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Elevated free fatty acids affect bovine granulosa cell function: a molecular cue for compromised reproduction during negative energy balance

Arpna Sharma, Vijay Simha Baddela, Frank Becker, Dirk Dannenberger, Torsten Viergutz and **Jens Vanselow**

Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

Correspondence should be addressed to J Vanselow: vanselow@fbn-dummerstorf.de

Abstract

High-yielding dairy cows postpartum face the challenge of negative energy balance leading to elevated free fatty acids levels in the serum and follicular fluid thus affecting the ovarian function. Here, we investigated effects of physiological concentrations of palmitic acid (PA), stearic acid (SA) and oleic acid (OA) on the viability, steroid production and gene expression in a bovine granulosa cell (GC) culture model. Treatment with individual and combined fatty acids increased the *CD36* gene expression, while no significant apoptotic effects were observed. Both PA and SA significantly upregulated the expression of *FSHR*, *LHCGR*, *CYP19A1*, *HSD3B1*, *CCND2* and increased 17β-estradiol (E2) production, while OA downregulated the expression of these genes and reduced E2. Interestingly, *STAR* was equally downregulated by all fatty acids and combination treatment. E2 was significantly reduced after combination treatment. To validate the effects of OA, *in vivo* growing dominant follicles (10–19 mm) were injected with bovine serum albumin (BSA) with/without conjugated OA. The follicular fluid was recovered 48 h post injection. As in our *in vitro* model, OA significantly reduced intrafollicular E2 concentrations. In addition, expression of *CD36* was significantly up- and that of *CYP19A1* and *STAR* significantly downregulated in antral GC recovered from aspirated follicles. The ovulation rates of OA-injected follicles tended to be reduced. Our results indicate that elevated free fatty acid concentrations specifically target functional key genes in GC both *in vitro* and *in vivo*. Suggestively, this could be a possible mechanism through which elevated free fatty acids affect folliculogenesis in dairy cows postpartum.

Key Words

- palmitic acid
- stearic acid
- oleic acid
- estradiol
- progesterone

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Introduction

High-yielding dairy cows suffer post-parturition from ovarian dysfunctions such as prolonged calving intervals and delayed cyclic resumption due to various factors (1). One of such risk factor is negative energy balance (NEB). Catabolism and mobilization of stored triglycerides lead to increased systemic concentrations of non-esterified fatty acids (NEFA) like palmitic acid (PA, C16:0), stearic acid (SA, C18:0), oleic acid (OA, C18:1) and of β-hydroxybutyric acid (BHBA) (2). Such excessive lipid mobilization has been associated with metabolic and reproductive disorders (3) affecting both the cumulus oocyte complex morphology and embryo viability (4). Elevated plasma levels of PA, SA, OA and of linoleic acid have been shown in dairy cows postpartum as well as their infiltration into the follicular fluid (5). Since granulosa cells (GC) are the most important somatic constituents

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of the ovarian follicle regulating follicle development along with the production of follicular fluid and plasma estradiol (6). Elevated NEFA levels have detrimental effects on bovine GC (7). In human GC, saturated fatty acids (PA and SA) are known to induce apoptosis, causing potential reproductive abnormalities such as amenorrhea, which is often observed in obese women (8). However, on the contrary, OA (monounsaturated fatty acid) supplementation has shown to protect exocrine pancreatic cells from PA-induced apoptosis thus reducing the rate of pancreatic diseases caused by obesity (9). Also, OA co-supplementation diminishes pro-inflammatory and cytotoxic effects of SA accumulation in human aortic endothelial cells (10). *In vitro* exposure of COCs to PA and SA induces endoplasmic reticular stress, mitochondrial damage and increased apoptosis in cumulus cells thus impairing oocyte developmental competence (11), while OA is harmless even at high concentrations (12, 13, 14). However, studying the effects of these major NEFAs, particularly of PA, SA and OA both individually and in combination on steroidogenesis, cell proliferation and apoptosis in an *in vitro* bovine GC culture model might contribute to our understanding of the collective effects of elevated free fatty acid concentrations during NEB on bovine reproduction. Thus, in the present study, we examined the effects of these fatty acids on GC functionality at elevated, but physiological concentrations as observed in follicular fluid during NEB. For solubilization, fatty acids were conjugated with bovine serum albumin (BSA) to mimic the physiological conditions as close as possible. Specifically, we studied the individual and combined effect of PA, SA and OA on proliferation, apoptosis and steroidogenesis in our well-established serum-free, estrogen-producing GC culture model and validated effects of OA in a bovine *in vivo* animal model, which allows to test the effects of individual fatty acids on the differentiation of individual follicles while largely avoiding superimposed systemic influences.

Materials and methods

In vitro primary GC culture

Primary GC culture was performed as previously described (15). For testing the effects of fatty acids, the media (supplemented α-MEM) were replaced after 48 h with the same media containing different concentrations of PA (50, 100 and 200 µM), SA (50, 100, 200 µM) or OA (100, 200 and 400 µM) as BSA conjugates or BSA as vehicle control (fatty acids and BSA from Sigma-Aldrich). For BSA conjugation, fatty acids were dissolved in 1–2 mL chloroform/methanol solution (v/v = 2/3) and evaporated to dryness under nitrogen atmosphere. Subsequently, a solution of fatty acid-free BSA (w/v = 10% in PBS) was prepared and added. The BSA/fatty acid mixtures were then vortexed, sonicated and subjected to a mild heat treatment (<40°C) until a clear solution was formed. Subsequently, BSA/fatty acid solutions were sterilized by filtration (0.02 µm) and kept at −20°C until addition to the cell culture media (v/v = 1/10). All control wells received the same volume of BSA as the test groups with highest fatty acid concentrations.

Steroid hormone estimation

Concentrations of 17β-estradiol (E2) and progesterone (P4) in the conditioned media were determined by competitive 3H radioimmunoassay (RIA) with rabbit-raised antibodies purified by affinity chromatography as described earlier (16).

