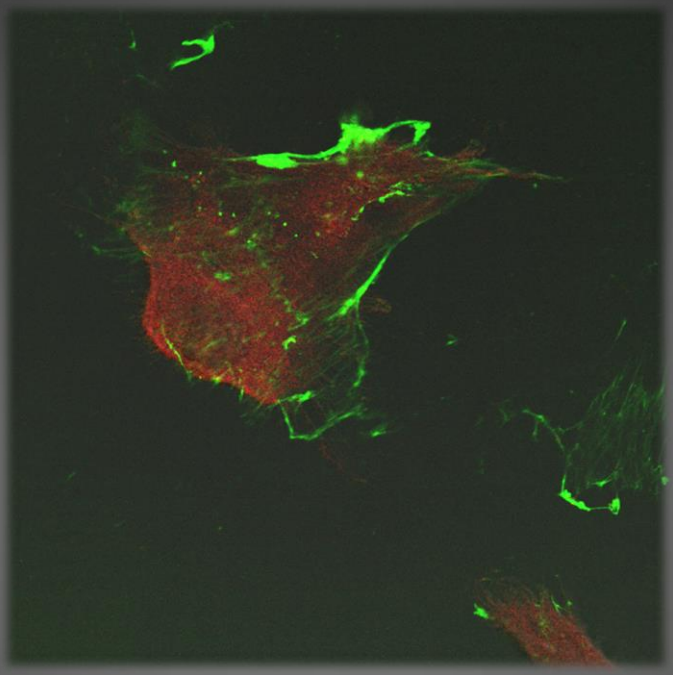

Effect of NEFA's on Cell Viability and Progesterone Production of Bovine Corpus Luteum Cells

Wondergem, M.A.

Bovine corpus luteum cells were cultured for 6 days. On day 6 medium was collected and NEFA's (100 and 250 µM of oleic and palmitic acid) were added. In the medium progesterone analysis is performed. Cells were fixated and colored using immunofluorescence. Images were made on Leica Confocal fluorescence microscope and cells were counted. 100 µM of oleic acid compared to 100 µM BSA and 100 and 250 µM palmitic acid compared to control group without BSA showed a negative effect on cell viability. Progesterone production per 100 cells showed no significant difference, however a trend is seen that the production per 100 cells increased when NEFA's were added.

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Internship: 1-9-2014 until 19-12-2014

29-4-2015

Contents

Introduction

In dairy husbandry, milk production has increased considerably in the past years (Figure 1). In the US and in Europe the production increased from approximately 7.000 lbs/cow to respectively over 20,000 and 14,000 lbs/cow (approximately 3.500 kg to 10.000 kg/cow).

Post-partum, cows have a high energy demand to meet the energy necessary for milk production. The first 60 days post-partum, cows use more energy for milk production than they obtain from feeding (Figure 2). As a result, a cow has a negative energy balance (NEB) over 5000 kcal. a day during the first 30 days post-partum (Collard 1999).

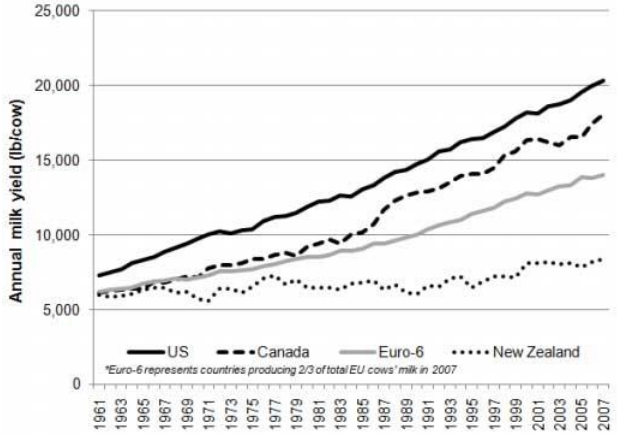

Figure 1. Increasing milk production during time (Capper, Cady & Bauman 2011)

From 1961 until 2007 the milk production is increased from approximately 6,000 until 17,000 LBS in Europe and from 7,000 until over 20,000 LBS in the US.

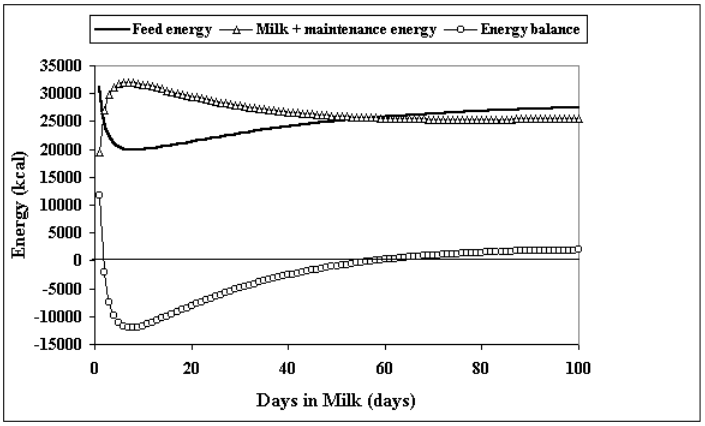

Figure 2. Energy intake, energy demand and NEB (Collard 1999)

Energy requirement in first 60 days is not equal to energy intake by feeding. Therefor the energy balance is negative, up to -12,500 Kcal/day, during the first 60 days of lactation.

Cows produce a high amount of milk post-partum. Since glucose is secreted into the milk by a GLUT1, an insulin-independent glucose transporter, cows need a lot of glucose. The glucose levels in the blood decrease and the cow starts glucogenisis. The substrates for the glucogenisis are taken in by feeding (acetate, propionate and butyrate). The energy required for the glucogenisis is obtained from bodyfat, released as NEFA's in the blood by the adipocytes. The liver uses the NEFA's for energy. This process is regulated by Growth hormone which has a stimulating effect on the lipolysis in adipocytes. Growth hormone has also a stimulating effect on the production of insulin-like growth factor (IGF), which has a stimulating effect on glucogenisis. During NEB, this system between growth hormone and IGF is disconnected. Therefore the growth hormone has a relatively high stimulating effect on lipolysis and the amount of NEFA's in the blood increases. The liver cannot use all the NEFA's as energy supply and therefor produces keton bodies and very low density lipids (VLDL) which are secreted into the milk. This increases the amount of fat in the milk. Besides the production of keton bodies and VLDL, the liver also accumulates fat as tri-acyl glycerol, which causes hepato steatosis. (Figure 3)

