Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes *in vitro*

J L M R Leroy, T Vanholder, B Mateusen, A Christophe[1], G Opsomer, A de Kruif, G Genicot[2] and A Van Soom

Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium, [1]Department of Internal Medicine, Division of Nutrition, Faculty of Medicine, Ghent University, De Pintelaan 185, 9000 Ghent, Belgium and [2]Institut des Sciences de la Vie, Unité des Sciences Vétérinaires, Catholic University of Louvain, Place Croix du Sud 5 box 10, B-1348 Louvain-la-Neuve, Belgium

Correspondence should be addressed to J L M R Leroy; Email: jo.leroy@UGent.be

Abstract

In this study concentration and composition of non-esterified fatty acids (NEFA) in follicular fluid (FF) of high-yielding dairy cows were determined during the period of negative energy balance (NEB) early post partum. NEFA were then added during *in vitro* maturation at concentrations measured previously in FF to evaluate their effect on the oocyte's developmental competence. At 16 and 44 days post partum, FF of the dominant follicle and blood were collected from nine high-yielding dairy cows. Samples were analysed for NEFA concentration and composition. NEFA concentrations in FF (0.2–0.6 mmol/l) during NEB remained ± 40% lower compared with serum (0.4–1.2 mmol/l). The NEFA composition differed significantly between serum and FF with oleic acid (OA), palmitic acid (PA) and stearic acid (SA) being the predominant fatty acids in FF. Based on these results, 5115 oocytes were matured for 24 h in serum-free media with or without (negative control) the addition of 0.200 mmol/l OA, 0.133 mmol/l PA or 0.067 mmol/l SA dissolved in ethanol or ethanol alone (positive control). Matured oocytes were fertilized and cultured for 7 days in SOF medium. Addition of PA or SA during oocyte maturation had negative effects on maturation, fertilization and cleavage rate and blastocyst yield. More (late) apoptotic cumulus cells were observed in cumulus–oocyte complexes matured in the presence of SA or PA. Ethanol or OA had no effect. These *in vitro* results suggest that NEB may hamper fertility of high-yielding dairy cows through increased NEFA concentrations in FF affecting oocyte quality.

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Introduction

Reduced fertility in high-yielding dairy cows has been reported world-wide during the last decades (Lucy 2001). Ovarian dysfunction early post partum (pp), leading to delayed resumption of cyclicity and prolonged calving intervals, is one of the major and thoroughly studied drawbacks of this high productivity (Opsomer *et al.* 1998, Shrestha *et al.* 2004). It is only recently that an important role has been attributed to the oocyte and embryo quality in determining the final fertility outcome. Some studies already suggested that the decline in fertility is mainly caused by an inferior oocyte and embryo quality rather than being related to an ovarian/endocrine dysfunction (Harrison *et al.* 1990, O'Callaghan & Boland 1999, Horan *et al.* 2005). A remarkable decline in first-service conception rates from around 65% in the fifties to well below 40% in 2001 has been reported by Butler (2003). A significant reduction in oocyte quality has been seen in high-yielding dairy cows (Kruip *et al.* 1995, Gwazdauskas *et al.* 2000, Snijders *et al.* 2000, Sartori *et al.* 2002, Walters *et al.* 2002) and can result in reduced conception rates or in a higher prevalence of early embryonic mortality (Boland *et al.* 2001, Lucy 2001, Silke *et al.* 2002). Britt (1994) hypothesized that follicles grown during the period of negative energy balance (NEB) early pp could be affected by unfavourable metabolic changes and may contain a developmentally incompetent oocyte. It has recently been shown that the composition of follicular fluid (FF) is subjected to these metabolic adaptations early pp (Leroy *et al.* 2004). Subsequently, after a growing and maturation phase of several weeks, this inferior oocyte

will be ovulated at the moment of first insemination (Britt 1994). One of the major metabolic changes during the period of NEB is the increased non-esterified fatty acid (NEFA) concentrations in serum which are strongly correlated with the depth of NEB.

Recently, it has been demonstrated that elevated NEFA levels are toxic for bovine (Vanholder *et al.* 2005) and human (Mu *et al.* 2001) granulosa cell growth and function *in vitro*. Similar cytotoxic effects were described in pancreatic β-cells (Cnop *et al.* 2001, Maedler *et al.* 2001), Leydig cells (Lu *et al.* 2003) and blood mononuclear cells (Lacetera *et al.* 2002).

Until now, knowledge about the influence of elevated NEFA levels as encountered during NEB *in vivo* on oocyte developmental capacity *in vitro* is very scarce or even absent. Furthermore, very little is known about the NEFA concentration and NEFA composition in the intrafollicular environment in relation to the serum composition. This knowledge is indispensable to investigate the effect of *in vivo* intrafollicular NEFA concentrations during a period of NEB in an *in vitro* maturation (IVM) model.

In the present study we wanted to clarify possible interactions between high NEFA concentrations and oocyte quality, being a potential contributing factor in the pathogenesis of subfertility in modern high-yielding dairy cows. Therefore, the aims of the present study were (1) to investigate the concentration and composition of NEFA in serum and in FF of the dominant follicle in high-yielding dairy cows during and shortly after the period of NEB; and (2) to imitate these NEB associated FF NEFA concentrations in an IVM model to test their effect on oocyte developmental competence.