Cell viability and cell cycle analysis

Attached GCs were thoroughly washed twice with 1× PBS and trypsinized using 250 µL TryplE solution (Thermo Fisher) followed by incubation (37°C, 20 min). After centrifugation the cells were merged with non-attached cells from spent media. All cells were again centrifuged, washed with 1 mL MEM and analyzed for apoptosis using an Annexin V FITC kit (Miltenyi Biotec). Cell pellets were re-suspended in 100 µL of 1× binding buffer to which 10 µL of Annexin V reagent was added. After gentle pipetting, the tubes were incubated in dark for 15 min followed by washing and resuspension in 500 µL 1× binding buffer. Then, 5 µL of PI (propidium iodide, 500 µg/mL) were added to the cells and gently mixed just prior to flow cytometric analysis. The fluorescence signal was quantified from single cells (10,000 counts) by a flow cytometer (Gallios, Beckman-Coulter) and the data obtained were analyzed using the Kaluza software (Beckman-Coulter).

For cell cycle analysis, the combined (attached and non-attached) cells were washed and dissolved in 300 µL of 1× PBS. Then, 70% ice cold ethanol was added dropwise into the cell suspensions and stored (−20°C, 2 h). Later, cells were pelleted (300 ***g***, 10 min, 4°C), re-suspended in 1 mL RNase solution (1 mg/mL) and incubated (37°C, 5% CO_2, 30 min). The PI reagent was added to the cells and further incubated in the dark (37°C, 30 min). Lastly, the fluorescence signal was quantified from single

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cells (10,000 counts) by a flow cytometer (EPICS-XL, Beckman-Coulter). Data were analyzed using the MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).

RNA isolation and cDNA synthesis

Total RNA preparation was done using innuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol and quantified using NanoDrop1000 Spectrophotometer (Thermo Scientific). Later, cDNA was prepared using SensiFAST cDNASynthesis Kit (Bioline, Luckenwalde, Germany) from 200 ng RNA as previously described (17).

Quantitative real-time PCR (qPCR) analysis

qPCR was performed using SensiFAST SYBR No-ROX (Bioline, London, UK) with gene-specific primers (Table 1) in a Light Cycler 96 instrument (Roche) as described previously (18). The abundance of transcripts was normalized relative to *RPLPO* as a suitable reference gene.

Animals, follicle injection and aspiration

Animal experiments were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (Aktenzeichen: 7221.3-1.1-068/17). Fourteen Holstein heifers aged 13–14 months, weighing 380–400 kg were kept indoors at our institute and fed a standard diet. Ultrasonographic follicular monitoring (transportable equipment with 5 MHz linear probe, Hitachi CS 9100 Picker) was carried out aside from the normal heat detection to verify the cycle stage. Special attention was given to the development of larger follicles about 2–3 days before intrafollicular injection to recognize growing and regressing follicles within the follicular population. An adapted amount of BSA without/with 400 µM conjugated OA was injected into the selected growing dominant follicle (10–19 mm). A sector scanner with a 6.5 MHz puncture transducer and a special designed carrier (VETEC GmbH, Rostock, Germany) to guide a needle with an outer diameter of 0.5 mm was used. Epidural anesthesia was induced by 5 mL of lidocaine-hydrochloride injectable-2% (Procamidor, WdT, Garbsen, Germany). To monitor effects on the ovulation of injected follicles 14 animal were further monitored for cycling activity. Sizes of the injected dominant follicles were measured and sample collection was done using transvaginal ultrasonographically guided follicle aspiration 48 h after injection. Recovered follicular fluid was immediately stored on ice (<15 min) and taken to the laboratory. Further, the follicular fluid was centrifuged for 3 min at 15,000 ***g*** to obtain clear follicular fluid and GC pellets. The GC pellets were instantly frozen in liquid nitrogen followed by storage at −80°C for gene expression analysis while the obtained follicular fluid was stored at −20°C for hormone estimation.

Lipid analysis and fatty acid analysis

Follicular fluid samples of approximately 200 µL were dropwise added to 8 mL chloroform/methanol (2:1, v/v) at room temperature. The solution contained C19:0 as an internal standard. The general sample preparation procedure is as earlier described (19). The fatty acid analysis of the follicular fluid was performed using capillary gas chromatography with a CP-Sil 88 CB column (100 m × 0.25 mm, Agilent) that was installed in

Table 1 List of primers used for gene quantification by q-PCR.

Gene	Sequence	Size (bp)	Accession no.
CD36	For: GCTCCTTAAGCCATTCTTGGAT Rev: CACCAGTGTCAACGCACTTT	151	NM_001278621.1
CYP19A1	For. GCTTTTGGAAGTGCTGAACCCAAGG Rev: GGGCCCAATTCCCAGAAAGTAGCTG	172	NM_174305
FSHR	For: TCACCAAGCTTCGAGTCATCCCAAA Rev: TCTGGAAGGCATCAGGGTCGATGTA	189	NM_174061
CCND2	For: CGCAGGGCCGTGCCGGACGCCAAC Rev: CACGGCCCCCAGCAGCTGCAGATGG	279	NM_001076372
PCNA	For: GTGAACCTGCAGAGCATGGACTCGT Rev: CGTGTCCGCGTTATCTTCAGCTCTT	192	NM_001034494
LHCGR	For: GCATCCACAAGCTTCCAGATGTTACGA Rev: GGGAAATCAGCGTTGTCCCATTGA	205	NM_174381
HSD3B1	For: TGTTGGTGGAGGAGAAGGATCTG Rev: GCATTCCTGACGTCAATGACAGAG	208	NM_174343
RPLP0	For: TGGTTACCCAACCGTCGCATCTGTA Rev: CACAAAGGCAGATGGATCAGCCAAG	142	NM_001012682

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a PerkinElmer gas chromatograph CLARUS 680 with a flame ionization detector and split injection (PerkinElmer Instruments). The detailed gas chromatography conditions were set as described (20).

Statistical analysis

All experiments were carried out in triplicates with different GC preparations. Data of tested groups were analyzed using one-way ANOVA (all pair wise multiple comparison procedures with Holm-Sidak method) or unpaired *t* test using the Sigma Plot 11.0 Statistical Analysis System (Jandel Scientific, San Rafael, CA, USA). Differences were considered significant at $P<0.05$.