During the first 30 days post-partum NEFA blood concentration increases to very high levels. Since NEFA's are cytotoxic (Aardema et al. 2013) the surplus of NEFA's are stored in peripheral tissues, such as liver, muscles and adipose tissues. However, relatively high levels remain present in the blood. High NEFA levels are known to have a detrimental effect of several body functions, such as reproduction and food intake (Gross et al. 2013). Since reproduction is controlled by the hypothalamus-pituitary-gonadal axis, it is likely that NEFA's exert their detrimental effects by interaction with this axis. Moreover, NEFA's also may influence reproductive cells directly. According to a review of Leroy and Opsomer, the fertility of cows is decreased due to the NEB (Leroy et al. 2008). Since NEB has a negative effect on fertility, many studies have investigated the relationship

between NEB and decreased fertility(Badinga et al. 1985, Erb et al. 1976, Sonderegger, Schurch 1977, Piccinato et al. 2010)

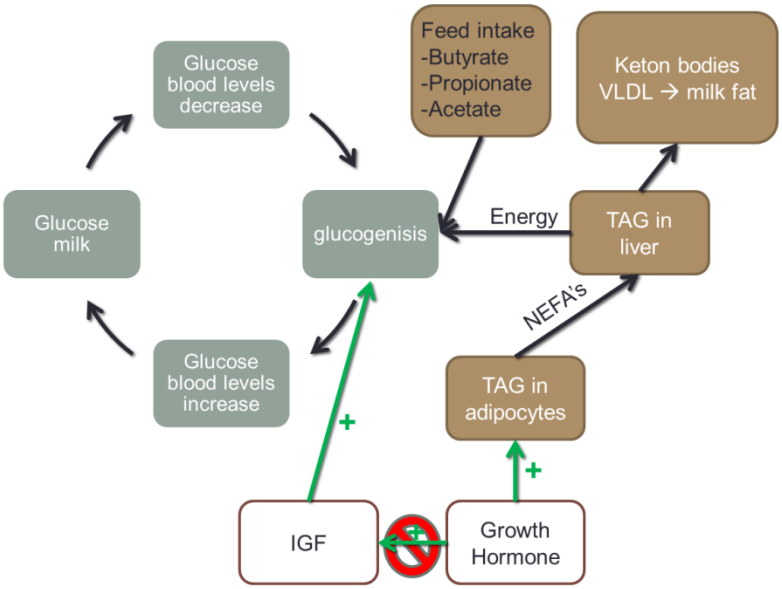

Figure 3. Production of NEFA's

Glucose in the blood is low since this is secreted into the milk by GLUT1. This induces glucogenisis wherefore energy is required. This energy is coming from TAG. This process is regulated by growth hormone and IGF. During NEB the stimulating effect of growth hormone on IGF is disconnected, resulting in a higher amount of lipolysis and an increased amount of NEFA's, keton bodies, VLDL and TAG in the liver.

The reproductive cycle in dairy cattle is regulated by the hypothalamus-pituitary-axis. The hypothalamus secretes gonadotropin releasing hormone (GnRH), which activates the pituitary to release follicle stimulating hormone (FSH) and luteinizing hormone (LH). These hormones stimulate the growth of follicles.

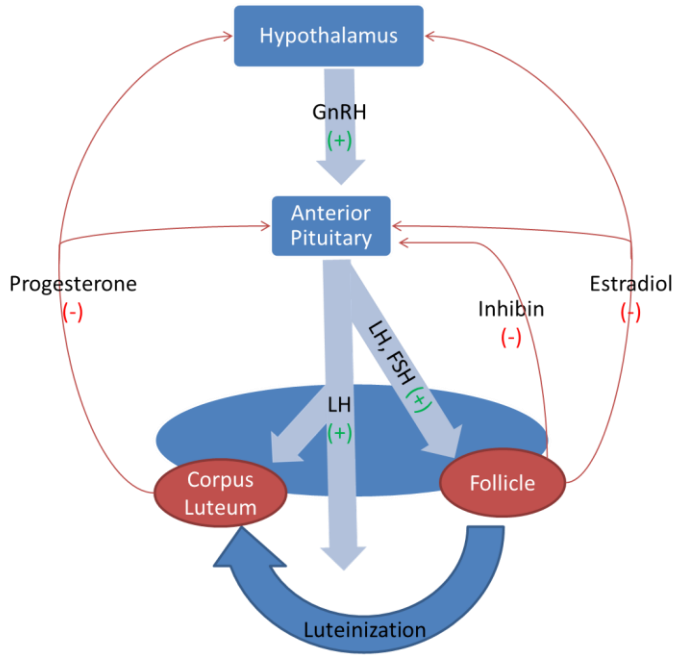

Figure 4. Hypothalamus-Pituitary-Ovaria axis

Hypothalamus excretes GnRH which stimulates the anterior pituitary to produces FSH which stimulates follicles to develop. The dominant follicle excretes estradiol which has an negative feedback on GnRH production. The dominant follicle also produces inhibin, which stimulates the pituitary to switch production from FSH to LH for what the follicle is sensitive. Ovulation will occur and the follicle tissue luteinizes into corpus luteum, which produces P_4, which has a negative effect on GnRH, FSH and LH production.

When a follicle has become a dominant follicle, it will be less dependent on FSH and more dependent on LH. Therefore the dominant follicle will produce inhibin. Inhibin causes the pituitary to produces more LH and less FSH and ovulation will occur. The post-ovulatory follicle will luteinize and produce progesterone (P4). This hormone inhibits the hypothalamus to produces FSH or LH whereby a new follicles do not develop. After ovulation, the remaining follicular tissue forms the CL. This CL produces P4 and functions among others as maintainer of the pregnancy, such as suppressing the local and general immunosuppression and enclosure of the cervix. Before ovulation a small amount of P4 helps the follicle to ovulate by a release of collagenase. (Senger 1997)

In this hypothalamus-pituitary-ovaria axis NEFA's may stimulate neurons that in turn stimulate the GnRH neurons. NEFA's in this way are indicative for the nutritional status of the animal and can in- or decrease the GnRH output of the hypothalamus and therefore influence the fertility of the animal. (Senger 1997)