Materials and Methods

NEFA concentration and composition in serum and FF of the dominant follicle

Animals

Nine healthy multiparous Holstein-Friesian cows were used in this study. All experimental work was performed at the research dairy farm of Ghent University (Biocentrum Agri-Vet, Melle, Belgium) following protocol approval by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University). Cows were milked on average 2.2 times a day by means of an automated voluntary milking system. The average milk yield per cow in the herd was 10 200 kg milk (4.1% fat and 3.4% protein) during 305 days of lactation. After an average dry period of 55 days, all cows calved normally between October 2003 and March 2004. During the experimental period (first 50 days of lactation), all cows were housed in a loose stable with cubicles and were fed according to their requirements for maintenance and milk production. The ration consisted of high quality roughages (corn silage and grass silage, sugar beet pulp), soybean meal and concentrates. All animals showed a normal puerperium and

uterine involution. One animal suffered from a mild mastitis in one quarter. After an intramammary treatment with antibiotics, the animal was cured within 3 days, well before the first ovarian puncture. Body condition scores (BCS) based on the notation of Edmondson *et al.* (1989), were recorded by the same experienced operator using a score on a scale of 1–5 (with 0.25 increments).

Blood and FF sampling

Blood samples were collected from each animal 7 days prior to the expected calving date, at the day of parturition and at days 16 (severe NEB) and 44 (improving NEB) pp. Blood was sampled from the jugular vein into two unheparinized, silicone coated tubes (Venoject, Autosep, Gel + Clot. Act.; Terumo Europe N.V., Leuven, Belgium). Any stress prior to blood sampling was avoided. Samples were taken between 1.00 pm and 3.00 pm, 2 h after automated milking at the latest and before any other handling of the animals was performed. The coagulated blood samples were centrifuged ($1400 \times \mathbf{g}$, 30 min) within 1.5 h after collection and the collected serum was stored under N_2 atmosphere at $-80\,°C$ until analysis.

On day 11 pp an ultrasound examination of the genital tract was performed in all cows to monitor uterine involution and follicular growth. On day 16 and 44 pp only dominant follicles with a diameter greater than 0.8 cm were subjected to ultrasound guided transvaginal aspiration as described previously (Leroy *et al.* 2004). Attention was paid to prevent blood contamination. FF samples with obvious blood contamination were omitted from further processing. The collected FF was cooled immediately (4 °C). Subsequently, FF samples were centrifuged ($10\,000 \times \mathbf{g}$ 10 min) and the supernatant was collected for analysis. Within 2 h after each session, the FF samples were frozen under N_2 atmosphere at $-80\,°C$ until analysis.

Analyses

To identify possible atresia of the punctured follicles, a progesterone (P_4) and estradiol-17β (E_2) analysis was carried out on each FF sample as previously described (Leroy *et al.* 2004). Follicular fluid with a E_2/P_4 ratio <1 was considered to originate from an atretic follicle and was omitted from biochemical analysis (Badinga *et al.* 1992, Landau *et al.* 2000).

The analyses for total NEFA concentration were done using wet chemistry techniques on a clinical automated analyser (Hitachi 911, Roche Diagnostics, Mannheim, Germany). A commercial kit was used (Wako Chemicals GmbH, Neuss, Germany) according to the manufacturer's instructions. The intra- and inter-assay coefficients of variation were below 5%.

The composition of the NEFA fraction in serum and FF samples was determined as follows. The total lipid fraction was extracted with methanol/chloroform according to a modified method of Folch *et al.* (1957). In brief, 100 μl of

1 M HCl, 1 ml of methanol and 2 ml of chloroform were added to 1 ml of serum or FF. After centrifugation at 4 °C, the upper phase and the interface were removed by aspiration and filtration respectively. The filtrate was evaporated to dryness under a N_2 flow and the residue was dissolved in chloroform. To avoid any fatty acid oxidation, the samples were kept under N_2 atmosphere. Non-esterified fatty acids were isolated by thin layer chromatography on rhodamine-impregnated silica gel plates using petroleum ether (bp 60–80 °C; Merck Belgolab, Overijse, Belgium) and acetone (85:15 by volume) as mobile phase. The free fatty acid band was scraped off and the fatty acids were converted into methyl esters by esterification using 2 ml of a mixture of methanol/chloroform/HCl (fuming 37%) (80:20:4 by volume) as methylating agent for 4 h at 95 °C. After cooling and addition of 2 ml of distilled water, the methyl esters were extracted with petroleum ether (bp 40–60 °C) and evaporated to dryness under a N_2 flow. The fatty acids were analysed by temperature-programmed capillary gas chromatography (Varian model 3500 gas chromatograph; Walnut Creek, CA, USA) on a 60 m × 250 μm (L × ID) × 0.2 μm film thickness 10% cyanopropylphenyl-90% biscyanopropyl polysiloxane column (Rtx-2330, Restek, Bellefonte, PA, USA). The injection and detection temperatures were set at 285 °C. The starting temperature of the column was 165 °C, which, after 1 min, was increased to 230 °C at a rate of 2 °C/min. The carrier gas was nitrogen with a linear velocity of 18.1 cm/s. Peak identification was done based on the retention times using authentic standards. Peak integration and calculation of the fatty acid compositions were automatically performed using appropriate software (Varian Star 5.52 1998). The results for individual fatty acids were expressed as percentage weight of the amount of total fatty acids.

