Elevated non-esterified fatty acid concentrations hamper bovine oviductal epithelial cell physiology in three different in vitro culture systems

Reference:

Jordaens Lies, Arias-Alvarez M., Pintelon Isabel, Thys Sofie, Valckx Sara, Dezhkam Y., Bols Peter, Leroy Jo.- Elevated non-esterified fatty acid concentrations hamper bovine oviductal epithelial cell physiology in three different in vitro culture systems Theriogenology: an international journal of animal reproduction - ISSN 0093-691X - 84:6(2015), p. 899-910

Full text (Publishers DOI): http://dx.doi.org/doi:10.1016/j.theriogenology.2015.05.030

Revised

Elevated Non-Esterified Fatty Acid concentrations hamper Bovine Oviductal Epithelial Cell physiology in three different *in vitro* culture systems

L. Jordaens[a*], M. Arias-Alvarez[b], I. Pintelon[c], S. Thys[c], S. Valckx[a], Y. Dezhkam[a], P.E.J. Bols[a], J.L.M.R. Leroy[a]

[a] *Laboratory for Veterinary Physiology and Biochemistry, Gamete Research Center, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium*

[b] *Dpto. de Producción Animal, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain*

[c] *Laboratory for Cell Biology and Histology, Core Facility for Biomedical Microscopic Imaging, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium*

**Corresponding autor: Lies Jordaens; Laboratory for Veterinary Physiology and Biochemistry, Gamete Research Center, University of Antwerp, Universiteitsplein 1, B-2610 Wilrijk, Belgium; +32 3 265 23 98; lies.jordaens@uantwerpen.be*

Abstract

Elevated non-esterified fatty acids (NEFAs) have been recognized as an important link between lipolytic metabolic conditions and impaired fertility in high yielding dairy cows. However, NEFA-effects on the oviductal micro-environment currently remain unknown. We hypothesize that elevated NEFAs may contribute to the complex pathology of subfertility by exerting a negative effect on Bovine Oviductal Epithelial Cell (BOEC)-physiology. Therefore, the objectives of this study were to elucidate direct NEFA-effects on BOEC-physiology in three different *in vitro* cell culture systems. BOECs (4 replicates) were mechanically isolated, pooled and cultured as conventional monolayers, as explants and in a polarized cell culture system (PCC) with DMEM/F12-based culture medium. BOECs were exposed to a NEFA-mixture of oleic (OA), stearic (SA) and palmitic acid (PA) for 24h at both physiological and pathological concentrations. A control (0µM NEFA) and a solvent control (0µM NEFA + 0.45% ethanol) group was implemented. BOEC-physiology was assessed by means of cell number and viability, a sperm binding assay, transepithelial electric resistance (TER) and a wound healing assay. BOEC morphology was assessed by scanning electron microscopy on cell polarity, presence of microvilli and cilia, and monolayer integrity. **BOEC-number** was negatively affected by increasing NEFAs, however **cell viability** was not. **Sperm binding affinity** significantly decreased with increasing NEFAs and tended (*P=0.051*) to be more affected by the direction of NEFA-exposure in the PCC. The absolute **TER**-increase post-NEFA-exposure in the control (110±11 $\Omega.cm^2$) was significantly higher than in all the other treatments and was also different depending on the exposure side. Bidirectional exposed monolayers were even associated with a significant TER-reduction (-15±10 $\Omega.cm^2$) (*P<0.05*). Cell **proliferation capacity** showed a decreased cell migration with increasing NEFA-concentrations, but was irrespective of the exposure side. BOEC **morphology** was not affected. In conclusion, in an *in vitro* setting NEFAs exert a negative effect on BOEC physiology, but not morphology. Ultimately, these physiological alterations in its micro-environment may result in suboptimal development of the pre-implantation embryo and a reduced reproductive outcome in dairy cattle.

Keywords: Bovine oviduct, fatty acids, maternal metabolism, subfertility

Introduction

In dairy cattle, decades of extensive genetic selection to promote milk yield coincided with a significantly lower fertility outcome, partly due to a decreased oocyte quality and an increased early embryonic mortality rate [1]. Typically reduced estrus expression, and a retarded postpartum onset of ovarian cyclicity result in a much longer calving interval and thus significant economic losses [2]. These observations are associated with specific metabolic and hormonal changes in the blood of modern dairy cattle [3,4].

Among these metabolic changes, elevated non-esterified fatty acid concentrations, due to upregulated lipolysis typically during periods of negative energy balance, have been recognized as important detrimental factors within the female reproductive tract at follicular, oocyte [5,6,7] and early embryonic level [8,9]. Recently, Valckx *et al*. [10] noticed that long term *in vitro* NEFA-exposure hampers murine early pre-antral follicular development, while Van Holder *et al*. [11] and Mu *et al*. [12] registered negative influences of NEFAs on bovine and human granulosa and theca cell proliferation, respectively, and on the steroidogenesis. Also, murine [10] and bovine [5,6,7,9] oocyte developmental competence is drastically reduced after NEFA-exposure during the final oocyte maturation, significantly affecting embryo quality and energy metabolism [8,9]. Embryos show a reduced blastocyst cell number, increased apoptotic cell ratio and altered gene expression patterns [8]. Whether elevated NEFAs also have an immediate effect on the oviductal micro-environment, is not yet known.

The oviduct is a crucial organ in reproduction [13-17] and specifically in early embryonic development [18,19]. Both Rizos *et al*. [20], Maillo *et al*. [21] and Matoba *et al*. [22] indicated that the conditions within the reproductive tract may impair its ability to support the early embryo and may contribute to early embryonic mortality. However, up until now the underlying causes to these observations remain unknown and studying oviduct physiology remains straining to perform [23-25]. *In vitro* research for morphological [24,26-28] and functional [29-32] characteristics of bovine oviductal epithelial cells (BOECs) has provided us with three different, well-established cell culture

systems allowing the acquisition of mechanistic insights and observation of cellular pathways: 1) monolayers grown at the bottom of culture plates [33], and 2) a BOEC suspension system with a high maintenance of physiological cellular characteristics [34-36], or 3) in hanging inserts [37-39] of a polarized cell culture (PCC) system allowing bidirectional cell exposure.

Ultimately, the objective of this study was to elucidate the direct effects of NEFAs on the oviductal micro-environment. We hypothesized that elevated NEFAs may influence BOEC-physiology, by possibly altering the oviductal micro-environment that hosts early development of the pre-implantation embryo, and thus may affect overall fertility. Hereto, BOEC-morphology, quality and functionality, was studied by assessing BOEC number, viability, proliferation capacity, monolayer integrity, morphological characteristics and sperm binding capacity after a 24h *in vitro* NEFA exposure period at physiologically relevant concentrations. Furthermore, BOECs were cultivated in three different cell culture systems to compare the outcome of the specific parameters observed and to estimate the biological relevance of the systems used.