Results

Effects of individual fatty acids on GC functionality *in vitro*

Cell morphology

As evident from the light microscopic photographs, treatment with increased PA, SA and OA concentrations resulted in morphological changes. GC changed their appearance from a regular fibroblast-like appearance under BSA vehicle control treatment to an increasingly round-shaped morphology with increased accumulation of intracellular vesicles at highest PA, SA or OA concentrations (Fig. 1).

Steroid hormone concentrations

Estimations of P4 and E2 concentrations within conditioned media after the 8-day culture period revealed that PA and SA significantly stimulated E2 production as compared to the BSA control, whereas OA strongly reduced E2 concentrations. In contrast, levels of P4 remained unaltered after PA and SA treatment, but were significantly reduced only at highest OA concentration (Fig. 2).

Gene expression analysis

qPCR data showed that transcript abundance of the cell cycle regulator *CCND2* was significantly up-regulated by PA and SA at high concentrations. In contrast, OA significantly reduced the *CCND2* transcripts at all concentrations. Transcript abundance of the proliferation marker *PCNA* however, was only affected by PA showing significant upregulation at the highest concentration (Fig. 3). Interestingly, we also found differential, in part opposing regulation of other functional key genes. Whereas the transcript abundance of the fatty acid transporter *CD36* was strongly upregulated by all three fatty acids (Fig. 4), key transcripts of estradiol and progesterone production, *CYP19A1* and *HSD3B1* as well as those of gonadotropin receptors, *FSHR* and *LHCGR*, showed increased upregulation after PA (Fig. 5A) and SA treatment (Fig. 5B), but clearly reduced expression in OA-treated cells (Fig. 6). In contrast, all fatty acids reduced transcript abundance of *STAR*, the rate-limiting factor of steroidogenesis (Fig. 7).

Cell viability and proliferation

Flow cytometry analysis of Annexin V-FITC/PI-stained cells showed no statistically significant differences in the proportions of apoptotic cells after treatment with highest concentrations of PA, SA or OA (200 µM, 200 µM and 400 µM, respectively) as compared to vehicle control. However, the percentage of apoptotic cells tended to be higher in the PA and SA treated groups,

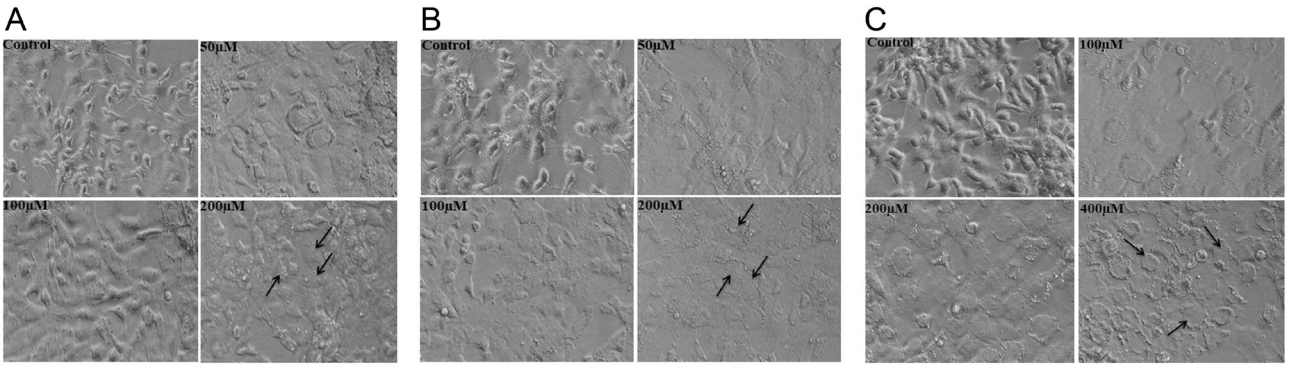

Figure 1
Effects of increasing concentrations of (A) palmitic acid (C16:0), (B) stearic acid (C18:0) and (C) oleic acid (C18:1*cis*-9) on *in vitro*-cultured GC morphology. Photomicrographs were obtained from Nikon TMS-F inverted microscope. Arrows indicate lipid droplet formation in the cells.

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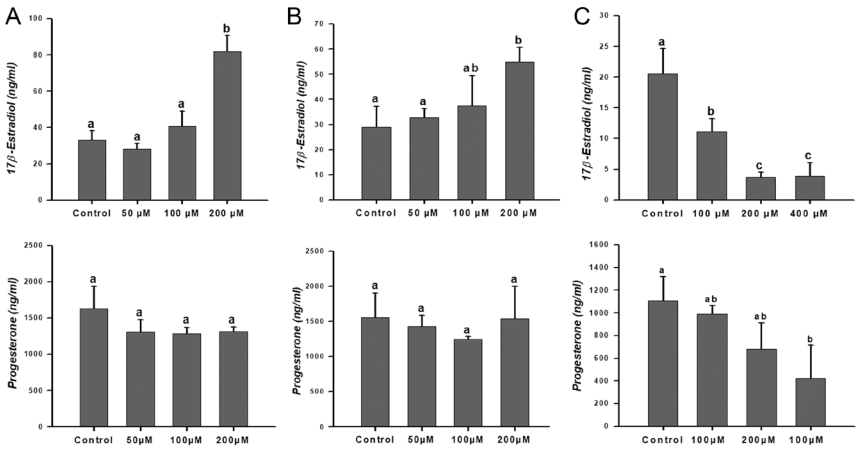

Figure 2
Effects of increasing concentrations of (A) palmitic acid (C16:0), (B) stearic acid (C18:0) and (C) oleic acid (C18:1*cis*-9) on steroid hormone concentrations conditioned media of cultured bovine GC. The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm–Sidak test: $n = 3$, $P < 0.05$).

but lower with OA (Table 2). The proportion of viable cells was virtually unchanged in all treatment groups, whereas the number of dead cells was significantly higher in OA group. Further, none of the fatty acids induced a significant shift of cell cycle phases in cultured GC. Flow cytometric analysis revealed similar proportions of cells in SubG0/G1, G0/G1, S and G2/M phases (Table 3).