Addition of oleic acid, palmitic acid or stearic acid during IVM of bovine oocytes

Materials and media

Chemicals and media were obtained from Sigma (Bornem, Belgium) and from Gibco/Invitrogen life technologies (Merelbeke, Belgium). A modified HEPES-buffered Tyrode's balanced salt solution, termed HEPES-TALP, consisted of 114 mmol/l NaCl, 3.1 mmol/l KCl, 2 mmol/l NaHCO₃, 0.3 mmol/l NaH₂PO₄, 10 mmol/l HEPES, 2.1 mmol/l CaCl₂, 0.4 mmol/l MgCl₂, 10 mmol/l sodium lactate, 0.2 mmol/l sodium pyruvate, 3 mg/ml fatty acid free bovine serum albumin (BSA) and 10 μg/ml gentamycine sulphate. Oleic acid (OA, cis C18:1), palmitic acid (PA, C16:0) and steric acid (SA, C18:0), were dissolved in pure ethanol (Vel/Merck Eurolab, Zaventem, Belgium) at a concentration of 50, 25 and 12.5 mg/ml respectively. Murine epidermal growth factor (EGF) was dissolved at a concentration of 1 μg/ml in bicarbonate buffered Medium 199 with Earle's and glutamine (TCM199) and with 0.1% w/v fatty acid-free BSA.

The serum-free maturation media (pH = 7.2) contained TCM199, one fatty acid dissolved in ethanol (cfr. Infra) and EGF (20 ng/ml). Fertilization medium consisted of Tyrode's balanced salt solution supplemented with 25 mmol/l NaHCO₃, 10 mmol/l sodium lactate, 0.2 mmol/l sodium pyruvate, 6 mg/ml fatty acid-free BSA, 10 μg/ml gentamycin sulphate and 10 μg/ml heparin. The embryo culture medium consisted of synthetic oviduct fluid (SOF) (Minitüb, Tiefenbach, Germany) supplemented with 40 μl/ml basal medium eagle (BME), 10 μl/ml minimum essential medium (MEM), 0.2 mmol/l sodium pyruvate and 50 μl/ml fetal calf serum (FCS) (N.V. HyClone, Europe S.A., Erembodegem, Belgium).

Percoll was purchased from Amersham Biosciences (Uppsala, Sweden), heparin from Leo Pharma (Zaventem, Belgium), ethanol from Vel/Merck Eurolab (Zaventem, Belgium), and Hoechst 33342 from Molecular Probes (Leiden, The Netherlands).

In vitro production of embryos

Ovaries and oocytes were collected as described by Tanghe *et al.* (2003). After collection, ovaries were rinsed in physiological saline (0.9% NaCl) with 0.5% kanamycin. The IVM was performed as follows. Immature cumulus–oocyte complexes (COCs) were aspirated from follicles 2–6 mm in diameter. Only grade I COCs were used for further culture following selection under a stereo microscope. After several washings in HEPES-TALP, the COCs were cultured in groups of 50–60 for 24 h at 38.5 °C in 500 μl of serum-free maturation medium in a humidified 5% CO₂ incubator.

After IVM, fertilization was performed as described by Tanghe *et al.* (2003). Briefly, all groups of COCs were co-incubated per 100–120 with spermatozoa at a final concentration of 10^6 sperm cells/ml for 20 h at 38.5 °C in fertilization medium, in a humidified 5% CO₂ incubator. For all experiments, frozen bull semen from the same ejaculate was thawed and live spermatozoa were selected by centrifugation on a discontinuous Percoll gradient (90 and 45%). The final sperm–egg ratio was adjusted to 5000:1.

After co-incubation with spermatozoa, the presumptive zygotes were vortexed for 4 min to remove excess sperm and cumulus cells. After several washings with HEPES-TALP and modified SOF medium, presumptive zygotes were cultured per 25 in 50 μl droplets of modified SOF medium with 5% FCS, under mineral oil (modular incubator: 39 °C, 5% CO₂, 5% O₂ and 90% N₂) until 8 days after fertilization. For each replicate, four drops of embryos were prepared per treatment.

Analyses

Maturation and fertilization rate After IVM or fertilization, COCs or presumptive zygotes were vortexed for 4 or 2 min respectively. The denuded matured oocytes/presumptive zygotes were fixed in 2% paraformaldehyde and 2% glutaraldehyde in PBS for at least 24 h (4 °C), and

stained for 10 min with 10 µg/ml Hoechst 33342 (Molecular Probes, Leiden, The Netherlands). The matured oocytes/presumed zygotes were mounted in 100% glycerol and evaluated by means of a Leica DMR fluorescence microscope (Van Hopplynus N.V., Brussels, Belgium) (400× magnification). To evaluate the maturation rate of the oocytes, the nuclear stage was recorded as being in first metaphase (MI), anaphase or telophase (AT) and second metaphase with extruded polar body (MII, successful nuclear maturation). To investigate the fertilization rate, following stages were distinguished: MII, the presence of 2 pronuclei (2PN, successful fertilization) and the presence of more than 2 pronuclei (>2PN, polyspermy).

Lipid content To investigate whether IVM in the presence of a fatty acid (PA or SA) influenced the lipid content in the matured and denuded oocytes, the selected oocytes were fixed, stained with 10 µg/ml Nile Red (Molecular Probes, Inc., Eugene, Oregon, USA) for 3 h and analysed as described before (Genicot *et al.* 2005). The emitted fluorescent light was evaluated at a wavelength of 582 ± 6 nm with an inverted fluorescence microscope (Excitation: 400–500 nm and Emission: 515LP) using a 10× objective. The fluorescence was amplified with a photomultiplier, quantified with a photometer attached to the microscope (MPV-SP, Leitz, Wetzlar, Germany) and calculated by the MPF Bio Software (Leitz). The results were expressed in arbitrary units of fluorescence.