Materials and methods

All chemicals were purchased from Life Technologies® (Carlsbad, California, USA), unless otherwise stated.

1. Collection, transport and harvesting of BOECs

For each experiment, four bovine, early luteal phase (days 3 to 5 of the estrous cycle) oviducts were obtained in a local slaughterhouse and selected on ovarian morphology accordingly to the method described by Ireland *et al.* [37,40]. The oviducts were dissected free of surrounding tissues and maximally 2 to 3h upon collection they were processed in the laboratory under controlled air conditions. The infundibulum and the lower part of the uterotubal junction were removed and the remaining oviductal parts were washed in Hanks Buffered Saline Solution (HBSS) (room temperature).

Bovine oviductal epithelial cells (BOECs) were mechanically isolated, by squeezing the oviducts between thumb and index finger, and pooled in warm HBSS. Following 2 rounds of centrifugation for

5 min at 971 x g, the BOECs were easily distinguishable from the overlaying erythrocytes. The supernatant was discarded and the BOECs were pipetted and resuspended in warm and equilibrated (38,5°C, 5% CO_2, 20% O_2) culture medium based on DMEM and Ham's F-12 nutrient mixture (1:1), and containing 0.75% BSA (essentially fatty acid free; Sigma-Aldrich, St-Louis, MO, USA), 5% serum (2.5% Fetal Bovine serum, Greiner Bio-One, Frickenhausen, Germany; and 2.5% Newborn Calf Serum, Sigma-Aldrich, St-Louis, MO, USA), 2.5% penicilline/streptomycine and 2% amphotericine B. Cell count and cell viability testing were performed with a Bürker Counting Chamber (W. Schreck, Hofheim) and Trypan Blue. BOECs were diluted to a density of 1 x 10^6 cells/mL and seeded in three different culture systems as described below. The culture medium was renewed after 24h and subsequently every 48h.

2. Assessment of epithelial cell purity and polarity

A. Immunocytochemistry

The epithelial origin of the isolated BOECs was confirmed by subjecting the cells to immunocytochemical staining for the expression of cytokeratins using monoclonal mouse anti-human cytokeratin antibodies (clone AE1/AE3), biotinylated goat anti-mouse antibodies, streptavidine / horseradish peroxidase and AEC substrate-chromogen (Dako, Denmark) according to the manufacturer's instructions. BOECs were grown in 4-well culture plates: 1) as freshly retrieved BOECs, 2) as first passage BOECs after a 7-day culture period in a culture flask (to increase cell purity), trypsinized and reseeded on glass inlets, and 3) as freshly retrieved BOECs cultured as monolayers on hanging inserts in a PCC-system.

Cytokeratin stained cells were compared to non-stained cells, and a fibroblast culture of fetal bovine cartilage was used as a negative control, and non-specific binding reactions of the secondary and tertiary antibodies were excluded. Morphologic evaluation was performed by an inverted light microscope, and the imaging software of CellD* was used to assess the ratio of cytokeratin positive and negative cells.

B. Scanning Electron Microscopy

Scanning Electron Microscopy was used to visualize BOEC polarity (i.e. monolayer formation, confluency, cell orientation) and differentiation level of BOECs grown on polyester membranes of hanging inserts (ThinCert, Greiner Bio-One, Frickenhausen, Germany). Hereto, BOEC-monolayers were washed twice with Krebs solution and fixed with 2.5% glutaraldehyde (in 0.1M Na^+-cacodylate) at 4°C for 24h. The cells were rinsed three times for 20 minutes in cacodylate buffer containing 7.5% saccharose after which the insert membranes (polyester) were removed from their polystyrene housing and placed in a clean well of a 12-well plate containing cacodylate buffer. Subsequently, cells were dehydrated in an ascending series of ethanol concentrations (70%, 90%, 95% each for 15 minutes at room temperature, and 100% for 3x 30 minutes) and submitted to critical point drying in a Leica EM CPD030 after which the monolayers were mounted on a stub and gold coated in a Sputter coater. SEM-imaging was performed with a SEM 515 (Philips, The Netherlands) at different magnifications. Monolayers were assessed on cell polarity, the presence of microvilli and cilia, monolayer integrity and cell growth [41]. For each treatment 2 monolayers were processed, and all results are descriptive, not quantified.

3. Preparation of NEFA-treatments

The types and concentrations of free fatty acids used are based upon the *in vivo* concentrations found in the serum of high yielding dairy cows in negative energy balance (NEB) [6]. The oviductal luminal fluid (OLF) is essentially an ultrafiltration from the serum [42], complemented with a cycle-dependent active secretion of the outlining epithelial cells [43,44], follicular fluid (FF) and cumulus cells released after ovulation [45,46]. Therefore, it is assumable that NEFAs can occur in the OLF like they occur in the serum. Preliminary data in our laboratory suggest a correlation between serum-NEFA-concentrations and those measured in the OLF, similar to the observations made between serum. Based on these findings we established 4 treatment groups:

1) Control group without NEFAs, 2) Basal physiological NEFA-concentrations of 72 µM, ranging up until 3) 360 µM in Moderate elevated though physiological concentrations, and 4) 720 µM in High pathological and potentially cytotoxic metabolic conditions.

Stearic Acid (SA), Palmitic Acid (PA) and Oleic Acid (OA) (Sigma-Aldrich, St-Louis, MO, USA) were dissolved in a stock solution of pure ethanol at concentrations of 25, 150 and 200 mM, respectively. These ethanol stock solutions were vortex-mixed for 4 minutes and diluted to obtain the desired final concentration in the culture medium. Prior to use, all media were vigorously shaken and filter-sterilized under aseptic conditions. NEFA solvability was checked in all NEFA-medium samples prior to exposure to determine whether the intended concentrations were actually achieved. Final NEFA concentrations were measured at the 'Algemeen Medisch Labo' (Antwerp, Belgium), using commercial photometric assays, RX Daytona (Randox Laboratories). Measurements were carried out according to manufacturers' instructions.

4. Experimental design:

To obtain optimal use of the beneficial characteristics of each culture system, three experiments each using a different *in vitro* approach, were set-up. Static monolayers are most suited to study morphological characteristics **(experiment 1)**, while explants **(experiment 2)** better maintain functional cell characteristics such as ciliary activity [33,34,47] and monolayers grown on hanging inserts **(experiment 3)** allow a more physiological relevant NEFA exposure on polarized cells [48].

