NEB. Hoogproductieve melkkoeien werden gedurende de postpartumperiode van één of meerdere lactaties nauwgezet opgevolgd. Dagelijks werden er melkstalen genomen voor progesteronanalyse en tweemaal
per week bloedstalen voor hormoon- en metaboliëtbepalingen. Eveneens werd tweemaal per week een echografisch onderzoek van de genitaltractus uitgevoerd om de follikelgroei en eventuele cystenvorming op te volgen. In 8/30 lactaties (26,7%) werd er een cyste gevormd, terwijl in 22 lactaties (73,3%) de eerste ovulatie optrad vóór dag 60 postpartum. Ovulatie en cystenvorming gebeurden op hetzelfde moment postpartum. De metabole en hormonale profielen tijdens de eerste 3 weken postpartum waren gelijkwaardig voor “ovulatoire” en “cysteuze lactaties”. Wanneer er werd gekeken naar de laatste week vóór ovulatie of cystenvorming, dan waren de insulineconcentraties van “cysteuze lactaties” beduidend lager dan deze van “ovulatoire lactaties”. Andere verschillen werden niet opgemerkt tijdens deze periode. In twee lactaties werd cystenvorming voorafgegaan door verhoogde progesteron- en oestradiolconcentraties. Deze resultaten tonen aan dat bij hoogproductieve melkkoeien cystenvorming vroeg postpartum geassocieerd is met lage insulineconcentraties, maar niet met andere duidelijke hormonale en metabole afwijkingen. Aan de hand van deze studie kunnen we echter niet uitsluiten dat subtiele hormonale en metabole verschillen al dan niet betrokken zijn bij de vorming van ovariële cysten. Suprabasale progesteronconcentraties, samen met afwijkende oestadiolprofielen, lijken een beperkte invloed te hebben op cystenvorming.

De afwezigheid van een duidelijk verband tussen cystenvorming en metabole afwijkingen behalve insuline, sluit de betrokkenheid van subtiele metabole verschillen niet uit. Enkele publicaties suggereren immers dat verhoogde NEFA-concentraties aanleiding geven tot het ontstaan van COF. Deze en andere metabole veranderingen, eigen aan de NEB, zouden de ovariële activiteit kunnen beïnvloeden door rechtsstreeks in te werken op follikelcellen. Om dit na te gaan, werd het effect van verhoogde NEFA-concentraties op follikelcellen in vitro onderzocht (Hoofdstuk 5). Granulosacellen (Hoofdstuk 5.1) en thecacellen (Hoofdstuk 5.2) werden gedurende 48 u gecultiveerd in de aanwezigheid van fysiologische concentraties van de 3 belangrijkste vrije vetzuren (palmitine-, stearine- en oleïnezuur) in follikelvocht en serum. De in vitro concentraties en ratios van de verschillende vrije vetzuren in cultuurmedium werden gebaseerd op in vivo waarnemingen.

De verzadigde vrije vetzuren palmitine- en stearinezuur hadden een nadelige invloed op het aantal granulosacellen, terwijl oleïnezuur alleen maar bij de hoogst geteste concentratie een inhiberend effect uitoefende. Een combinatie van deze drie vrije vetzuren had eveneens een duidelijk negatieve invloed. Thecacellen daarentegen waren minder gevoelig voor de effecten van de vrije vetzuren, aangezien alleen maar bij de hoogste
Samenvatting

palmitinezuurconcentratie een reductie van het cellenaantal werd waargenomen. Wanneer echter thecacellen werden blootgesteld aan een combinatie van de drie vrije vetzuren, was er een duidelijk negatief effect op het cellenaantal merkbaar. Deze inhiberende effecten waren het gevolg van inductie van apoptose/necrose door de vrije vetzuren in zowel granulosa- als thecacellen. Wat betreft de steroïdgenese, werd de oestrogeenproductie in granulosacellen gestimuleerd door palmitine-, stearine- en in mindere mate door oleinezuur. Ook een combinatie van de drie vetzuren had een stimulerend effect. De steroïdgenese in thecacellen werd nauwelijks beïnvloed door de vrije vetzuren. Aan de hand van deze in vitro resultaten kunnen we besluiten dat tijdens periodes van NEB, NEFA’s vermoedelijk de ovariële functie beïnvloeden door een direct toxisch effect op follikelcellen en door modulatie van de steroïdgenese.