Effects of fatty acid combinations

To test combined effects of PA, SA and OA, highest effective concentrations of these fatty acids (200 µM, 200 µM and 400 µM) were mixed and added to cultured GC. As in case of individual fatty acids the morphology of the cells dramatically changed as compared to BSA vehicle controls (Fig. 8A). The results also revealed that co-supplementation had no influence on P4 production, but significantly reduced E2 levels (Fig. 8B). qPCR quantification of key transcripts showed no significant regulation of *CYP19A1*, *HSD3B1*, *FSHR*, *LHCGR CCND2* and *PCNA*, but highly upregulated *CD36* transcripts and significantly downregulated *STAR* (Fig. 9). Results from flow cytometry by Annexin V-FITC/PI staining revealed that the percentage of apoptotic cells was unchanged, whereas that of viable cells was significantly reduced by the combined fatty acid treatment. In particular, the proportion of dead cells was remarkably increased by the combined treatment as compared to the BSA controls (Table 4). However, we did not find significant difference in cell cycle stages (Table 5).

Effect of intrafollicular OA injection on GC functionality *in vivo*

To validate the data obtained from cultured GC, we tested effects of OA in an animal model by directly injecting growing dominant follicles. This approach will expose antral GCs directly to increased OA concentrations while largely avoiding systemic effects. In some animals, post-injection follicle differentiation and ovulation rate was also monitored, whereas in other animals injected follicles were aspirated 48 h later in order to collect follicular fluid and cells therein for hormone production and gene expression analysis.

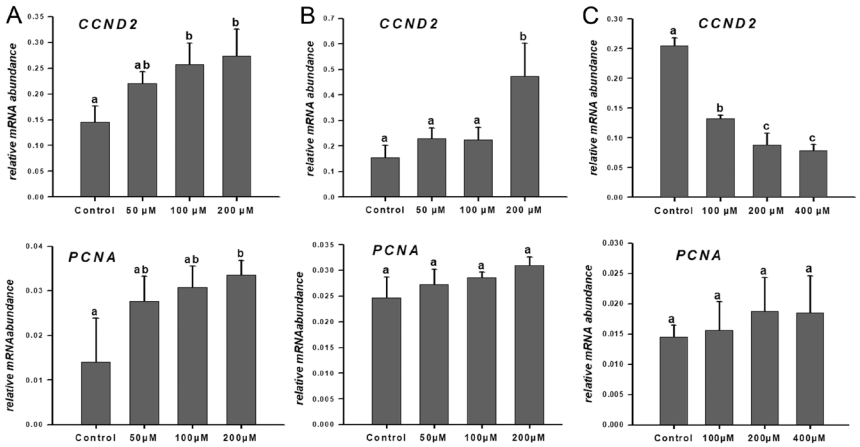

Figure 3
Effects of increasing concentrations of (A) palmitic acid (C16:0), (B) stearic acid (C18:0) and (C) oleic acid (C18:1*cis*-9) on transcript abundance of the cell proliferation marker genes *CCND2* and *PCNA*. Transcript abundance was normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm-Sidak test: $n = 3$, $P < 0.05$.).

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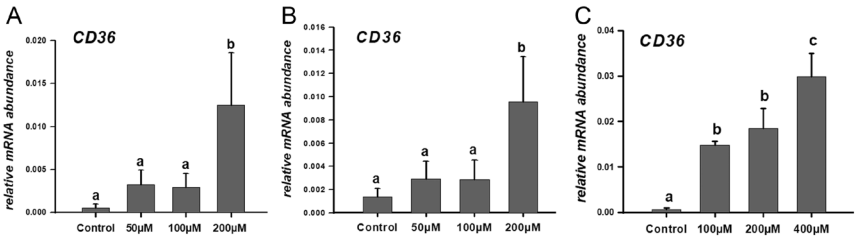

Figure 4
Effects of increasing concentrations of (A) palmitic acid (C16:0), (B) stearic acid (C18:0) and (C) oleic acid (C18:1*cis*-9) on transcript abundance of the fatty acid translocase/*CD36* gene. Transcript abundance was normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm–Sidak test: $n = 3$, $P < 0.05$).

Ovulation rates

From 14 injected growing dominant follicles (one per animal) that were kept for monitoring the ovulatory competence, nine were injected with BSA, out of which seven follicles advanced to the ovulatory stage (77%), while only one out of five follicles injected with OA ovulated (20%) within 96 h post injection. Although not significant yet the ovulatory competence tended to be reduced following the OA as compared to the BSA injections.

Fatty acid analysis by gas chromatography

Gas chromatography analysis of follicular fluid samples obtained 48 h post intrafollicular injections (BSA with/without conjugated OA) revealed varying proportions of different fatty acids. However, the percentage of OA (46.51 ± 5.8%) in OA-injected follicles was significantly higher as compare to BSA (13.59 ± 2.5%) injected follicles (Supplementary Table 1, see section on supplementary data given at the end of this article).

Steroid hormone estimation

Follicular fluid obtained from aspirated follicles was subjected to RIA for estimating the concentration of P4 and E2, respectively. The results revealed that intrafollicular OA injection remarkably reduced the production of E2 compare to the vehicle control (BSA), while P4 level remained largely unaffected (Fig. 10A).

Gene expression analysis

qPCR analysis showed significantly higher levels of *CD36* and lower levels of *CYP19A1* and *STAR* transcripts in GC recovered after intrafollicular OA injection. However, the transcript level of *CCND2* remained unaltered throughout (Fig. 10B).