Reports have shown the effect of NEFA's on follicle growth and composition of follicular fluid in vivo. Increased plasma NEFA levels are mirrored in the composition of the follicular fluid (Jorritsma et al. 2004). It was shown that these high NEFA levels in follicular fluid have a lipotoxic effect on granulosa cells (Aardema et al. 2013). Not only poor development of follicles can cause decreased fertility, but also imperfect development of the CL could effect it. Leroy et al. (2008) have investigated the effect of P4 concentrations in blood on the fertility of high-yielding dairy cows due P4 supplementation. This study has shown that high P4 concentrations in plasma have a positive effect on early embryonic development, interferon Tau production and therefore a positive effect on the fertility. Unpublished investigations have shown that cows in induced NEB have higher P4 concentration in blood plasma (Oossterhuis et al, unpublished results). This increased P4 concentration in blood plasma might be explained by lipotoxicity of NEFA's on liver cells, resulting in reduced clearance of P4, or by increased P4 production by CL cells as a result of exposure to elevated NEFA levels. Bouts (2013, unpublished results) has done bovine CL cultures *in vitro* and has shown an increase P4 concentration in culture medium when oleic acid (100 µM) was added, palmitic acid has a decreasing effect on P4 concentration measured in culture medium. Hence, the aim of this study is to investigate the effect of NEFA's on the P4 production and cell viability of CL cells *in vitro*.

Materials and Methods

Fibronectin coating cover slips

In order to increase anchoring of luteal cells, the cells were cultured on fibronectin coated coverslips (circular, 12 mm diameter x 0.015 mm thick, Thermo Fisher Scientific, Waltham, MA) in 24-well plates (Corning Inc., Corning, NY). Coverslips were coated in the wells by adding 0.4 ml 10 µg/ml fibronectin (Sigma-Aldrich) and incubated 4 hours in a humidified incubator at 37°C, 5% CO_2. Next, the fibronectin solution was aspirated and the plates were placed back in the humidified incubator at 37°C, 5% CO_2 to dry until use (approximately 24-26 hours).

Cell isolation and culture

For this research CL cells were cultured as previously described (Bouts, 2014; Robinson et al. (2008). Briefly, ovaries were obtained from an abattoir. Ten to fifteen ovaries were collected, approximately 10-20 minutes after slaughtering, and transported to the laboratory in Transport Buffer (1x PBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone (all Life Technologies) on ice. Based on their macroscopic appearance, corpora lutea of 1-10 days old where collected (Ireland, Murphee & Coulson 1980). Ovaries from cows with uterine pathologies or general pathologies as determined by the Nederlandse Voedsel en Waren Autoriteit (NVWA) were excluded.

At the culture lab, CL's were dissected from the ovaries in a sterile glass petri dish. First the CL was cut in half with sterile scissors. The CL was on cutting surface selected based criteria formulated by Ireland, Murphee & Coulson (1980), the CL was dissected with a sterile nr. 24 scalpel using a sterile tweezers. The dissected CL's were put in a 50 ml tube with 15 ml Transport Buffer of 4°C. For each experiment 4-5 CL's were dissected. The dissected CL tissue was placed on a sterile gauze to dry off the transport buffer and 6-8 grams of CL tissue was chopped with scissors into small pieces and transferred to a 50 ml tube with 15 ml Transport Buffer. The sample was centrifuged for 5 minutes at 950 rpm (Hettich Zentrifugen) (152 xg) at room temperature. Supernatant was aspirated and 10 ml Rinse Medium (DMEM/F12 1:1 (Sigma Aldrich), 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml fungizone) of 4°C was added to the pellet, the pellet was resuspended and centrifuged for 5 minutes at 950 rpm (152 xg) at room temperature and supernatant was aspirated. 10 ml Dissociation Medium (DMEM/F12 1:1, 2000 units/g tissue collagenase type 1 (Worthington Biochemicals), 0.005% deoxyribonuclease 1 (Sigma Aldrich) and 0.5% Bovine Serum Albumin (Life Technologies)) (at 37°C) was added to the pellet, resuspended and incubated in a shaking waterbath at 37°C for 1 hour. Every 15 minutes the suspension was resuspended with a 25 ml pipet.

After 1 hour, the suspension was once more resuspended and centrifuged for 5 minutes at 950 rpm (152 xg) at room temperature. Supernatant was aspirated and the supernatant was centrifuged for 5 minutes at 1200 rpm (242 xg)at room temperature. Supernatant was aspirated again and 10 ml Rinse Medium (at 37°C) was added to the pellet, the pellet was resuspended and centrifuged for 5 minutes at 1200 rpm (242 xg) at room temperature. Supernatant was aspirated and 15 ml Culture Medium (DMEM/F12 1:1, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite (ITS; Life Technologies (Gibco)) and 2% heat inactivated Bovine Serum) was added to the pellet and resuspended. In order to remove fibroblasts from the cell suspension, the suspension was pipetted into a 75 cm^2 culture flask (Corning Inc., Corning, NY) and incubated for 15 minutes in a humidified incubator at 37°C and 5% CO_2. The suspension was placed into a new 75 cm^2 culture flask and incubated again at 37°C and 5% CO_2. This was done once more. The suspension was aspirated and put in a 50 ml tube. A drop of cell suspension was put in a Eppendorf tube with a pipette, together with 1 drop of trypan blue and 3 drops of 1x PBS and cells were counted with a Fuchs-and-Rosenthal hemocytometer. Only

suspension with a cell viability was > 85% were used in this experiments. The 50 tube with cellsuspension was placed in the humidified incubator (37°C and 5% CO_2) while cell counting was performed.

Medium with 22 x 10^6 cells was aspirated, centrifuged for 5 minutes at 1200 rpm (242 xg) at room temperature. Supernatant was aspirated and Culture Medium (DMEM/F12 1:1, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite and 2% heat inactivated Bovine Serum) (at 37°C) was added to the pellet to a final concentration of 2.5 x 10^6 cells/ml. 0.4 ml with 1x10^6 cells was added to each well, with fibronectin coated coverlips, and incubated in a humidified incubator at 37° C with 5% CO_2.