All experiments were performed in 4 replicates.

Experiment 1: NEFA-toxicity on BOEC monolayers in conventional culture plates

In a first experimental set-up an overall sensitivity of BOECs to NEFAs was assessed. Hereto, BOECs were seeded in 12-well culture plates (wells n = 192; 3 wells per treatment and outcome parameter in each of 4 replicates) (Nunclon, Roskilde, Denmark) at 1 x 10^6 cells/mL and incubated at 38.5°C, 5% CO_2 in humidified air. After 24h in culture the cells were washed and the non-adherent cells were removed. Subsequently, culture medium was changed every 48h. Confluency of the monolayers (>95%) was determined by phase contrast inverted light microscopy (Olympus, CKX41) and was reached at Day 5 of culture, after which exposure to different NEFA concentrations started for 24h in 5 experimental groups: 1. Control group (0µM NEFA), 2. Solvent Control group (0 µM NEFA + 0.45% ethanol), 3. Basal group (72 µM NEFA), 4. Moderate group (360 µM NEFA), and 5. High group

(720 µM NEFA). After 24h NEFA-exposure BOEC cell number, cell viability, sperm binding capacity, and cell migration capacity were determined (see below).

Based on the results of experiment 1 and the lack of effects of the solvent on the outcome parameters assessed, the solvent control treatment group was excluded from further experiments.

Experiment 2. NEFA-toxicity in a suspension culture system

To estimate the immediate effects of NEFAs on BOEC-explants, BOECs were seeded in 4-well plates (Nunclon, Roskilde, Denmark) at 1×10^6 cells/mL and incubated for 2h at 38.5°C, 5% CO_2 in air, in normal culture medium as described above, in 4 replicates (total observations n = 160; n = 10 per treatment group and per replicate). After 1h BOECs slowly started to adhere to the bottom of the recipient, while some cells remained in suspension in their natural coherence forming BOEC-explants [33,49]. Approximately 100 BOEC-explants per well were size selected using a stereomicroscope and a hand-held glass pipette. After selection, the BOEC-explants were transferred in new, sterile 4-well plates, and divided in the same experimental groups as described for experiment 1. After 24h NEFA-exposure the explants were removed from the NEFA containing medium and replaced in normal culture medium, after which sperm cell co-incubation was performed as described below. The sperm binding capacity of the BOEC-explants was determined by inverted light microscopy and Cell*-imaging software where only explants between 10 000 and 40 000 μm^2 were taken into consideration to limit binding variances due to differences in explant size [50].

Experiment 3. NEFA-toxicity on BOEC monolayers in a Polarized Cell Culture system

BOECs were directly seeded at 1×10^6 cell/mL on laminin coated, polyester membranes of hanging inserts (wells n = 192; 12 wells per treatment in 4 replicates) (ThinCert, Greiner Bio-One, Frickenhausen, Germany; 12 wells, 0.4 µm pores, pore density $2 \times 10^{-6} cm^2$), to create a two compartment, PCC-system to support cell growth. Monolayer confluency was estimated visually by inverted light microscopy, and through daily measurements of the transepithelial electric resistance (TER) using an Avometer (Millicell-ERS®, Millipore, Massachusetts, USA). Monolayer formation was considered confluent and free of leakage between both compartments, when the TER-recordings

reached approximately 700 $\Omega.cm^2$ [51] at Day 7-9. As a control physiological serum NEFA-concentrations of 72 µM were applied, while to provoke negative effects on the cells, pathological serum NEFA-concentrations of 720 µM were used, exposing the monolayers for 24h as depicted in figure 1.

5. Outcome parameters

BOEC morphology: Scanning Electron Microscopy (experiment 3)

BOEC-morphology was assessed using Scanning Electron Microscopy for monolayers on inserts for each of the 4 treatment groups in experiment 3. Cell polarity, the presence of microvilli and cilia, monolayer integrity and cell growth were assessed. SEM was performed only in experiment 3 to illustrate the added value of the hanging inserts on the preservation of BOEC natural characteristics.

BOEC Number and Viability: Hemocytometer and Trypan Blue staining (experiment 1&3)

After 24h NEFA-exposure, three wells per experimental group were washed with 500µl of pre-warmed DPBS (without Ca^{2+}, Mg^{2+}). For 10 minutes 200µl trypsin was added to each well at room temperature, until all cells were detached. The enzymatic reaction was stopped by adding 700µl normal culture medium containing both serum and BSA, after which the cell suspension was homogenized. A hemocytometer (Bürker Counting Chamber, W. Schreck, Hofheim) and a trypan blue dye-based exclusion method was used to determine cell number and cell life/death-ratio. Briefly, the cell suspension and trypan blue 0.4% were mixed in a 1:1 ratio and BOECs were counted while a distinction could be made between the unstained live cells and the blue dye containing dead cells [52]. Results were expressed in cells/µl and % of live to total cells, respectively.

BOEC quality: Sperm Binding Capacity (experiment 1, 2, 3)

The same batch of frozen bovine semen, pooled from three bulls of proven fertility, was used in all 3 experiments. Straws were thawed in a water bath at 37°C for 30 seconds. Live and motile spermatozoa were selected by centrifugation on a discontinuous Percoll gradient (90% and 45%, Amersham Biosciences, Roosendaal, The Netherlands). Three wells in each experiment were co-incubated with 1×10^6 motile sperm cells in 0.5 mL TALP + BSA (without heparin) for 30 min,

according to Lefebvre and Suarez [53], and Gualtieri and Talevi [30]. The non-bound sperm cells were washed off with prewarmed DPBS. The monolayers or explants with bound sperm were fixed in 4% paraformaldehyde (VWR international, Radnor, PA, USA) for 30 minutes at room temperature. The number of bound sperm cells was counted according to a modification of Gualtieri and Talevi [30] in 4 fields of a predetermined dimension in 2 or 3 wells per time point in experiment 1 and 3, and in 10 size selected (10 000 - 40 000 μm^2) explants per treatment in experiment 2 using an inverted microscope (×400) (Olympus CKX41). All counts were performed blinded to treatment.

BOEC functionality: Wound Healing Assay / *in vitro* scratch assay (experiment 1&3)

In experiment 3, a wound healing assay was performed to study cell migration capacity. Hereto, an artificial gap was made in the center of each monolayer using a 200µl pipette tip. Loose cells were washed away using standard culture medium. Cells at both sides of the scratch migrated toward each other in order to re-establish confluency. Immediately after creating the scratch and at hourly intervals, images were captured during 8 consequent hours using inverted light microscopy (Olympus, CKX41). To obtain the same field of interest during the observations, etches were made on the bottom of each well (using a needle) used as microscopic reference points to assure measurements were always carried out at the exact same spot. Cell D* imaging software was used to measure the wound diameter and to determine cell migration rate by comparison of the consecutive images and diameters over time [54].