Morphology of COCs after IVM After IVM, COCs were evaluated morphologically for cumulus expansion by means of a binocular microscope (40× magnification). The presence of apoptosis in cumulus cells of COCs matured in the control group (with ethanol) and in the test group (SA or PA) was evaluated by means of propidium iodide (PI) and annexin V staining (Vybrant Apoptosis Assay kit #3, Molecular Probes, Eugene, Oregon, USA). Positive control COCs were incubated during the last 12 h of IVM with 1 µM staurosporine to induce apoptosis. After 24 h of IVM, COCs were first washed for 20 seconds in annexin binding buffer at 37 °C and incubated for 15 min in the presence of FITC conjugate of annexin V (25 µl/ml) and PI solution (3 µg/ml) according to the manufacturer's recommendations for the Vybrant Apoptosis Assay kit #3. Then COCs were washed for 20 sec in annexin binding buffer and transferred per three to a drop of pre-warmed PBS (37 °C) on a microscopic slide. The stained samples were examined with a Leica TCS SP2 laser scanning spectral confocal system (Leica Microsystems GmbH, Heidelberg, Germany) linked to a Leica DM IRB inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany). An Argon laser was used to excite FITC (488 nm) and PI (586 nm) fluorochromes. Positive labelling for annexin V on the outer surface membrane was observed as bright yellow to green staining. Late apoptotic and necrotic cells displayed a PI positive nucleus (red).

The total COC was evaluated by multiple cross sections set at 3 µm intervals. Analysis of the images was performed with Leica confocal software.

Experimental design

Each fatty acid in the IVM medium was tested for its effect on cleavage rate (48 h after fertilization) and blastocyst yield (8 days after fertilization). To explain possible observed effects on the developmental competence, fertilization and maturation rates were investigated in separate replicates. Per experiment, one fatty acid was tested and a negative and positive control group were included. The negative control group consisted of TCM199 and EGF (20 ng/ml). The sole difference in the positive control group was the addition of an equal volume of ethanol as used in the fatty acid group. In the fatty acid group, OA, PA or SA dissolved in ethanol were added to reach a final concentration of 200, 133 or 67 µM respectively. The fatty acid concentrations tested in this IVM model were based on the results of the *in vivo* experiment where the highest NEFA concentration observed in the FF during the NEB was 0.6 mmol/l and the average relative importance of OA, PA and SA at that time was 33%, 23% and 13% respectively. In total 5115 oocytes were cultured. The number of oocytes and replicates per experiment are shown in Table 1.

To evaluate the effect of maturation in the presence of one fatty acid on lipid content, 144 oocytes were evaluated (two replicates, 9 to 20 oocytes per group). Per replicate, four groups were compared: immature oocytes, oocytes matured in the presence of PA or SA; and oocytes matured in positive control medium.

To detect the presence of apoptosis/necrosis, ten COCs from each group (positive control, negative control and fatty acid group) were stained as described earlier (two replicates).

As an extra control of the described IVM model, also the effect of basal NEFA concentrations during IVM was investigated: 66.7 µM OA, 44.3 µM PA and 22.3 µM SA. These concentrations are based on the basal concentrations observed in the FF at day 44 pp, well after the period of NEB (total NEFA concentration of 0.2 mmol/l, see below).

Statistical analyses

Data are expressed as means± S.E.M. All statistical procedures were carried out with SPSS 11.0 for Windows,

Table 1 Number of bovine oocytes (and number of replicates) per experiment (one fatty acid tested per experiment including a negative and positive control group).

Experiment	Maturation rate	Fertilization rate	Cleavage and blastocyst yield
Oleic acid (C18:1)	338 (2)	437 (2)	752 (3)
Palmitic acid (C16:0)	450 (2)	487 (2)	845 (3)
Stearic acid (C18:0)	478 (2)	476 (2)	852 (3)

(Chicago, IL, USA). Values of $P < 0.05$ were considered statistically significant.

NEFA concentration and composition in serum and FF of the dominant follicle

The absolute NEFA concentrations in serum and in FF early and late pp were compared with a paired sample *t*-test (paired samples within the same animal in a different compartment (serum vs FF) or in a different time frame (early vs late pp)). There were no departures from normality. The different fatty acids, expressed as percentages in the NEFA fraction, were compared between serum and FF by a non parametric Wilcoxon Signed Ranks test.

Addition of oleic acid, palmitic acid or stearic acid during IVM of bovine oocytes

The proportion of oocytes that cleaved at 48 h after fertilization and the proportion of oocytes and cleaved zygotes that developed up to the blastocyst stage at day 8 after fertilization were calculated for each culture droplet (experimental unit). Four droplets were used per replicate and per treatment. No data transformations were necessary for inequality of variance between groups or for normality reasons. Data were analysed using a two-way ANOVA and a post-hoc Scheffé test. Treatment was inserted as fixed factor and replicate as random factor together with the interaction term (treatment × replicate) (mixed model). In the absence of a significant interaction term, the term was left out from the final model.

The proportion of oocytes that had reached the MI, AT or MII stage and the proportion of oocytes/zygotes that were in the MII, 2PN or >2PN stage, were calculated per treatment group and per replicate. Data were analysed using a binary logistic regression model in which treatment, replicate and the interaction of these two factors were included. In the absence of a significant interaction term, the term was left out from the final model.

The data of the lipid determination (arbitrary units of emitted fluorescence) were normally distributed and were analysed using a two-way ANOVA with treatment as fixed factor and replicate as random factor.

Results

NEFA concentration and composition in serum and FF of the dominant follicle

From 7 days prior to the expected parturition date (varying between 18 and 3 days prior to the real day of parturition) up to 44 days pp, all cows displayed a loss in BCS (on average 0.83 ± 0.15 points) ($P < 0.05$). From day 16 up to day 44 pp, the average daily milk yield increased by 5.6 kg, from 35.9 ± 1.8 kg to 41.5 ± 2.0 kg.