Naast gestegen NEFA-concentraties, wordt de NEB ook gekenmerkt door verhoogde ketonenconcentraties, met als belangrijkste β-hydroxybutyraat. Aangezien hoge ketonenwaarden in het bloed worden geassocieerd met een uitgestelde ovariële activiteit post partum, werd het effect van dergelijke β-hydroxybutyraatconcentraties op follikelcellen onderzocht in vitro (Hoofdstuk 6). Zowel bij granulosa- als bij thecacellen werd er geen nadelig effect op de celviabiliteit waargenomen. In aanwezigheid van lage glucoseconcentraties had β-hydroxybutyraat namelijk een positief effect op het overleven van granulosacellen, vermoedelijk door als alternatieve energiebron te dienen. De steroïdproductie van de granulosacellen werd echter onderdrukt door de β-hydroxybutyraatbehandelingen. Eén en ander kan verklaard worden door het hogere cellenaantal in de β-hydroxybutyraatgroepen waardoor er per cel een relatief tekort aan molecules noodzakelijk voor de steroïdproductie ontstaat. Concluderend kunnen we stellen dat verhoogde β-hydroxybutyraatconcentraties geen nadelige invloed hebben op follikelcellen in vitro.

In Hoofdstuk 7 wordt een onderzoek beschreven naar de hoeveelheden mRNA’s die coderen voor de insulinereceptoren A (IR-A) en B (IR-B), de insulin-like growth factor-receptoren 1 (IGFR-1) en 2 (IGFR-2) en de LH receptor (LHR), bepaald in granulosa- en thecacellen. Er werd een vergelijking gemaakt tussen COF en dominante follikels (DF). Gepaarde ovaria werden in het slachthuis verzameld en de granulosa- en thecacellen werden gecollecteerd per follikel of cyste. Aan de hand van de oestrogeen- en progesteronconcentraties in het follikelvocht, werden cysten geclasseificeerd als oestrogeen-producerend (COF-E₂) of als progesteron-producerend (COF-P₄). Tevens werden aldus
Samenvatting


In Hoofdstuk 8 worden de belangrijkste resultaten van het onderzoek samengevat, besproken en gerelateerd aan de huidige kennis omtrent het in dit proefschrift behandelde onderwerp.

Uit het onderzoek kunnen de volgende conclusies getrokken worden:

1. Verlaagde insulineconcentraties tijdens de finale stadia van ontwikkeling en maturatie van de dominante follikel houden verband met cystenvorming bij hoogproductieve melkkoeien postpartum. Geen andere duidelijke hormonale en metabole veranderingen gaan het ontstaan van een cyste vooraf.


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Tom
CURRICULUM VITAE


Kort daarna trad hij in dienst bij de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde, eerst met een tijdelijk mandaat en vanaf 1 oktober 2001 als doctoraatsbursaal met een 4-jarig mandaat gefinancierd door het Bijzonder Onderzoeksfonds van de Universiteit Gent. Hij verrichtte een onderzoeksstudie getiteld: “De rol van het ovarium in de pathogenese van cysteuse ovariële follikels bij hoogproductieve melkkoeien post partum”. Gedurende de 5 jaren op de vakgroep was hij eveneens actief in de buitenpraktijk waar hij deelnam aan de dag-, nacht- en weekenddiensten en mee instond voor de opleiding van studenten.

Tom Vanholder is auteur of mede-auteur van 14 wetenschappelijke publicaties in internationale en nationale tijdschriften.
Publicaties in internationale wetenschappelijke tijdschriften


Publicaties in nationale wetenschappelijke tijdschriften