BOEC-monolayer integrity: Transepithelial Electric Resistance (experiment 3)

During the entire culture period in experiment 3 monolayer integrity was assessed daily through TER-measurements, both prior and post NEFA-exposure. Hereto, a Millicell-ERS (Millipore, Massachusetts, USA) was used according to the manufacturer's instructions. Monolayers were considered to be confluent when TER-values exceeded 700 $\Omega.cm^2$ [51].

$$TER = (R_{sample} - R_{blank}) \times \text{effective membrane area}$$

6. Statistical analysis

Data are expressed as means ± SD. Trial data were analyzed as a completely randomized design using

SPSS 19.0 for Windows, (Chicago, IL, USA). Values of $P<0.05$ were considered statistically significant. Mean differences of cell viability (percentage live cells relative to total cell number), cell count, sperm binding, wound closure (change expressed as percentage relative to the initial wound diameter) and TER changes during exposure (change expressed as percentage relative to the TER before exposure) among experimental groups were compared with ANOVA and a post-hoc Scheffe test including the fixed effect of the treatment, the random effect of the replicate and the interaction of these two factors. Only when the interaction term was not significant, it was left out from the final statistical model. TER and wound size before and after closure were first compared using a paired samples t-test to identify changes significantly different from zero. For normality and equality of variance reasons, TER data were log transformed before statistical analysis.

Results

Assessment of epithelial cell purity and polarity

1. Immunocytochemistry

Cells positive for cytokeratins were stained red (figure 2), while negative cells remained unstained. Overall analysis of the immunocytochemical staining showed >95% positive cells for anti-cytokeratin antibodies, thus confirming the epithelial origin and purity of the cells isolated and cultured in both conventional monolayers, and monolayers grown on hanging inserts. Also, freshly retrieved monolayers and monolayers from a first cell passage did not differ significantly in cell purity.

2. Scanning Electron Microscopy

The efficacy of the isolation and cultivation protocol was critically assessed by scanning electron microscopy. All monolayers showed signs of cellular polarity (figure 3. A) and differences in cell height. The apical side of the monolayers showed the presence of both microvilli and cilia (figure 3. B), though the latter were confined to specific regions within the monolayers and seemed less oriented when compared to *ex vivo* images [32]. Several interruptions in monolayer integrity were seen (figure 3. C), but might have been artificially induced during sample preparations. The presence of white flakes in between the cells (figure 3. A,C,D) may indicate cellular secretions [55].

Experiment 1: NEFA-toxicity on BOEC-monolayers in conventional culture plates

BOEC-number (figure 4A) was significantly lower after 24h exposure in treatment groups exposed to increased NEFA-concentrations (*P<0.05*). **Cell viability** (figure 4B) was comparable between the different treatment groups, regardless of the aplied NEFA-concentrations. The **number of bound spermatozoa** to BOECs was compared between the different treatment groups (figure 4C), indicating that increased NEFA-concentrations (Moderate and High NEFA-groups) induce a significantly reduced BOEC-sperm binding capacity (*P<0.05*). Basal NEFA-concentrations at 72µM did not affect the outcome of this parameter compared to the control (0 µM NEFA). Looking at the BOEC **migration capacity** in figure 4D, only the High NEFA-group showed a reduced ability to close an artificial wound within the monolayers (*P<0.05*). All other treatment groups showed comparable results. The solvent did not affect the results in the outcome parameters tested and showed results comparable to the control group in which no NEFAs were added.

Experiment 2. NEFA-toxicity in a suspension culture system

24h NEFA exposure to BOECs in a suspension culture system showed a statistically significant reduced **sperm binding** for the Moderate and the High NEFA groups, of 59.19% and 68.05% respectively, when compared to the Control group (figure 5). Also, there was a significant difference in sperm binding between the Basal (196.25 ± SD 30.92 spermatozoa/0.05mm^2) and the High (148.75 ± SD 50.23 spermatozoa/0.05mm^2) treatment group *(P<0.05)*.

No statistical differences were found in average explant surface area within the different replicates nor treatment groups.

Experiment 3. NEFA-toxicity on BOEC monolayers in a Polarized Cell Culture system

BOEC morphology, as assessed on cell polarity, presence of microvilli and cilia, cellular outlining and secretion, was not affected by a 24h exposure time to the different NEFA treatments.

As indicated in figure 6 **BOEC number** (figure 6. A) was significantly higher in treatment 1 (Control) (34.92 ± 12.13x10^6 cells/mL) when compared to all the other treatments. Also, cell number in treatment 4 (21.33 ± 10.26x10^6 cells/mL) was significantly lower as compared to all other treatments.

The average **cell viability** varied between 90 and 95%, but no significant differences were detected between the different treatments (data not shown).

Sperm binding capacity (figure 6. B) in treatment 1 (97.90 ± 10.76 spermatozoa/0.05mm^2) was significantly higher than in all other treatments. Also treatment 2 (68.55 ± 15.38 spermatozoa/0.05mm^2) showed significant higher sperm binding capacity than treatment 4 (31.28 ± 6.16 spermatozoa/0.05mm^2) (*P<0.05*) and tended to be higher than treatment 3 (39.95 ± 19.30 spermatozoa/0.05mm^2) (*P=0.051*). Overall, sperm binding was negatively affected by bidirectional NEFA-exposure when compared to unidirectional exposure, and tended to be affected by the exposure side where NEFA-exposure in the upper compartment tended to reduce monolayer sperm binding affinity more than when exposed from the lower compartment. Sperm binding appeared to be less clustered when monolayers were bilaterally exposed to pathological NEFA-concentrations, however this is merely an estimation and these observations were not objectified.

Cell migration capacity (figure 6. C), through comparison of the wound diameter at T0 and T8, showed a significant closure of the gap in all treatments and the percentage of closure in treatment 1 (41.64 %) was significantly different from the other treatment groups (treatment 2 = 28.3 %; 3 = 31.62 %; 4 = 30.9 %) (*P<0.05*). No differences could be observed between uni- and bidirectionally exposed monolayers, nor resulting from alternating the exposure side.