On average, 1.54 ± 0.2 ml FF was aspirated from 1.14 ± 0.15 follicles per cow and per session. Nine percent of all FF samples were excluded from further analysis

due to atresia, based on an E_2/P_4 ratio < 1, or because of blood contamination. In the FF samples which were analysed, the average E_2/P_4 ratio was 13.15 ± 2.17.

In serum the NEFA concentration increased significantly around parturition and was still high at 16 days pp (0.4–1.2 mmol/l). At 44 days pp, the serum NEFA concentrations were again at the basal level (0.1–0.3 mmol/l). Similarly, a significant decrease was also found in the FF from day 16 to day 44 pp. The FF NEFA concentrations early pp (day 16) ranged from 0.2 to 0.6 mmol/l and were on average $47 \pm 6.4\%$ lower than those in serum. Later pp (day 44) there was no significant difference in NEFA concentrations between serum (0.1–0.3 mmol/l) and FF (0.1–0.3 mmol/l) (Fig. 1).

Both in serum and in FF, OA, PA and SA were the three predominant free fatty acids (Fig. 2). The NEFA composition differed significantly between the two compartments. Early pp the relative concentration of SA in FF was significantly lower compared with serum. Linoleic acid (LA, C18:2), as a percentage of the NEFA, on the other hand was higher in FF than in serum. At 44 days pp, almost all investigated fatty acids differed in relative concentration in serum compared with FF. Parallel with the decrease of the NEFA concentration from early to later pp, there was a change in the composition of the NEFA fraction both in serum and in FF. In serum, the relative concentrations of SA and LA increased and the concentrations of PA and OA decreased significantly. In FF similar significant changes for OA and LA were observed as in serum.

Addition of oleic acid, palmitic acid or stearic acid during IVM of bovine oocytes

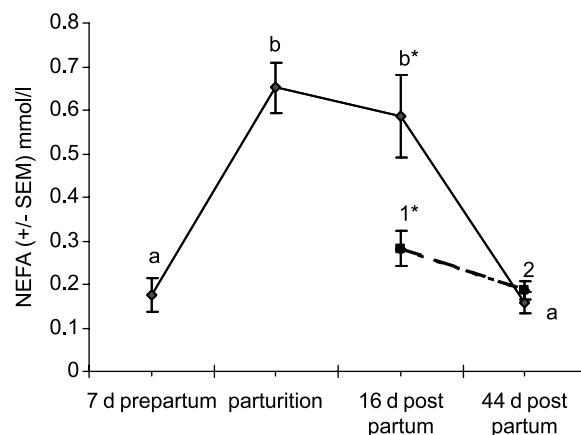Maturation in the presence of OA had no significant effect on the oocyte developmental capacity in terms of

Figure 1 Mean non-esterified fatty acid (NEFA) concentrations ($\pm$ s.e.m.) in bovine serum (black line) and in follicular fluid (dotted line) at different time points relative to parturition. Serum NEFA concentrations with different letters differ significantly between different time points. Follicular fluid NEFA concentrations with different numbers differ significantly between different time points. *Non-esterified fatty acid concentrations differ significantly between serum and follicular fluid at the same time point ($P < 0.05$).
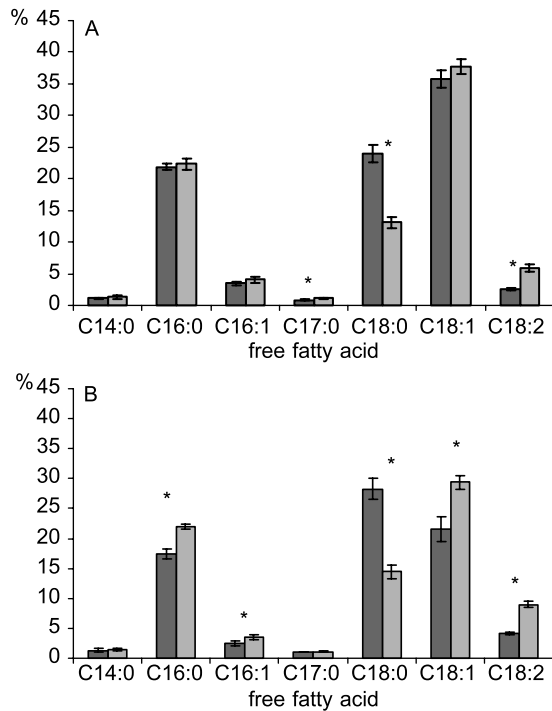
Figure 2 Mean percentage ($\pm$ S.E.M.) of the predominant fatty acids in the non-esterified fatty acid lipid fraction in serum (dark bars) and in FF (pale bars) early (day 16; A) and late (day 44; B) post partum: myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), margaric acid (C17:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). *Fatty acids with significantly different relative concentrations in serum compared with follicular fluid ($P < 0.05$).

cleavage or blastocyst yield (data not shown). However, addition of SA resulted in a significantly lower cleavage rate and subsequent blastocyst yield (Table 2) ($P < 0.05$). Similarly, there was a strong tendency for a reduced cleavage rate ($P = 0.07$) and blastocyst yield relative to the number of cultured oocytes ($P = 0.06$) or to the number of cleaved zygotes ($P = 0.12$) after maturation in the presence of PA (Table 3). The fertilization rate was

significantly reduced for the oocytes matured in the presence of PA or SA ($P < 0.05$). Moreover, the presence of PA or SA during the IVM delayed the progression through meiosis, expressed as a significantly higher number of oocytes still in MI and a concomitant lower relative number of oocytes in MII (Tables 2 and 3) ($P < 0.05$).