The Culture Medium was aspirated after 24 hours and replaced by Complete Culture Medium containing LH (DMEM/F12 1:1, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml sodium selenite, 2% heat inactivated Bovine Serum and 0.02 U/ml LH (Sioux Biochemical Inc., Sioux Center, IA)). Every 48 hours this medium was changed by fresh Complete Culture medium (On day 3 and day 5). After 6.5 days of culture the medium was collected and stored at -20 °C for P4 measurements. Medium was replaced by Complete Culture Medium containing 100 µM + 100 µM BSA (100 µM) or 250 µM + 250 µM BSA (250 µM) of oleic or palmitic acid(Sigma Aldrich). For the control groups Complete Culture Medium was used with 100 µM BSA (BSA "C100") and 250 µM BSA (BSA "C250") or without addition (Control). Exactly 24 hours after addition of fatty acids the medium was collected and stored at -20 °C for P4-analaysis. Figure 4 summarizes the culture scheme.

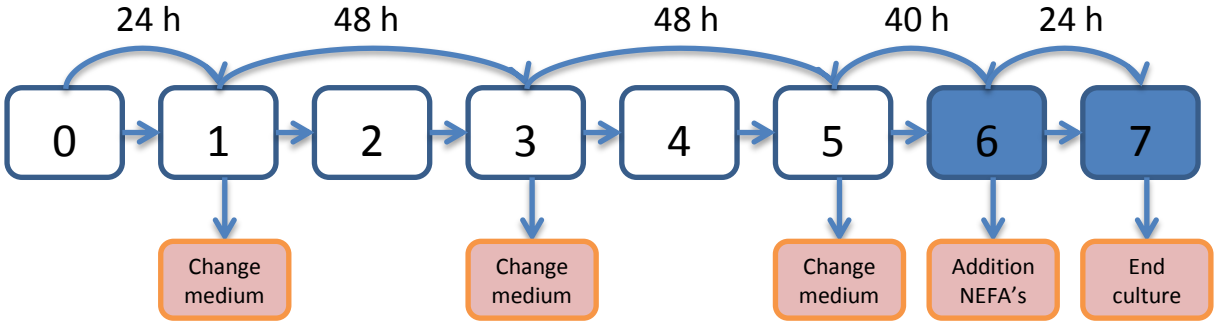

Figure 5. Changing medium scheme

t0= day of start culturing, t1= day of changing medium, t3= day of changing medium, t5= day of changing medium, t6= collecting medium for P_4 determination and addition of new medium with additions of BSA or oleic acid or palmitic acid, t7= collecting medium and end of culture.

Live-dead staining and immunofluorescence

After collection of the culture medium on day 7, cells were washed 2 times with 1x PBS for 5 minutes at room temperature. Next, 0.4 ml of 1 µl/ml fixable live/dead stain (LIVE/DEAD® Fixable Green Dead Cell Stain Kit, Life Technologies) was added to each well and cells were incubated for 30 minutes at room temperature in the dark. Subsequently, cells were washed with 1x PBS for 5 minutes, fixed in buffered 4% paraformaldehyde (Klinipath BV) (30 minutes, room temperature) and washed with 1x PBS (2x 5 minutes). Cells were stored in 1x PBS overnight at 4 °C. Next, cells were permeabilized in 0.05% Triton X-100 in PBS (10 minutes, at room temperature) and incubated in 10% Normal Donkey Serum (Sigma Aldrich) in 1x PBS for 30 minutes and placed on a shaking table at room temperature. The Normal Donkey Serum was aspirated and, without washing, cells were incubated for 1 hour in monoclonal mouse-anti-human 3β-HSD (Abcam) (10 µg/ml in 1x PBS, 0.05% Triton X-100, 1% BSA) at room temperature in the dark. After incubation cells were subsequently washed with 1X PBS (5 minutes) and 2 times with 1x PBS with Triton X-100 and 1% BSA for 5 minutes. Then cells were incubated, with Cy3-conjugated donkey-anti-mouse IgG (1:200) and DAPI (300 nM) (Invitrogen) in 1x PBS, 0.05% Triton X-100, 1% BSA) for 1 hour room temperature in the

dark. Finally, cells were washed with 1x PBS, 1x PBS with 0.05% Triton X-100 (5 minutes), 1x PBS (5 minutes) and Mili-Q (2x 5 minutes). Coverslips were mounted with fluorsave on object glasses and stored in the dark at 4°C for later use.

Cell counting

Cells were counted by using a Leica Confocal Fluorescence microscope using a 20x objective (0.13 mm^2/image). For counting, a systemic random sampling technique was used in which the first window is chosen at random on the coverslip and this determines the position of all other windows (figure 6). The distance between sampling windows was set at 3 mm, resulting in 10-13 sampling windows/coverslip, depending on position of first window. Each window was imaged with the same setting and cells in each window were scored for live/dead and 3β-HSD positive/negative.
Cells were counted based on the live dead staining whether they were dead or alive. Alive cells were labeled green at the outside of the cell, fluorescence has bound on proteins on the cell membrane. Dead cells colored green, however also on the inside, since the fluorescence also could bind to the proteins in the cytoplasm and nucleus. The difference between P4-producing cells was based on the red colored 3β-HSD. Immunofluorescence controls were used as a set point for imaging. Based on an immunofluorescence control the labeling of cells who showed red fluorescence during imaging were compared with the immunofluorescence control (figure 7 A & B)

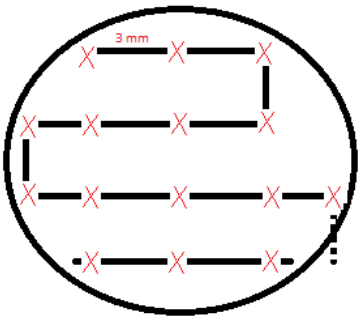

Figure 6. Randomized way of cell counting

Start imaging on a random site. Every shift 3 mm shift horizontally, if the end of the coverslip is reached, shift is vertically 3 mm. Next shift will be in opposite direction 3 mm horizontally.