In all treatment groups the **TER** (figure 6. D) at T0 (prior to NEFA exposure) and T1 (post NEFA-exposure) were very well correlated. The average relative TER increase in treatments 1 and 2 was significantly different from 0 (*P<0.05*) and tended to decrease in treatment 4 (*P = 0.05*). However, the absolute TER-values in that group were still on average 944.13 ± 49.18 Ω.cm^2, meaning the monolayers were still 100 % confluent preventing leakage from one compartment to another [51]. Furthermore, the absolute TER-increase after NEFA-exposure in treatment 1 (110 ± 11 Ω.cm^2) was significantly higher than the TER in all the other treatment groups. Also, treatment 2 (29 ± 8 Ω.cm2) and 3 (3 ± 6 Ω.cm^2), and 3 and 4 (-15 ± 10 Ω.cm^2) showed TER values that were statistical significantly different from each other *(P < 0.05),* meaning that bidirectional exposure reduces TER-

values more than unidirectional exposure, and also exposure from the upper compartment affects monolayer TER more than exposure from the lower side.

Discussion

We hypothesized that elevated NEFAs may affect BOEC quality and functionality, thereby potentially creating a less than favorable developmental environment for the pre-implantation embryo which may negatively affect overall fertility. NEFAs are suggested to be introduced in the oviductal lumen via 2 pathways: 1) a rather small fraction through the ovulation of a NEFA-containing follicle [56], and 2) a larger fraction through the natural formation of the OLF, which is essentially an ultrafiltrate from the blood [42]. Preliminary data from our laboratory have suggested a close correlation between serum NEFA concentrations and these in the OLF, making the effects of alternating unidirectional NEFA-exposure but also bidirectional exposure physiologically relevant to study. The NEFA-concentrations added during our experimental setting are therefore closely resembling the serum NEFA-concentrations of dairy cattle in periods of peak lactation, during which NEFA concentrations are typically elevated due to up-regulated lipolysis [6]. Also, the combination of fatty acids used is our experiments are based on the three predominant NEFAs occurring in both serum and FF [57].

In previous experiments BOECs were mostly a means to support embryo production, but they have rarely ever been the primary subject in scientific research in relation to maternal metabolic disorders and subfertility as a base to study different pathways. Our study, hereto, aimed to focus on the direct in vitro effects of NEFAs on BOECs. Therefore, we selected three different, specifically designed *in vitro* cell culture techniques, each to exhibit specific cell features to best suit our research aims. Static monolayers are most suited to study morphological characteristics **(experiment 1)**, while explants **(experiment 2)** better maintain functional cell characteristics such as ciliary activity [33,34,47] and monolayers grown on hanging inserts **(experiment 3)** allow a more physiological relevant NEFA exposure on polarized cells [48, 58].

NEB and elevated serum NEFAs *in vivo* in dairy cows occur over an extended period of time up to 10-12 weeks pp with a maximum at 2 weeks pp [59]. Nonetheless, to really visualize direct NEFA-

toxicity-effects without interference of any compensatory mechanisms, we chose to implement a 24h exposure period. This was also recommended by Ricchi *et al*. [60], who studied NEFA-toxicity in rat hepatocytes, since shorter incubations induced little effects that were difficult to detect, while a prolonged incubation time was associated with a significant decrease in cell viability.

The main findings of this study were that BOEC-physiology was negatively affected by elevated NEFA-concentrations, and that the intensity of the effects observed depended on the exposure side and thus on cell polarity. These data might partly elucidate the *in vivo* findings of Rizos *et al*. [20], Maillo *et al*. [21] and Matoba *et al*. [22] where the reproductive tract of lactating dairy cows showed an impaired ability to support early embryo development compared to their non-lactating counterparts. In our experimental design we aimed to establish these negative conditions by mimicking metabolic stress and thus elevated NEFA-concentrations, and observed a hampered BOEC-quality and functionality but not morphology. Decreased BOEC viability, cell numbers, and cell migration capacity may alter the pre-implantation embryo micro-environment, may therefore create suboptimal growth conditions for the early embryo and can thus be proposed as a contributing factor in the complex pathogenesis of sub- and infertility in high yielding dairy cattle. Hampered BOEC sperm binding capacity may also contribute to sub- and infertility since natural processes like sperm capacitation and selection may be hindered, possibly leading to a less successful fertilization.

Looking at the NEFA-effects in closer detail by assessing the individual parameters, we noticed a decreasing ***BOEC/sperm binding capacity*** with increasing NEFA-concentrations. BOEC/sperm-binding is essentially a proteoglycan bridge formation between the head of the sperm cells and cilia or microvilli [61]. A successful BOEC/sperm-binding reaction requires a viable BOEC with an intact membrane, functional cilia and specific secretory activity [32]. Decreased sperm binding affinity may therefore be explained by a corruption of the cellular membranes after NEFA-exposure. Fatty acids are known to alter cell membrane composition and fluidity, depending on the chemical structure of the NEFAs added. Membranes will become more rigid when straight chained, saturated fatty acids, such as PA and SA, are incorporated in the cell membranes. While integration of unsaturated fatty

acids, such as OA, will rather induce an increase in membrane fluidity [62]. Altering BOEC-membranes might prevent the establishment of proper sperm binding reactions.

NEFA-induced alterations to BOEC-membrane properties might also explain the divergent results on **BOEC-viability** we have observed. Since the enzymatic reaction of the trypsin added to estimate this parameter might very well rupture the NEFA-impaired cell membranes, causing lysis of otherwise viable cells and thus induce falsely high viability percentages and masking the effects of the NEFAs added. To verify these data in situ viability assessment and quantification techniques, such as a Neutral Red assay [63], can be performed in order to distinguish enzyme induced damages from the actual NEFA-effects.

We also noticed a decreased **cell number** with increased NEFA-concentrations. As NEFA-toxicity is primarily attributed to elevated Reactive Oxygen Species (ROS)-production and as ROS target high fat cell membranes, lipid radicals could be generated in BOECs as seen in other cell types [64]. Thus affecting the BOEC-membranes might also attribute to the diminished BOEC/sperm binding capacity, but may also induce apoptosis, a reduced proliferation rate and therefore decreasing cell numbers.

In the PCC with hanging inserts, however, these NEFA-toxic effects on cell number do not seem to compromise the overall continuity of the epithelial outlining of the oviduct. TER-values are constituted by differences in electric potential between the 2 compartments and can be translated to the tightness of the intercellular interactions, such as tight junctions. Fatty acids have the ability to modulate tight junction composing proteins, such as occludins [65], possibly allowing intercellular transfer of larger molecules and even disruption of the cell layer. However, in our experiments the TER-values continued to increase during the NEFA-exposure period in unidirectionally exposed monolayers, while they decreased during bidirectional exposure. This decrease, however, was never to the extent that leakage between the two compartments would exist.