Discussion

Considerable changes in the serum metabolite profile of post-partum dairy cows are approximately mirrored in the follicular fluid of dominant follicles to which pre-ovulatory

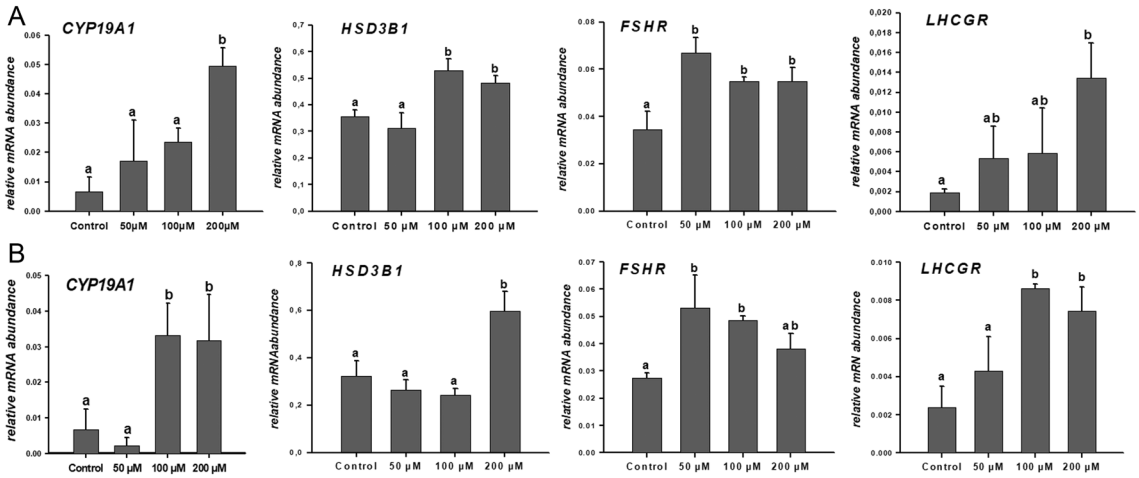

Figure 5
Effects of increasing concentrations of (A) palmitic acid (C16:0) and (B) stearic acid (C18:0) on key transcripts of estradiol and progesterone production (*CYP19A1*, *HSD3B1*) and gonadotrophin receptors (*FSHR*, *LHCGR*). Transcript abundance was normalized to the reference gene *RPLPO.* The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm–Sidak test: $n = 3$, $P < 0.05$).

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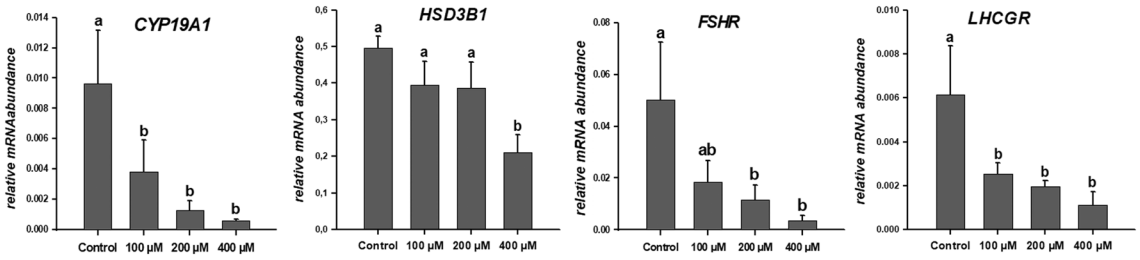

Figure 6
Effects of increasing concentrations of oleic acid (C18:1*cis*-9) on key transcripts of estradiol and progesterone production (*CYP19A1*, *HSD3B1*) and gonadotrophin receptors (*FSHR*, *LHCGR*). Transcript was abundance normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm–Sidak test: $n = 3$, $P < 0.05$).

oocytes and GC are exposed (21). Thus, to determine if elevated intrafollicular concentrations of free fatty acids affect GC functionality and thus may contribute to declined fertility rates as experienced by cows during NEB, in the present study, we analyzed the *in vitro* effects of major free fatty acids both separately and in combination on GC functionality. The tested concentrations of PA, SA and OA were based on earlier *in vivo* experiments which provided estimated physiological concentrations of free fatty acids in the follicular fluid of short-term fasted cows (22). Additionally, we knew from earlier reports that concentrations of OA is highest in the follicular fluid of cows and the present data showed that GC functionality is adversely modulated *in vitro*; thus, we specifically selected OA for intrafollicular injection to validate whether the effects of OA observed in our GC culture model can be transferred to the *in vivo* situation. In contrast to our previous studies (23) and to those of others (11), where the fatty acids had been solubilized by using ethanol, we solubilized the fatty acids by conjugation with BSA as previously published (24). On one hand, this certainly mirrors the physiological situation in the follicular fluid more closely, and on the other hand, it enabled us to reproducibly solubilize not only the monounsaturated fatty acid OA, but also the saturated fatty acids PA and SA. According to our previous (unpublished) observations, the effects of ethanol solubilized PA or SA, but not those of OA, on cultured GC showed extreme variability. Very likely, this might be due to different physicochemical properties of saturated vs unsaturated fatty acids. In any case, this let us eventually change our experimental procedure. However, it is also obvious from our present data that the effects of OA on GC were very similar independent of the method of solubilization.

PA, SA and OA alter cell morphology of cultured bovine GC by inducing lipid accumulation

In vitro supplementation of fatty acids separately and in combination induced significant alterations in the morphology and physiological activity of cultured GC. Their morphology closely resembled the morphology of foam cells as observed in OA-treated smooth muscle cells and macrophages (25, 26, 27). In addition, these morphological changes were associated with a highly induced expression of fatty acid translocase *CD36* (FAT) known to mediate fatty acid uptake (28). This clearly suggests that all three fatty acids induce their own uptake by the cells. Our results are quite consistent with other studies in primary bovine mammary epithelial cells where mRNA abundance of *CD36* was markedly increased when the cells were cultured with PA, SA, OA, linoleate or an conjugated linoleic acid isomer (*trans*-10, *cis*-12 18:2) (29, 30) and in rat type II pneumocytes, where FAT/CD36 is expressed mediating the uptake of PA (31). This is well in line with data of the present study showing that PA, SA and OA induce massive accumulation of intracellular vesicles previously (23) identified as lipid droplets in cultured bovine GC.