Maturation of oocytes in the presence of PA or SA had no effect on the lipid content of single bovine oocytes. The arbitrary units of emitted fluorescent light were similar in the four groups (data not shown).

After IVM in PA or SA, COC morphology was evaluated and compared with control COCs. Poor expansion of the COCs cultured in the presence of PA or SA was obvious (Fig. 3). After staining and evaluation with laser scanning confocal microscopy all COCs in the SA or PA group displayed a high proportion of apoptotic or late apoptotic/necrotic cells ($>40\%$ of the cells were positive). In the positive control group only few cells of the COCs ($<10\%$ of the cells) were apoptotic (Fig. 4).

No effect of ethanol during the IVM could be observed on all evaluated outcome variables. Similarly, IVM of oocytes in the presence of positive energy balance associated concentrations of the three tested fatty acids, had no effect on any of the tested variables.

Discussion

In the present study it was hypothesized that possible toxic effects of NEFA on oocyte quality may be a partial explanation for the fertility decline in modern high-yielding dairy cows. Therefore, we aimed first to determine the NEFA concentration and composition in FF of high-yielding dairy cows in relation to serum early and later pp. Secondly, the three predominant NEFA in the FF of the dominant follicle, were added in an IVM model at concentrations observed *in vivo*, to investigate their effect on the developmental capacity of the oocyte.

The results of the *in vivo* study show a significant increase in serum NEFA concentrations around parturition

Table 2 Effect of stearic acid (C18:0) added to the maturation medium on maturation and fertilization rate, cleavage rate ($\pm$ S.E.M.) at 48 h after fertilization (pi) and number of blastocysts ($\pm$ S.E.M.) at 8 days pi relative to the number of bovine oocytes put in culture or relative to the cleaved zygotes.

	Negative control	Positive control	Stearic acid (C18:0)
Maturation rate (%)			
Metaphase I	9.2[a]	18.6[b]*	26.0[b]*
Ana-/Telophase[1]	16.1[a]	11.6[a]	18.4[a]
Metaphase II	74.8[a]	67.8[a]	54.0[b]
Fertilization rate (%)			
Metaphase II	10.7[a]	8.8[a]	23.4[b]
2 Pronuclei	69.7[a]	72.2[a]	55.6[b]
$>$ 2 Pronuclei	12.5[a]	12.1[a]	12.5[a]
Cleavage rate at 48 h pi (%)	76.9 $\pm$ 3.2[a]	77.4 $\pm$ 2.7[a]	57.9 $\pm$ 3.6[b]
% blastocysts from oocytes	33.3 $\pm$ 3.6[a]	34.4 $\pm$ 2.1[a]	21.3 $\pm$ 3.5[b]
% blastocysts from cleaved zygotes	43.1 $\pm$ 4.3[a]	44.4 $\pm$ 2.1[a]	39.6 $\pm$ 7.0[a]

[a,b]Data within a row marked with different superscripts, differ significantly ($P < 0.05$). *$P = 0.1$. [1]Significant interaction term "treatment X replicate".

Table 3 Effect of palmitic acid (C16:0) added to the maturation medium on maturation and fertilization rate, cleavage rate ($\pm$s.e.m.) at 48 h after fertilization (pi) and number of blastocysts ($\pm$s.e.m.) at 8 days pi relative to the number of bovine oocytes put in culture or relative to the cleaved zygotes.

	Negative control	Positive control	Palmitic acid (C16:O)
Maturation rate (%)			
Metaphase I	9.1[a]	12.5[a]	24.1[b]
Ana-Telophase	15.9[a,b]	10.5[a]	19.9[b]
Metaphase II	75.0[a]	77.1[a]	63.2[b]
Fertilization rate (%)			
Metaphase II	21.6[a]	20.2[a]	33.5[b]
2 Pronuclei	64.0[a]	59.2[a]	43.4[b]
> 2 Pronuclei[1]	7.0[a]	5.8[a]	11.6[a]
Cleavage rate at 48 h pi (%)	76.6 $\pm$ 2.3[a]	74.5 $\pm$ 2.6[a,b*]	66.6 $\pm$ 3.2[b*]
% blastocysts from oocytes	22.4 $\pm$ 2.0[a]	24.6 $\pm$ 1.5[a†]	17.2 $\pm$ 3.0[a†]
% blastocysts from cleaved zygotes	29.1 $\pm$ 2.4[ab§]	33.2 $\pm$ 1.8[a]	22.7 $\pm$ 4.1[b§]

[a,b]Data within a row marked with different superscripts, differ significantly ($P < 0.05$). [1]Significant interaction term "treatment X replicate".
*$P = 0.07$; †$P = 0.06$; §$P = 0.12$.

and elevated levels are maintained up to two weeks pp. At 44 days pp the NEFA concentrations had returned to prepartum levels. This change in NEFA concentration with time pp is in accordance with other studies and is a major characteristic of the NEB early pp. The NEB together with low insulin concentrations and the release of stress associated catecholamines increases the degree of lipolysis and decreases the rate of re-esterification of free fatty acids in the adipose tissue (Chilliard *et al.* 1998, Vernon 2002). Moreover, all animals displayed a significant loss in body condition early pp, confirming the presence of NEB. Several studies have associated the NEB with delayed resumption of ovarian activity and reduced conception rates, finally leading to suboptimal fertility (Zurek *et al.* 1995, Beam & Butler 1999, de Vries & Veerkamp 2000).