These data are also supported by our findings on the **cell migration capacity**. Cell migration contributes to tissue repair and regeneration [66]. High concentrations of PA and SA are suggested to

cause inhibitory effects on cell growth preventing closure of an artificially induced gap, while OA is suggested to provide the opposite effect [67]. OA also, has previously been suggested as being able to compensate the detrimental effects of saturated fatty acids [7] and might also be accountable for the less pronounced effects observed here.

Comparison of the culture systems used can only be done by assessing the results of the sperm binding assay in the three experiments, since this is the only outcome parameter consistently used in all three the culture systems. In experiment 2, using a short term suspension BOEC-culture, the BOEC-explants/sperm binding capacity is much higher than in the other 2 experiments, and similar to the data have been reported by Sostaric *et al*. [32]. BOEC-monolayers tend to dedifferentiate and lose the expression of cilia at the cell surface. BOEC/sperm-binding is therefore not established with cilia but rather with microvilli. This binding reaction, however, appears to be less efficient [32].
In experiment 1, the reduced sperm binding capacity, the lower cell numbers and the lack of the ability for maintaining the cells 'fresh' over a longer period of time, may provide us with sufficient indications that BOEC-monolayers in the hanging inserts in experiment 3 are more suited to observe *in vitro* reactions within the oviductal epithelium. Therefore, because of the possibility to expose the cells via two compartments as seen *in vivo* and the use of cells that maintain their natural characteristics more closely, the PCC will be preferred in future experiments.

In conclusion, our study is the first to use three different *in vitro* culture systems each to obtain specific information on the immediate effects of NEFAs on BOECs. Elevated NEFAs have a negative impact on *in vitro* BOEC physiology with decreased cell number, migration capacity and sperm binding ability, indicating lower BOEC-quality and functionality. This less than favorable environment for the oocyte, spermatozoa and the pre-implantation embryo may ultimately partake in the complex pathogenesis of sub- and infertility of high yielding dairy cattle. Future experiments should, however, focus on BOEC lipid metabolism to further elucidate some of the phenomena seen in this study.

Acknowledgements

The authors acknowledge Els Merckx and Silke Andries, of the University of Antwerp Gamete Research Center, for their outstanding technical assistance, and also Lieve Svensson, of the University of Antwerp, Laboratory for Cell Biology and Histology for excellent guidance in scanning electron microscopy.

References

[1] Butler ST. Nutritional management to optimize fertility in dairy cows in pasture-based systems. *Animal* 2014; **1**: 15-26.

[2] de Kruif A, Leroy JLMR, Opsomer G. Reproductive performance in high producing dairy cows: practical implications. *Tieraertzl prax* 2008; **36**: S29-S33.

[3] Leroy, JLMR, Opsomer G, Van Soom A, Goovaerts IG, Bols, PEJ. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part I. The importance of negative energy balance and altered corpus luteum function to the reduction of oocyte and embryo quality in high-yielding dairy cow. *Reprod. Domest. Anim.* 2008; **43**, 612-622.

[4] Leroy JLMR, Van Soom A, Opsomer G, Goovaerts IG, Bols PEJ. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part II. Mechanisms linking nutrition and reduced oocyte and embryo quality in high-yielding dairy cows. *Reprod. Domest. Anim.* 2008; **43**, 623-632.

[5] Jorritsma R, César ML, Hermans JT, Kruitwagen CLJJ, Vos PLAM, Kruip TAM. Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes *in vitro. Anim Reprod Sci* 2004; **81**: 225-235.

[6] Leroy JLMR, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, et al. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes *in vitro. Reproduction* 2005; **130**, 485-495.

[7] Aardema H, Vos PLAM, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, et al. Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. *Biol. Reprod.* 2011; **85**, 62-69.

[8] Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, et al. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *Plos One* 2011*;* **6**, e23183.

[9] Van Hoeck V, Leroy JLMR, Arias-Alvarez M, Rizos D, Gutierrez-Adan A, Schnorbusch K, et al. Oocyte developmental failure in response to elevated nonesterified fatty acid concentrations: mechanistic insights. *Reproduction* 2013; **145**, 33-44.

[10] Valckx S, Van Hoeck V, Arias-Alvarez M, Maillo V, Lopez-Cardona AP, Gutierrez-Adan A, et al. Elevated non-esterified fatty acid concentrations during *in vitro* murine follicle growth alter follicular physiology and reduce oocyte developmental competence. *Fertil. Steril.* 2014; in press DOI: 10.1016/j.fertnstert.2014.08.018

[11] Vanholder T, Leroy JLMR, Van Soom A, Opsomer G, Maes D, Coryn M, et al. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation *in vitro. Anim. Reprod. Sci.* 2005; **87**, 33-44.

[12] Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M. Saturated FFAs, palmitic acid and stearic acid, induce apoptosis in human granulosa cells. *Endocrinology* 2001; **142**: 3590-3597.

[13] Suarez SS. Formation of a reservoir of sperm in the oviduct; *Reprod. Domest. Anim. 2002;* **37**, 140-143.

[14] Dobrinski I, Smith TT, Suarez SS, Ball BA. Membrane contact with oviductal epithelium modulates the intracellular calcium concentration of equine spermatozoa *in vitro*. *Biol. Reprod.* 1997; **56**, 861-869.

[15] Smith TT and Nothnick WB. Role of direct contact between spermatozoa and oviductal epithelial cells in maintaining rabbit sperm viability. *Biol. Reprod.* 1997; **56**, 83-89.

[16] Fazeli A, Elliott RMA, Duncan AE, Moore A, Watson PF, Holt WV. *In vitro* maintenance of boar sperm viability by a soluble fraction obtained from oviductal apical plasma membrane preparations. *Reproduction* 2003; **12**, 509-517.

[17] Kidson A, Schoevers E, Langendijk P, Verheijden J, Colenbrander B, Bevres M. The effect of oviductal epithelial cell co-culture during *in vitro* maturation on sow oocyte morphology, fertilization and embryo development. *Theriogenology* 2003; **59**,1889-1903.

[18] Ellington JE. The bovine oviduct and its role in reproduction: a review of the literature. *Cornell Vet.* 1991; **81**, 313-328.

[19] Hunter RH. Reflections upon sperm-endosalpingeal and sperm-zona pellucida interactions *in vivo* and *in vitro*. *Reprod. Domest. Anim.* 2003; **38**,147-154

[20] Rizos D, Carter F, Besenfelder U, Havlicek V, Lonergan P. Contribution of the female reproductive tract to low fertility in postpartum lactating dairy cows. *J. Dairy Sci.* 2010; **93**, 1022-1029.