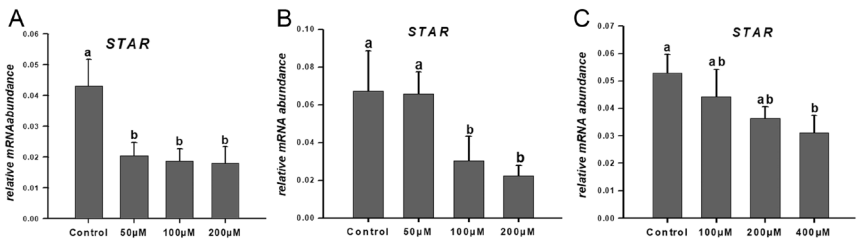

Figure 7
Effects of increasing concentrations of (A) palmitic acid (C16:0), (B) stearic acid (C18:0) and (C) oleic acid (C18:1*cis*-9) on gene expression of *STAR*. Transcript abundance was normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (Holm–Sidak test: $n = 3$, $P < 0.05$).

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Table 2 Cell viability was determined by Annexin V-FITC staining assay as described in the Materials and methods section.

Tested groups	Parameters		
	Viable cells (%)	Apoptotic cells (%)	Dead cells (%)
(I) Control	79.27 ± 1.2	13.72 ± 2.4	5.9 ± 1.2[a]
(II) Palmitic acid (200 µM)	74 ± 2.9	19.2 ± 3	5.8 ± 0.2[a]
(III) Stearic acid (200 µM)	76.9 ± 4.2	15.8 ± 2.6	6% ± 1.9[a]
(IV) Oleic acid (400 µM)	67.8 ± 3.5	11.3 ± 0.8	20.4 ± 3.6[b]

The data are shown as means ± S.E.M. (Holm–Sidak test: $n = 3$). Significant differences are indicated by letters a and b ($P < 0.05$).

In vitro cell viability and proliferation

In spite of dramatic morphological alteration of cultured GC under PA and SA supplementation, even at their highest concentrations these fatty acids did not elicit significant apoptotic effects. As mentioned earlier saturated fatty acids, particularly PA and SA, induce apoptosis in human GC (8) and lipoapoptosis in human β-cells, while the unsaturated fatty acids OA and linoleate have no such effects (32). But it is equally important to consider the fact that in cows, follicles that secrete higher E2 levels are selected for continued growth up to the ovulatory stage (33, 34) and higher E2 level is known to increase both GC proliferation and follicle cell differentiation (35, 36, 37). A positive correlation between elevated E2 levels and GC survival has been reported, where higher E2 levels not only protected GC from Fas ligand-induced apoptosis, but also increased the percentage of cells entering from G1 to S phase of the cell cycle, in addition to an increased cyclin D2 protein expression (38). In the present study, both PA and SA stimulated the E2 production significantly along with upregulation of the *CCND2* gene. This could effectively mediate the protective effect of high estradiol levels in PA- and SA-treated GC despite of their apoptotic impacts in other mammalian cells as described earlier. As per OA, there were no signs of apoptosis in cultured GC even at its highest tested concentration. OA has been previously reported to have no apoptotic effects on CHO cells and β-islet cells even at high concentrations. This is in line with the observation that OA causes lipid accumulation more effectively as compared to saturated fatty acids such as PA (39). The cells ability to effectively incorporate fatty acids into cytoplasmic triglycerides (TG) might serve as a protection against their pro-apoptotic effects. OA is more readily incorporated into triglyceride and is therefore considered less apoptotic as compared to saturated fatty acids (40), which are poor substrates for TG synthesis. In addition, OA is known to even mediate protection against PA-induced apoptosis (41). In response to FSH, *CCND2* mRNA expression rapidly increased in GC (42, 43), while FSH β-null mutant mice display decreased *CCND2* mRNA levels (44). This is well in line with our observation that PA and SA increased the FSHR transcript levels, and as a consequence probably increased the responsiveness of the GC toward this gonadotropin, which in turn may have indirectly induced elevated *CCND2* transcript abundance. In contrast, OA steadily decreased the *FSHR* expression, thus leading to reduced FSH responsiveness. The observed steep downregulation of *CCND2* transcript abundance might be therefore a consequence of reduced FSH signaling under OA treatment. Both observations thus are indicative for the important role of FSH signaling for GC viability and proliferation.

Our results showed that combined PA, SA and OA treatment did also not induce apoptotic effects and more interestingly, significantly lowered the E2 production, thus suggesting that OA effects seem to dominate those of PA and SA in the mixture. This could be partially due to the observation that OA effectively rescues saturated fatty acid-induced apoptosis under the activity of the enzyme stearoyl-CoA desaturase (SCD1), catalyzing the conversion of saturated fatty acid into monounsaturated fatty acids, thus directing the saturated fats into TG synthesis and away from pathways leading to apoptosis.

Steroidogenesis is modulated differentially *in vitro* by saturated and monounsaturated fatty acid

To determine the physiological effects of PA, SA and OA on GC we looked upon the transcriptional activity of key

Table 3 Cell proliferation was determined by flow cytometer analysis.

Tested groups	Cell cycle phase			
	SubG0/G1 (%)	G0/G1 (%)	S Phase (%)	G2/M (%)
(I) Control	13.23 ± 6.4	91.2 ± 1.5	6.8 ± 1.2	1.9 ± 0.25
(II) PA 200 µM	10.16 ± 3.2	88.6 ± 1.0	8.1 ± 1.4	3.2 ± 0.4
(III) SA 200 µM	24.3 ± 9	89.2 ± 1.3	6.8 ± 0.4	3.8 ± 1
(IV) OA 400 µM	11.9 ± 4.07	92 ± 2.9	4.1 ± 1.7	3.8 ± 1.25

PA, SA, OA represent palmitic acid, stearic acid and oleic acid, respectively. The data are shown as means ± S.E.M., Holm–Sidak test, $n = 3$).