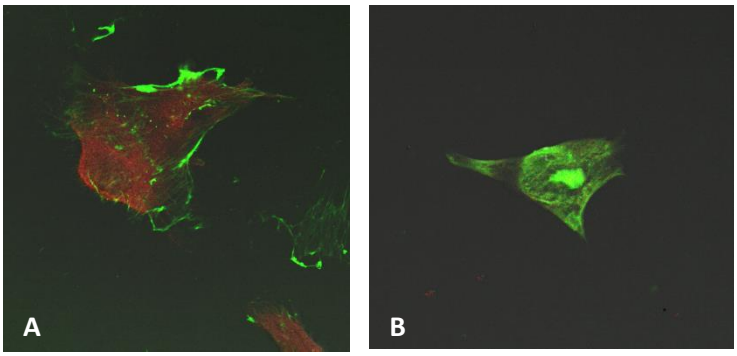

Figure 7 A & B. Live and Dead CL cell

Figure 7A: showing a live CL cell, outside membrane is colored green, showing it is alive. Insite is red, showing it is a CL cell and not showing green, showing it was alive during fixation. Figure 7B: showing a dead CL cell, outside and insite (nucleus) is colored green showing it is dead. Insite is also red, showing it is an CL cell. (magnification 40X)

P4-analysis

Medium was collected at day 6.5, before NEFA's were added, and on day 7,5, 24 hours after NEFA's were added. Samples were stored at -20°C until analysis. Analyzing is done by the University Veterinary Diagnostic Laboratory (Utrecht University, Utrecht), by using a solid-phase [125I] radioimmunoassay.

Results

In this study steroid genic corpus luteum cells were isolated from slaughterhouse corpora lutea and cultured in vitro during 6 days after which they were subjected to different concentrations of NEFA's i.e. palmitic and oleic acid. After 24 hrs of incubation in NEFA's, culture medium was removed and analyzed for progesterone (P4) levels and the remaining cells were counted and the effect of palmitic and oleic acid on cell viability was measured. Two experiments were performed in which the effect of palmitic and oleic acid was measured.

Figure 7 shows the difference in number of surviving cells between the two experiments in which the effect of two concentrations of oleic acid was tested. It appears that the average cell count in experiment 2 is approximately 3-10 times lower than in experiment 1. Therefore the whole data from experiment 2 is excluded from this research. In addition, the 250 µM of Oleic acid condition is excluded from this study since the standard deviation is too high (± 5624; SD 5672).

The results from experiment 1 show that incubation of CL cells for 24 hrs in 100 µM of oleic acid has a more profound effect on surviving cell number (average 2500 cells/well) that incubation with higher concentrations or with BSA alone. The addition of 100 µM BSA resulted in an average of 5,752 surviving cells/well, a decrease of 61% compared with the addition of 100 µM of oleic acid (P= 0.1).

Figure 8 shows the difference in surviving cell numbers as a result of exposure to two concentrations of palmitic acid. This shows an average of 2710 cells/well when 100 µM of palmitic acid was added, 250 µM of palmitic acid resulted in 949 cells/well. Compared to the control group addition of 100 (P= 0.03) and 250 (P= 0.009) µM of palmitic acid resulted in a decrease of respectively 50% and 83% in cells/well.

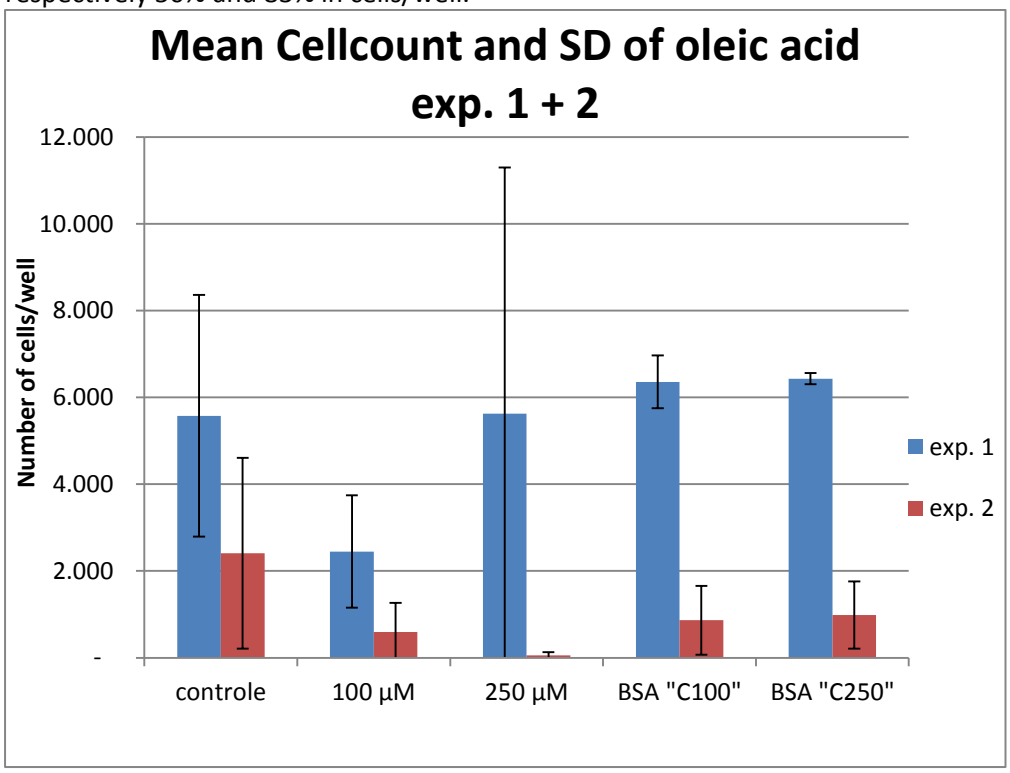

Figure 7. Cell count of oleic acid, difference between exp. 1 and 2

X-axis: Additions to medium in experiment. Y-axis: Number of cells/well. Figure shows the number of cells counted per well between experiment 1 and 2 with addition of oleic acid.