[21] Maillo V, Rizos D, Besenfelder U, Havlicek V, Kelly AK, Garrett M, Lonergan P. Influence of lactation on metabolic characteristics and embryo development in postpartum Holstein dairy cows. *J. Dairy Sci.* 2012; **95**, 3865-3876.

[22] Matoba S, O'Hara L, Carter F, Kelly AK, Fair T, Rizos D, Lonergan P. The association between metabolic parameters and oocyte quality early and late post-partum in Holstein dairy cows. *J. Dairy Sci.* 2012; **95,** 1257-1266**.**

[23] Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG. Metabolism of the viable mammalian embryo: quietness revisited. *Mol. Hum. Reprod.* 2008; **14**, 667-672.

[24] Ulbrich S, Zitta K, Hiendleder S, Wolf E, Ulbrich SE. In vitro systems for intercepting early embryo-maternal cross-talk in the bovine oviduct. *Theriogenology* 2010; **73**: 802-816.

[25] Velazquez MA, Parrilla I, Van Soom A, Verberckmoes S, Kues W, Niemann H Sampling techniques for oviductal and uterine luminal fluid in cattle. *Theriogenology* 2010; **73**, 758-767.

[26] Abe H. The mammalian oviductal epithelium: regional variations in cytological and functional aspects of the oviductal secretory cells. *Histol. Histopathol.* 1996; **11** (3): 743-768.

[27] Reischl J, Prelle K, Schol H, Neumuller C, Einspanier R, Sinowatz F. Factors affecting proliferation and dedifferentiation of bovine oviduct epithelial cells *in vitro*; *Cell Tissue Res.* 1999; **296**: 371-383.

[28] Yaniz JL, Lopez-Gatius F, Santolaria P, Mullins KJ. Study of the Functional Anatomy of Bovine Oviductal Mucosa. *Anat. Rec.* 2000; **260**: 268-278.

[29] Abe H and Hoshi H. Bovine oviductal epithelial cells: their cell culture and applications in studies for reproductive biology. *Cytotechnology* 1997; **23**: 171-183.

[30] Gualtieri R and Talevi R. In Vitro-Cultured Bovine Oviductal Cells Bind Acrosome-Intact Sperm and Retain This Ability upon Sperm Release. *Biol.Reprod.* 2000; **62**: 1754-1762.

[31] Buhi WC. Characterization and biological roles of oviduct-specific,oestrogen-dependent glycoprotein. *Reproduction* 2002; **123**: 355-362.

[32] Sostaric E, Dieleman SJ, Van De Lest CHA, Colenbrander B, Vos PLAM, Garcia-Gil N, et al. Sperm Binding Properties and Secretory Activity of the Bovine Oviduct Immediately Before and After Ovulation. *Mol. Reprod. Dev*. 2008; **75**: 60-74.

[33] Witkowska E. Tissue culture of isolated epithelial cells of the bovine oviduct. *Bull. Acad. Pol. Sci. Biol.* 1976; **24** (6): 373-376.

[34] Walter I. Culture of Bovine Oviduct Epithelial Cells (BOEC). *Anat. Rec.* 1995; **243** (3): 347-356.

[35] Rottmayer R, Ulbrich SE, Kölle S, Prell K, Neumueller C, Sinowatz F, et al. A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. *Reproduction* 2006; **132**: 637-648.

[36] Gualtieri R, Mollo V, Braun S, Barbato V, Fiorentino I, Talevi R. Long-term viability and differentiation of bovine oviductal monolayers: Bidimensional versus three-dimensional culture. *Theriogenology* 2012; **78**: 1456-1464.

[37] Schoen J, Bondzio A, Topp K, Einspanier R. Establishment and characterization of an adherent pure epithelial cell line derived from the bovine oviduct.; *Theriogenology* 2008; **69**: 536-545.

[38] Miessen K, Sharbati S, Einspanier R, Schoen J. Modelling the porcine oviduct epithelium: a polarized *in vitro* system suitable for long-term cultivation. *Theriogenology* 2011; **76**: 900-910.

[39] Chen S, Einspanier R, Schoen J. Long term culture of primary porcine oviduct epithelial cells: Validation of a comprehensive in vitro model for reproductive science. *Theriogenology* 2013; **80**: 862-869.

[40] Ireland JJ, Murphee RL, Coulson PB. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *J Dairy Sci* 1980; **63**: 155-160.

[41] Rottmayer R, Ulbrich SE, Kölle S, Prelle K, Neumueller C, Sinowatz F, et al. A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. *Reproduction* 2006; **132**(4): 637-648.

[42] Leese H.J. The formation and function of oviduct fluid. *J. Reprod. Fertil.* 1988; **82**, 843-856.

[43] Buhi WC, Vallet JL, Bazer FW. De novo synthesis and release of polypeptides from cyclic and early pregnant porcine oviductal tissue in explant culture. *J Exp Zool.* 1989; **252**(1):79-88.

[44] Kilian GJ, Chapman DA, Kavanaugh JF, Deaver DR, Wiggin HB. Changes in phospholipids, cholesterol and protein content of oviduct fluid of cows during the oestrous cycle. *J Reprod Fert* 1989; **86**: 419-426.

[45] Hunter RH, Nichol R. Capacitation potential of the fallopian tube: a study involving surgical insemination and subsequent incidence of polyspermy. *Gamete Res.* 1988; **21**(3):255-266.

[46] Hansen C, Srikandakumar A, Downey BR. Presence of follicular fluid in the porcine oviduct and its contribution to the acrosome reaction. *Mol. Reprod. Dev.* 199; **30**(2): 148-153.

[47] Lefebvre R, Chenoweth PJ, Drost M, LeClear CT, MacCubbin M, Dutton JT, et al. Characterization of the oviductal sperm reservoir in cattle. *Biol. Reprod.* 1995; **53**: 1066-1074.

[48] Artursson P and Borchardt RT. Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond. *Pharm. Res.* 1997; **14**(12): 1655-1658.

[49] Freshney RI. Culture of Animal Cells: A manual of Basic Technique and Specialized Applications; Wiley-Blackwell 2000.

[50] De Pauw I, Van Soom A, Laevens H, Verberckmoes S, de Kruif A. Sperm binding to epithelial oviduct explants in bulls with differeny non-return rates investigated with a new *in vitro* model. *Biol. Reprod*. 2008; **67**: 1073-1079.

[51] Tahir MZ, George F, Donnay I. Comparison of different membrane supports for monolayer culture of bovine oviduct epithelial cells; *BMC Proceedings* 2011; **5**(8): 117.