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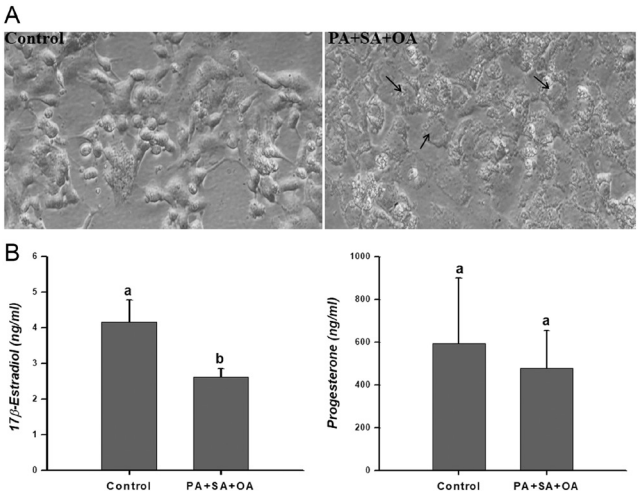

Figure 8
(A) Effects of combination treatment with PA + SA + OA (200 µM + 200 µM + 400 µM) on *in vitro* cultured GC. Photomicrographs were obtained from Nikon TMS-F inverted microscope. Arrows indicate lipid droplet formation in the cells. (B) Effects of combination treatment with PA + SA + OA (200 µM + 200 µM + 400 µM) on the steroid hormone concentration in conditioned media of cultured bovine GC. The data are shown as means ± SEM. Different letters indicate significantly different means (unpaired *t* test; $n = 3$, $P < 0.05$).

genes involved in gonadotropin signaling such as *FSHR* and *LHCGR* and in steroidogenesis such as *CYP19A1*, *STAR* and *HSD3B1*. Since both folliculogenesis and steroidogenesis are closely dependent on the coordinated actions of FSH and LH with their receptors on GC and thecal cells of ovarian follicles (45). *CYP19A1* expression has been found to be elevated by SA supplementation in murine GC (46). This is in line with our data showing that both PA and SA increased the expression of *FSHR*, *LHCGR*, *CYP19A1* and *HSD3B1*, and thus, stimulated E2 production. In contrast, all these genes and E2 production were significantly downregulated by OA. Interestingly, the expression of *STAR* was significantly downregulated by all three fatty acids and by the combined treatment. However, only OA and the combined treatment also resulted in a significantly reduced E2 production, whereas E2 levels were increased by PA and SA treatment. Most likely, in case of OA treatment E2 reduction could be largely due to the reduced transcription of *HSD3B1* and *CYP19A1*, whereas higher levels of E2 in PA and SA treated GC can be attributed to upregulation of *CYP19A1* (47). Nevertheless, a clear effect of reduced transcription of *STAR* that is considered as the rate-limiting factor of steroid hormone synthesis (48) might be obscured by the presence of androstenedione that is a preferred substrate for *CYP19A1* to produce E2 in GC (49) in our cell culture medium.

The presented results on OA effects are in accordance with our previous study (23), but are contradictory to data reported by others (7). These authors concluded that OA despite of inhibiting proliferation stimulates the production of E2 in cultured bovine GC. However, our results on stimulatory effects of PA and SA on E2 production are in line with the same study and also with other reports showing that exposure of

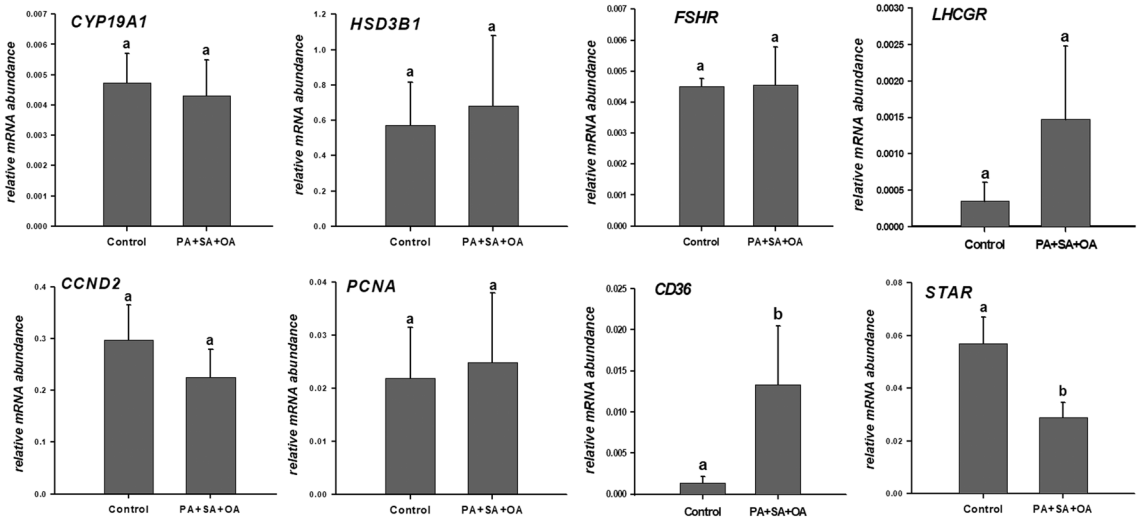

Figure 9
Effects of combination treatment with PA + SA + OA (200 µM + 200 µM + 400 µM) on the abundance of key transcripts of estradiol, progesterone production (*CYP19A1*, *HSD3B1*), gonadotrophin receptor genes (*FSHR*, *LHCGR*), cell proliferation markers (*CCND2*, *PCNA*), *CD36* and *STAR*. Transcript abundance was normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (unpaired *t* test: $n = 3$, $P < 0.05$).

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Table 4 Cell viability was determined by Annexin V-FITC staining assay as described in the Materials and methods section.