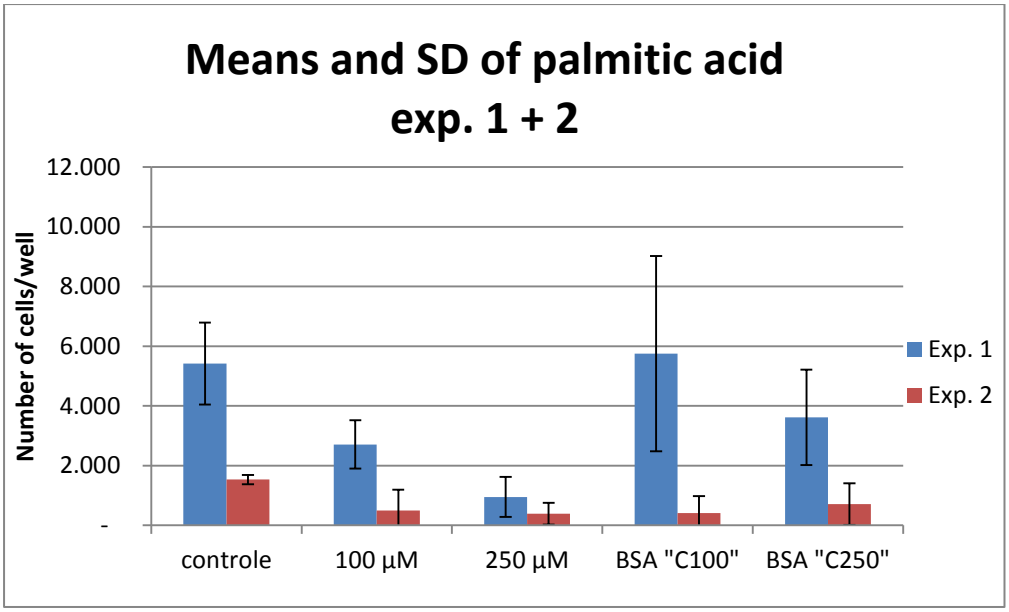

Figure 8. Cell count of palmitic acid, difference between exp. 1 and 2

X-axis: Additions to medium in experiment. Y-axis: Number of cells/well. Figure shows the number of cells counted per well between experiment 1 and 2 with addition of palmitic acid.

Nevertheless, figure 7 shows that that both BSA control groups do not differ from the blanco control group. Seen is that the addition of 100 µM of oleic acid reduces the measured cell population (p= 0.1). The same trend is found in figure 8 which shows that the control groups with BSA do not differ from the control group without addition. Addition of 100 µM and 250 µM of palmitic acid has a negative effect on the surviving cell number. This data does not show significant difference when compared to control groups where BSA was added (P= 0.11 and P=0.20). When compared to the control group without addition of BSA a significant decrease is found (P= 0.03 and P=0.009). No difference is found between the control groups with and without addition of BSA.

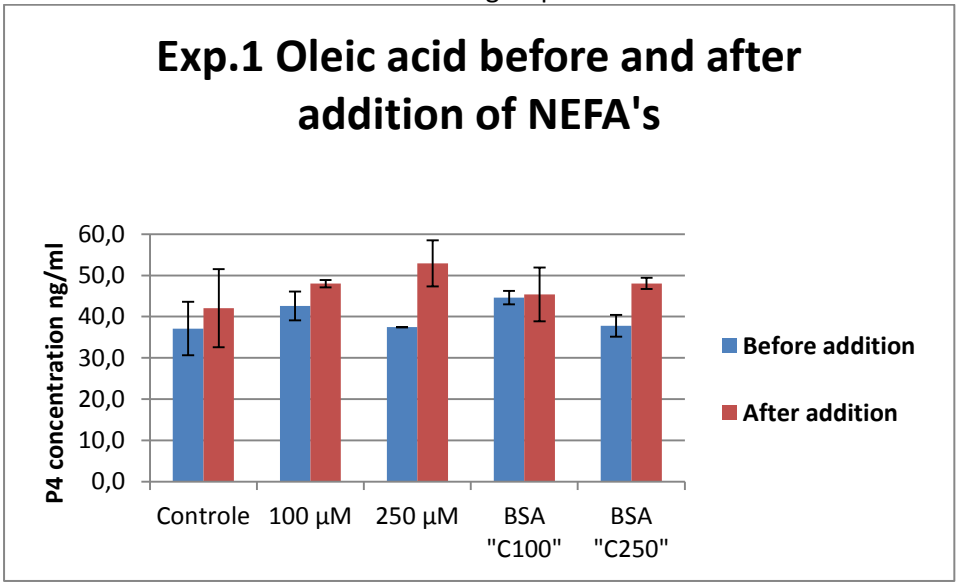

Figure 9. P4 concentration before and after addition of NEFA's

X-axis: Additions to medium in experiment. Y-axis: P4 concentration in ng/ml. Figure shows the concentration of P_4 in ng/ml before and after the addition of oleic acid.

The aim of this study was to investigate the effect of NEFA exposure on luteal cell viability and P4 production. In figure 9, the P4 production before and after replacement of the medium with NEFA's is shown. It is clear that luteal cells produced P4 in the absence of NEFA's. It also shows that

the production was nearly equal within all wells. After addition of NEFA's a trend is observed in which addition of 100 µM and 250 µM of oleic acid increases the P4 concentration in the medium. Exposure of cells to 100 µM of oleic acid resulted in a P4 concentration almost equal to the concentration in medium of cells exposed to 100 µM of BSA (C100). However this concentration is produced by a lower number of cells as mentioned above. However, no statistically significant difference is found between the production before and after addition of NEFA's.

Figure 10 shows the P4 production per 100 cells after the addition of oleic acid. It can be observed that the P4 production per 100 cells is not significant higher after exposure to both concentrations of oleic acid when compared to the control with and without BSA. Addition of BSA has no effect on the P4 production per cell. The P4 production of 250 µM oleic acid is based on one data, since one of the data was not valid.

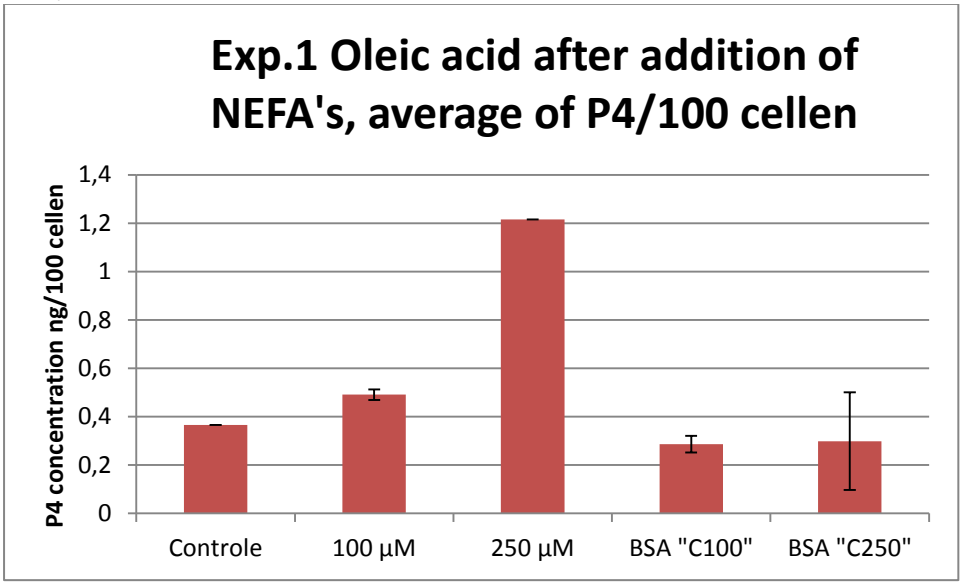

Figure 10. P4 production per 100 cells after addition of NEFA's

X-axis: Additions to medium in experiment. Y-axis: P4 concentration in ng/100 cells. Figure shows the concentration of P_4 in ng/100 cells after addition of oleic acid.