[52] Studzinski GP. Cell growth, differentiation and senescence: a practical approach 1999.

[53] Lefebvre R and Suarez SS. Effect of capacitation on bull sperm binding to homologous oviductal epithelium. *Biol. Reprod.* 1996; **54**: 575-582.

[54] Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols* 2007; **2** (2): 329-333.

[55] Kölle S. Live cell imaging in the female genital tract – new aspects for improving cow fertility; ASAS - http://vimeo.com/97823750. 2014.

[56] Kim M, Hong M, Kim J, Lee SJ, Kang SG, Cho DJ. Bovine follicular fluid and serum share a unique isoform of matrix metalloprotease-2 that is degraded by the oviductal fluid. *Biol. Reprod*. 2001; **65**: 1726-1731.

[57] Leroy JLMR, Vanholder T, Delanghe JR, Opsomer G, Van Soom A, Bols PEJ, et al. Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows. *Anim. Reprod. Sci*. 2004; **80**: 201-211.

[58] Palma-Vera S, Einspanier R, Schoen J. Bovine oviductal epithelial cells: Long term culture characterization and impact of insulin on cell morphology. *Reprod. Biol*. 2014; **14**: 206-2012.

[59] Butler ST, Marr AL, Pelton SH, Radcliff RP, Lucy MC. Insulin restores GH responsiveness during lactation induced negative energy balance in dairy cattle: effects of expression of IGF-I and GH receptor-1A. *J.Endocrinol.* 2003; **176**: 205-217.

[60] Ricchi M, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, et al. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J.Gastroenterol. Hepatol.* 2009; **24**: 830-840.

[61] Talevi R and Gualtieri R Molecules involved in sperm-oviduct adhesion an release. *Theriogenology* 2010; **73**: 796-801.

[62] MacDonald RC and MacDonald RI. Membrane surface pressure can account for differential activities of membrane-penetrating molecules. *J Biol. Chem.* 1987; **263** (2): 10052-10055.

[63] Repetto G, del Peso A, Zurita JL. Neutral Red uptake assay for the estimation of cell viability/cytotoxicity. *Nature protocols* 2008; **3**(7):1125-1131.

[64] Nonogaki T, Noda Y, Goto Y, Kishi J, Mori T. Developmental blockage of mouse embryos caused by fatty acids. *J Assist. Reprod. Genet.* 1994; **11**(9): 482-488.

[65] Roche HM, Terres AM, Black IB, Gibey MJ, Kelleher D. Fatty acids and epithelial permeability: effect of conjugated linoleic acid on CaCo-2 cells. *Gut* 2001; **48**: 797-802.

[66] Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. *Science Magazine* 2003; **302**: 1704:1709.

[67] Wicha MS, Liotta LA, Kidwell WR. Effects of free fatty acids on the growth of normal and neoplastic rat mammary epithelial cells. *Cancer Res.* 1979; **39**: 426-435.

Figure legends

Figure 1. Experimental design NEFA-toxicity on BOECs in three cell culture systems: A = experiment 1: BOEC-monolayers in conventional culture plates; B = experiment 2: BOEC-explants in a suspension culture system; C = BOEC-monolayers in a PCC-system. The dotted line indicates the washing step which was performed in each experiment prior to assessment of the outcome parameters to limit the exposure period to 24h exactly and especially to avoid NEFA-contact with the sperm cells as we only wanted to observe the NEFA-effects on BOEC-physiology without any additional and confounding effects of high NEFA on sperm cells.

Figure 2. Immunocytochemistry images (x 200) using anticytokeratin antibodies in which A= non-stained BOECs; **B=** BOECs stained without the primary antibody; **C= negative control,** stained fibroblast culture from fetal bovine cartilage; **D=** stained freshly retrieved BOECs after a 4-day-culture and monolayer formation; **E=** stained freshly retrieved BOECs on hanging inserts in a PCC-system after a 7-day-culture; **F=** first passage BOECs after a 7-day culture period in a culture flask (to possibly increase cell purity), trypsinization and reseeding on glass inlets in a 4-day-culture; **G=** first passage

608 BOECs after a 7-day culture period in a culture flask (to possibly increase cell purity), trypsinization
609 and reseeding on hanging inserts in a 4-day-culture.

610 **Figure 3.** The epithelial characteristics of the cultivated cells were assessed by SEM with attention on
611 cell polarity (A), the presence of both microvilli and cilia (B), monolayer integrity (C), and cellular
612 secretion (A,C,D). Big arrow indicates cilia, small arrow = cellular secretions, asterisk = monolayer
613 interruptions and star represents microvilli.

614 **Figure 4.** Representation of the BOEC number, BOEC viability, BOEC sperm binding and wound
615 healing capacity (±SD) in **experiment 1** after 24h co-incubation with different NEFA-concentrations.
616 Results are shown ± SD. a,b,c Different superscripts per bar indicate statistical significant differences
617 (*P<0.05*). **A** = Absolute **cell number** shown as number of cells per mL; **B** = **Cell viability** is the ratio of
618 live compared to total cell number ; **C** = **Sperm binding capacity** shown as number of sperm cells
619 bound per 0.05mm^2 of cell culture surface; and **D** = relative **BOEC migration capacity** where the
620 wound diameter was compared at T0 (at the start of the assay) and T8 (8h into the assay) and is
621 expressed as percentage of closure.

622 **Figure 5.** BOEC-explant sperm binding (± SD) in **experiment 2** expressed as the number of bound
623 sperm cells per 0.05 mm^2 of cell culture surface, showing a statistically significant reduced sperm
624 binding in the Moderate and High treatment groups when compared to the Control group. Also,
625 there was a significant difference in sperm binding between the Basal and the High treatment
626 groups. a,b,c Different superscripts per bar indicate statistical significant differences (*P<0.05*).

627 **Figure 6**. Representation of BOEC number, BOEC sperm binding capacity; BOEC migration capacity
628 and monolayer integrity by means of TER-values (±SD) in **experiment 3** on monolayers in a PCC
629 system after 24h NEFA-exposure. a,b,c Different superscripts within the bar chart indicate statistical
630 significant differences (*P<0.05*). **A** = Absolute **cell number** shown as number of cells per mL cell
631 suspension; **B** = **Sperm binding capacity** expressed as number of sperm cells bound per 0.05mm^2 of

632 cell culture surface (* = a tendency; *P = 0.051*); **C** = relative **BOEC migration capacity** where the
633 wound diameter was compared at T0 (at the start of the assay) and T8 (8hrs into the assay); **D** =
634 relative average **TER** increase during 24h NEFA-exposure period expressed as percentages.