Focussing on the FF early pp, the NEFA concentrations were elevated but still significantly lower than in serum. This remarkable concentration gradient confirms what has been suggested in earlier work (Leroy *et al.* 2004). Later on pp, both serum and FF NEFA concentrations were basal again and no concentration differences were present. These findings suggest that, at least to some extent, the vulnerable oocyte and granulosa cells are protected from too high and possibly toxic NEFA concentrations during the NEB in high-yielding dairy cows. Elevated

NEFA concentrations in serum and in FF have also been described in heifers and lactating cows that were subjected to an acute dietary restriction (Comin *et al.* 2002, Jorritsma *et al.* 2003). Our results also demonstrate that OA, PA and SA are the three predominant free fatty acids both in serum and in FF. This was also shown by Yao *et al.* (1980) in pigs. Moallem *et al.* (1999) however, found that LA dominated in the NEFA fraction of bovine FF. Furthermore, we observed that the NEFA composition in serum early pp differs from that later on pp.

Differences in serum or FF albumin concentration, on which NEFA are bound and transported, has been suggested to account for the observed NEFA gradient (Yao *et al.* 1980). We only found a 7% lower albumin concentration in FF compared with serum early and later pp (data not shown). Therefore, it is unlikely that this small albumin gradient is the only factor responsible for the observed differences in NEFA concentrations. Literature about the properties of the follicle–blood barrier and their effects on albumin and thus NEFA concentrations is contradictory (Zamboni 1974, Wise 1987).
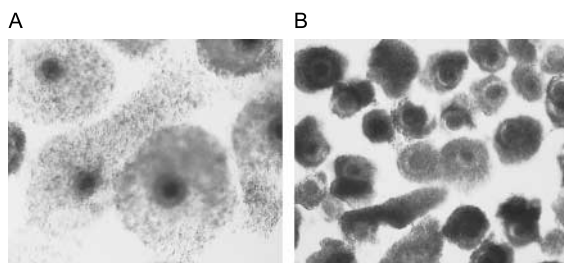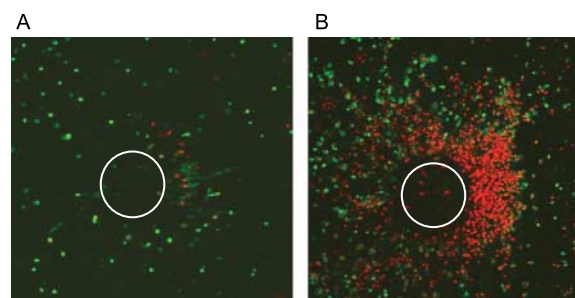
Figure 4 Cumulus–oocyte complexes from the positive control group (A) and the stearic acid group (B) after staining with Annexin V and propidium iodide for detection of apoptotic (green cell membranes) or late apoptotic/necrotic cells (green cell membranes and red nucleus) (100 × magnification). The white circle represents the position of the oocyte. A relative higher abundance of annexin V and PI positive cells can be appreciated in the stearic acid group.

Figure 3 Cumulus–oocyte complexes after 24 h of maturation in positive control medium (well expanded) (A) and in medium with added stearic acid (poor expansion) (B) (40 × magnification).

In the presence of high NEFA levels, a substantial portion of the NEFA can be partitioned to low density lipoproteins (LDL) (Chung *et al.* 1995). Especially since the saturated fatty acids are bound on LDL, while the unsaturated ones are preferably bound on albumin (Chung *et al.* 1995). The fact that LDL are absent in FF (Brantmeier *et al.* 1987, Wehrman *et al.* 1991), may explain the observed differences early pp in the concentration and composition of NEFA in FF compared with serum in our study. Indeed, the results show a lower fraction of SA (saturated) and a higher fraction of LA (unsaturated) in the NEFA present in FF compared with serum. Also active transport, desaturating enzymes and selective uptake or metabolisation by intrafollicular cells (Yao *et al.* 1980) could be responsible for the observed differences in NEFA concentration and composition in the two compartments early and later pp. Conclusively, it can be stated that mimicking NEB associated NEFA concentrations in IVM models should be based on the intrafollicular rather than on the serum concentrations.

After investigating the NEFA fraction in the FF of high-yielding dairy cows during NEB we were able to test the effect of elevated concentrations of the three major unbound NEFA on *in vitro* oocyte maturation. Although NEFA in FF are mainly bound to albumin, the unbound fraction is directly involved in the fatty acid uptake by cells (Berk & Stump 1999). The importance of the albumin bound fatty acids in this process remains a matter of discussion. It does seem as though both forms of fatty acids are taken up by the cells, suggesting the physiological significance of the total NEFA concentration (McArthur *et al.* 1999, Synak *et al.* 2003). In preliminary experiments with fatty acid free albumin and with albumin bound OA, albumin itself exerted a negative effect on the oocyte's developmental competence (Leroy *et al.* 2003). To avoid such effects, we used unbound fatty acids dissolved in ethanol, as has been done by others (Hinckley *et al.* 1996, Hirabara *et al.* 2003, Vanholder *et al.* 2005).

Supplementation of the medium with elevated concentrations of PA or SA resulted in a negative effect on the progression of meiosis. The subsequent fertilization and cleavage rates and blastocyst formation were significantly reduced. OA had no effect on any on the outcome of the variables which confirms that maturation and fertilization proceeded normally (Rizos *et al.* 2002). Two other studies which have investigated the effect of fatty acids on oocyte maturation differ from ours in the fact that they added fetal calf serum and applied albumin bound fatty acids in supraphysiological concentrations (Homa & Brown 1992, Jorritsma *et al.* 2004).