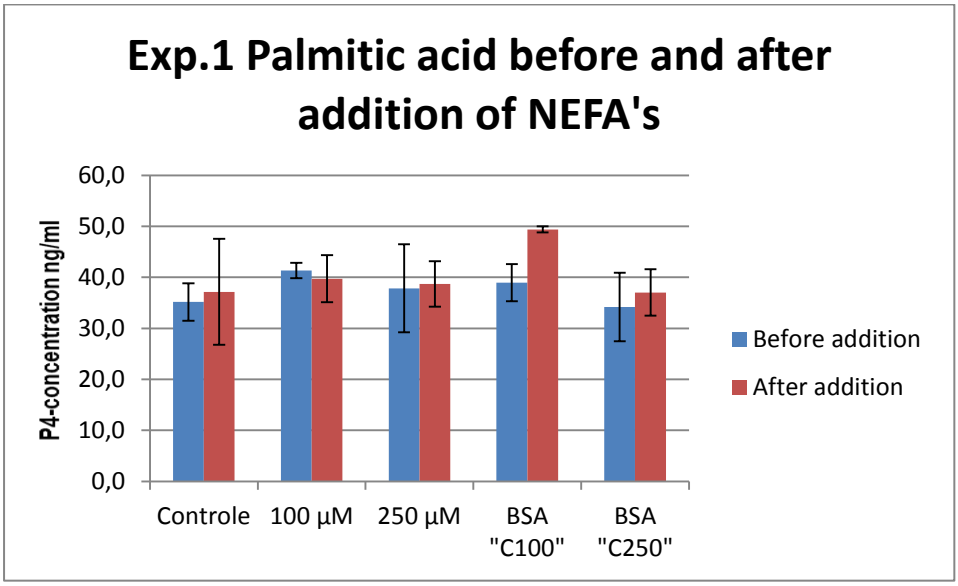

Figure 11. P4 concentration before and after addition of NEFA's

X-axis: Additions to medium in experiment. Y-axis: P4 concentration in ng/ml. Figure shows the concentration of P_4 in ng/ml before and after the addition of palmitic acid.

Figure 11 displays the similar pattern as figure 9 for palmitic acid; the concentrations before adding NEFA's show that these concentrations where approximately equal. The results are indicative that the addition of BSA (C100) increases the production of P4. This figure also shows that the production during 40 hours is approximately equal to the P4 production during 24 hours during which NEFA's are present.

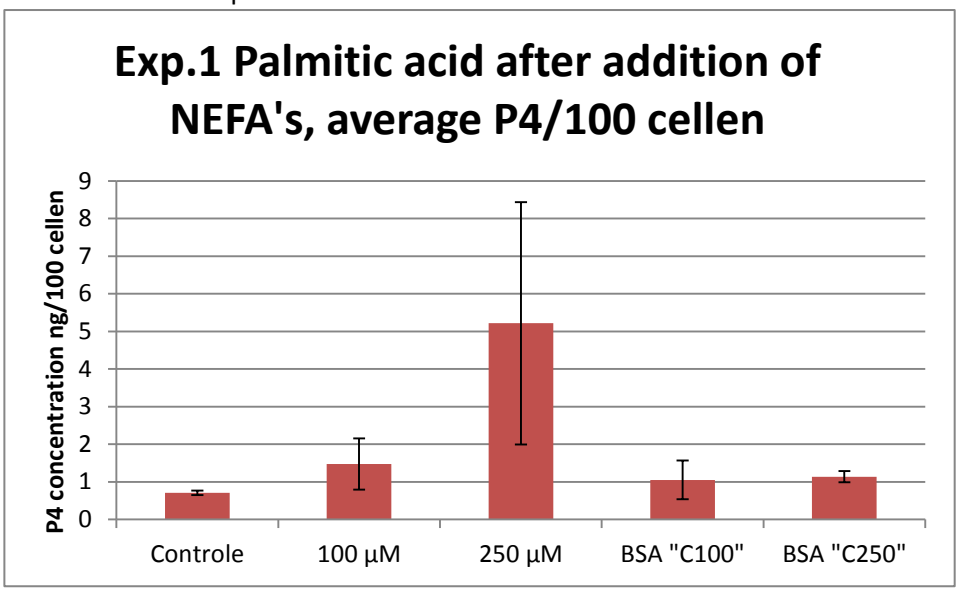

Figure 12. P4 production per 100 cells after addition of NEFA's

X-axis: Additions to medium in experiment. Y-axis: P4 concentration in ng/100 cells. Figure shows the concentration of P_4 in ng/100 cells after addition of palmitic acid.

Figure 12 shows the mean P4 production per 100 cells after addition of palmitic acid to the culture medium. It shows that there might be a higher production per 100 cells when 250 µM palmitic acid was added, compared to the control group. The standard deviation is quiet high (±5.22; SD3.22). Other additions do not differ from each other. All conditions do not significantly differ from other groups.

Discussion

The aim of this study was to investigate the effect of Non-Esterified Fatty Acids (NEFA's) on progesterone (P4) -production and on viability of corpus luteum (CL) cells. For this, an in vitro luteal cell culture system was developed in which luteal cells, obtained from slaughter house ovaries, were cultured for up to six days. P4 production and cell viability were determined after six days of culture in the absence or presence of different concentrations of oleic and palmitic acid. Our results showed a 61% decrease in cell viability after addition of 100 µM of oleic acid compared with 100 µM of BSA. The effect of 250 µM of oleic acid could not be measured, since the data was not reliable. Incubation in the presence of 100 and 250 µM palmitic acid resulted in a decrease of respectively 50% and 83% when compared to the control group. When addition of 100 and 250 µM of palmitic acid was compared to addition of respectively 100 and 250 µM of BSA, no de- or increase in cell viability was observed. The effect of NEFA's on P4 production was also measured. P4 production was measured before and during incubation with palmitic and oleic acid. P4 production was already present at the start of NEFA incubation. Luteal cells appeared to produce 37-49 ng/ml P4 before the addition of the NEFA's. P4 production per 100 cells was calculated. No statistically significant difference in P4 production per 100 cells could be demonstrated between conditions (figure 10 & 12). Standard deviations of measurements appeared to be large, indicative for a large inter-assay variability. Nevertheless, a trend could be observed in which P4 production per 100 cells is (non-significant) higher after addition of 100 µM of oleic acid and after addition of 100 and 250 µM of palmitic acid. However, when the total production of P4 is calculated (figure 9 & 11), it seems like a self-limiting-system. The P4 production in 24 hours is nearly equal to the P4 production in 40 hours. Since cells have produced an equal amount of P4 in 24 hours as they could in 40 hours, cells could be limited in P4 production caused by the P4 concentration in the environment or most likely by increased cell death due to lipotoxicity. From these findings we may surmise that both 100 and 250 µM palmitic acid and 100 µM oleic acid may have a lipotoxic effect and also a stimulating effect on the P4 production per cell.

Our results indicate an increased P4 production per cell when NEFA's were added. NEFA's may have a stimulating effect on the P4 production. Since the production of P4 in 24 hours is equal to the production in 40 hours, it indicates that cells will not produce more P4 when a certain amount of P4 concentration is reached. No reasonable arguments are found to elucidate these findings, considering the facts that no progesterone receptors are found in CL cells if progesterone would influence its own production and half-life time of progesterone (several hours) to induce a steady state.

In this study, cell viability during incubation with 100 µM of oleic- and 100 and 250 µM of palmitic acid was decreased, Aardema et al. (2013) have shown a lipotoxic effect of NEFA's on cells. This is according to the 100 µM oleic acid and 100 and 250 µM of palmitic acid found in this study. Bouts (2013) has shown a higher P4 concentration when 100 µM of oleic acid was added and a lower P4 concentration was measured when palmitic acid was added (Bouts 2013). This suggest that viability of cells will be lower when palmitic acid is added, or the progesterone production per cell will be inhibited. In this study we have shown that a possible trend is present such that the viability per cell is lower after addition of NEFA's and the production per cell may be higher. Research has shown that palmitic acid has a stimulating effect on the steroidogenesis through the MAP-Kinase pathway (Bellanger et al. 2012). However, in cardiomycytes, MAP-Kinase is important for survival signaling (Drosatos, Schulze 2013). In this way NEFA's could have a stimulating effect through MAP-Kinase on steroidogenesis and furthermore an effect on the survival signaling and thus the survival and cell viability of CL cells. In unpublished data from Bouts (2013) it is shown that there is no saturation level of P4 concentration. This is in contradiction to the results of this study. To investigate this discrepancy between the outome of both studies more studies have to be performed.

The immediate cause of this research was the observation that P4 concentration in the blood increased when cows were fastened (Oosterhuis 2009). They proposed that the NEFA´s could have a stimulating effect on the P4 production, that the liver does not clear the blood optimally because of the NEFA storage or, alternatively, progesterone mobilization from the adipose tissue. This is partly corroborated in this study by the observation that NEFA's could increase the production of P4. Other studies have indicated that elevated P4 levels as a result of fastening could be due to a release of P4 from the fat storage. When the cow is in fastening it uses the fat storages and during lipolysis P4 stored in the fat is released into the blood, which may result in an increased P4 concentration in the blood during fastening. (Hamudikuwanda et al. 1996)

Experiment 1 was a well performed experiment, with sufficient number of cells per well. There was no monolayer cultured, and therefore the range (min. 1,533 – max. 9,635 cells/well) between the data was way too high. Experiment 2 had less cells per well and therefore needed to be excluded from the investigation. Since a monolayer is more reliable and reproducible (Melero-Martin, Santhalingam & Al-Rubeai 2009), for further research it is recommended to culture more cells per well to create a monolayer. In order to create a monolayer 20-40 cells per microscope view (0.13 mm2) is recommended to incubate 60,000 cells per well. The average cell count in experiment 1 was 4,500 cells per well and in experiment 2,850 cells per well. This implies that for experiment 1 thirteen times more cells per well need to be cultured and for experiment 2 71 times more. The big difference between the two experiments cannot be explained by mistakes, since the cultures were processed the same way. Maybe the culture medium was not optimal, since this came out of the same badge, but was two weeks older and opened already. When imaging cultures with few cells, shifts of 2 mm instead of 3 mm per shift can be chosen to increase the reliability of imaging. In this study the SD was often too high. For further investigation it is recommended to repeat the conditions four time instead of two times and focus on one fatty acid at the time.

Conclusion

The addition of oleic acid was indicative of an increased progesterone production per 100 corpus luteum cells in vitro. However, the survival of corpus luteum cells cultured with 100 µM oleic acid seemed to be lower compared to control group with 100 µM BSA. This observed lower cell survival has also been seen after addition of palmitic acid (compared to control group without addition of BSA), although the progesterone production seemed to increase after addition of 250 µM of palmitic acid. However the standard deviation of this data is too high to be reliable. All differences seen in this research must be noted as trend, since the differences were not significantly.

Acknowledgement

I would like to thank my supervisors Theo van Haeften, Peter Vos and Carla Zijlstra for the supervision during my internship. Without their help I could not manage to culture cells, write a report in English nor give a scientific presentation. From the JDV I would like to thank Leni Tol for the provision and the help concerning luteinizing hormone. From the JDV I would also like to thank Christine Oei for the progesterone analysis in my culture media. The CCI has helped me with getting around with the Leica Confocal microscope and therefore I would like to thank Esther van ‘t Veld, Richard Wubbolts and Rob Bleumink. From department farm animal health I would like to thank Jamal Afkir and Nordine Aharam for the trips to and the help in the slaughterhouse Gosschalk in Epe. Therefor I would like to thank this slaughterhouse for providing me with ovaria. At last I would like to thank all the people in department Biochemistry and Cell Biology for all the help and the fun time during my internship.

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