The reduced fertilization rate and hampered *in vitro* development are most likely carry-over effects of the delayed or blocked maturation. Therefore, based on the present study, it is impossible to give evidence on how maturation in the presence of PA or SA directly influenced the oocyte's developmental capacity after maturation. Only IVM in the presence of PA tended to have a negative effect on the rate of blastocyst formation relative to the cleaved zygotes. It is clear, however, that the major impact of PA and SA is on the oocyte maturation itself. A combination of the three fatty acids in one IVM set up, also negatively affected oocyte quality. Unfortunately, because there was a tendency for subtle aggregation and precipitation of the added fatty acids, data were not fully reliable and hence are not shown.

Parallel with the results of the present study, it has been shown earlier in our lab that PA and SA and not OA exert a toxic effect on bovine granulosa cell growth and function *in vitro* (Vanholder *et al.* 2005). Similar results were observed in human granulosa cells (Mu *et al.* 2001) and in rat Leydig cells *in vitro* (Lu *et al.* 2003). These studies demonstrated the induction of apoptosis by PA and SA, probably through ceramide production or through a down-regulation of the apoptosis inhibitor Bcl-2 and the up-regulation of an apoptosis mediator such as Bax. Our observations of the poorly expanded COCs after maturation in the presence of PA or SA seem to be due to the induction of apoptosis as well, since a massive degree of late apoptotic and even necrotic cumulus cells were detected. Iseki *et al.* (1995) documented the presence of fatty acid binding proteins in rat granulosa cells, illustrating the possibility of fatty acid uptake. The existence of such receptors in the cell membrane of bovine cumulus cells, however, has never been described. Others found that saturated fatty acids can induce peripheral insulin resistance and thus blocking of glucose uptake in muscle cells (Hirabara *et al.* 2003). Furthermore, insulin depletion in pancreatic β-cells can also be triggered by an increased prevalence of apoptosis and necrosis after incubation with saturated fatty acids (Mason *et al.* 1999, Cnop *et al.* 2001, Maedler *et al.* 2001). Jorritsma *et al.* (2004) suggested that changes in membrane properties of the oocyte could be responsible for the observed negative effects of albumin bound OA in the IVM medium. Whatever the mechanisms, our results clearly indicate that exposure of COC to PA or SA during 24 h has a deleterious effect on cumulus cell health and survival. Because a healthy cumulus investment is indispensable for correct oocyte maturation (Tanghe *et al.* 2002), the oocyte is most likely indirectly affected by these fatty acids.

Oocytes are said to be able to accumulate fatty acids from their environment, potentially changing their lipid content and composition (Kim *et al.* 2001, Adamiak *et al.* 2005). Lipid accumulation in oocytes and embryos can reduce their quality and cryotolerance (Abe *et al.* 2002). But, in contrast with xenopus oocytes (Zhou *et al.* 1994), a fatty acid binding protein on the oolemma of bovine oocytes has never been described. Shimabukuro *et al.* (1998) attributed the lipotoxicity of added NEFA in β-cell cultures to the accumulation of intracellular lipids, inducing ceramide and NO production, finally resulting in apoptosis. To test the possibility of such lipid accumulation in the oocyte, we analysed the lipid content of mature oocytes after IVM in the presence of PA or SA. No

lipid accumulation, however, could be detected. This suggests that lipid accumulation in oocytes is probably not involved in the observed negative effects of the free fatty acids in this study.

The findings of the present study support the hypothesis of Britt (1994), confirming that metabolic changes during a period of NEB (*in casu*: high NEFA concentrations) may have detrimental effects on the developmental capacity of the oocyte. It is however important to mention that the combined *in vitro* and *in vivo* model used in this study was not entirely appropriate in investigating the described carry-over effect on oocyte quality. Our results only document on the FF composition in the dominant follicle during the NEB which was mimicked *in vitro*. Quiescent follicles, which embed the oocytes of interest, however, provide a much poorer isolation of the oocyte from the extrafollicular environment and blood serum, probably exposing the growing oocyte to even higher NEFA concentrations (Zamboni 1974, Fair 2003). Furthermore, in this study the COCs were exposed to elevated NEFA levels for only 24 h, whereas *in vivo* the oocytes are exposed to such levels for weeks. The ideal model should cultivate primordial follicles in high NEFA conditions for several days or even weeks. Moreover, extrapolating *in vitro* results from this well defined IVM model to the real *in vivo* situation should always be done with caution. Being the only practical approach, the model used in the present study revealed for the first time possible toxic effects of high intrafollicular NEFA concentrations on the developmental competence of bovine oocytes *in vitro*. Acute fatty acid mobilization caused by food restriction or reduced appetite (illness or lameness) later pp also involves a fast NEFA rise both in serum as well as in FF (Comin *et al.* 2002, Jorritsma *et al.* 2003). The present study demonstrates that even a very short (24 h) exposure to elevated NEFA levels just prior to ovulation can be detrimental to the developmental capacity of the pre-ovulatory oocyte.

It can be concluded that even though FF NEFA levels are high during the period of NEB early pp, the concentration remains remarkably lower than in serum. Furthermore, the NEFA composition in FF differs from that of serum. *In vitro* oocyte maturation in the presence of NEB associated concentrations of PA and SA is hampered, leading to reduced fertilization rate and developmental competence. The data of the present study suggest that toxic effects of elevated FF NEFA concentrations on oocyte quality may be one of the factors through which NEB exerts its negative effects on fertility in high-yielding dairy cows.

Future research should concentrate on the cellular mechanisms through which fatty acids can exert a toxic effect on COCs.

Acknowledgements

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