Tested groups	Parameters		
	Viable cell (%)	Apoptotic cells (%)	Dead cells (%)
(I) Control	55.7 ± 9.5[a]	34 ± 8.3	9 ± 0.9[a]
(II) PA + SA + OA	29.7 ± 1.09[b]	32.3 ± 3.2	36.9 ± 4.1[b]

Palmitic acid (PA, 200 µM), stearic acid (SA, 200 µM) and oleic acid (OA, 400 µM) were co-supplemented to cultured GC and were compared to control. The data are shown as means ± S.E.M., unpaired *t* test: ($n = 3$). Significant difference is indicated by letters a and b ($P < 0.05$).

androgen-producing cells to saturated fatty acids significantly increased androgen production (50). In this context, it is important to point out that PA and SA clearly increase the expression of gonadotropin receptors *FSHR* and *LHCGR*, whereas OA showed opposite effects. FSH is known to induce the expression of *LHCGR* via protein kinase A (PKA) and phosphoinositide3-kinase (PI3K) pathways in rat GC (51). *LHCGR* is highly expressed in GC during the pre-ovulatory stage to enable responsiveness to the LH surge thus leading to ovulation, oocyte maturation and corpus luteum formation (52). The downregulation of both *FSHR* and *LHCGR* might therefore affect the FSH signaling pathway leading to compromised GC functionality due to elevated NEFAs specifically by OA. Both PA and SA upregulated *CYP19A1* expression with subsequent increase of the E2 production, while OA downregulated the *CYP19A1* expression while reducing E2 production. These data, but in particular, our results on the effects of combined fatty acids (PA+SA+OA) may give some hints to better understand the *in vivo* situation. *STAR* expression is downregulated by all three fatty acids as well as by the combined treatment. This suggests that elevated *in vivo* concentrations of fatty acids during NEB might cause cumulative effects on *STAR* expression in steroidogenic cells similar to our *in vitro* tested combination. However, it has to be considered as well that about 10–15% of steroid biosynthesis is known to occur via *STAR* independent mechanisms (53, 54) as some other proteins like MLN4 have been known to possess *STAR*-like activity in promoting cholesterol flux into mitochondria (55). From our data, it became very clear that the saturated fatty acids tested (PA, SA) elicited very different, partly opposing effects in GC as compared with effects of OA as a monounsaturated fatty acid.

Intrafollicular OA injection modulates GC function *in vivo* and impedes ovulation

To validate the *in vitro* data obtained from our GC culture model we used an *in vivo* approach by directly injecting the antral cavity of growing dominant follicles. This approach will expose antral GC directly to the injected agent while largely avoiding superimposing systemic effects. *In vivo* effects of Insulin-like Growth Factor 1 (IGF-1) has been also studied by intrafollicular injection to reveal the mechanisms underlying follicle deviation in heifers and mares (56). However, to our knowledge no functional *in vivo* study on the role of fatty acids on GC function and hormone concentration by direct injection of follicles in heifers has been carried out so far. Our *in vivo* data clearly provided an affirmation of the results obtained *in vitro*. As discussed above *in vitro* data indicated that OA has a dominant effect as compared to other fatty acids *in vitro*, thus, we specifically selected this fatty acid for intrafollicular injection. Fatty acid analysis of follicular fluid samples by gas chromatography revealed significantly higher proportions of OA in OA injected as compared to BSA injected follicles even 48 h post-injection, but also demonstrated that proportions of most of the other fatty acids was not markedly altered.

The results are consistent with those reported *in vitro*, with a reduced E2 concentration detected in follicular fluid recovered from OA injected follicles. Additionally, only 22% of OA injected follicles reached the ovulatory stage as compared to 77% of BSA injected follicles achieving ovulation. This indicates a tendency of OA to inhibit ovulation. Since, dominant follicles secrets sufficient E2 to induce the pre-ovulatory LH surge for successful ovulation (57), reduced E2 concentrations as observed in

Table 5 Cell proliferation was determined by flow cytometer analysis.

Tested groups	Cell cycle phase			
	SubG0/G1 (%)	G0/G1 (%)	S phase (%)	G2/M (%)
(I) Control	13.36 ± 3.7	94.8 ± 0.5	3.8 ± 0.8	1.5 ± 0.2
(II) PA + SA + OA	14 ± 3.1	94.5 ± 0.5	3.9 ± 0.8	1.75 ± 0.45

Palmitic acid (PA, 200 µM), stearic acid (SA, 200 µM) and oleic acid (OA, 400 µM) were co-supplemented to cultured GC and were compared to control. The data are shown as means ± S.E.M., unpaired *t* test: $n = 3$.

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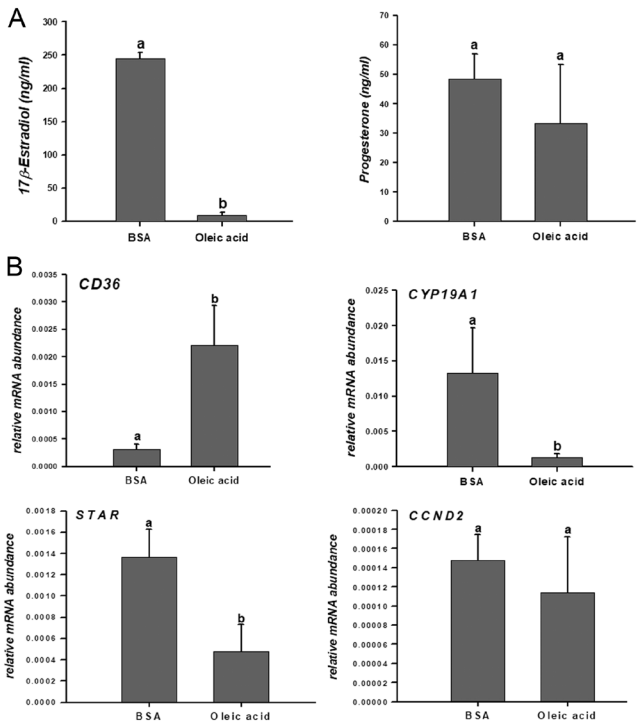

Figure 10
(A) Effects of intrafollicular oleic acid (C18:1*cis*-9) injection on the steroid hormone concentration in follicular fluid recovered 48 h post injection. The data are shown as means ± S.E.M. Different letters indicate significantly different means (unpaired *t* test; $n = 3$, $P < 0.05$). (B) Effects of intrafollicular oleic acid (C18:1*cis*-9) injection on transcript abundance of *CD36*, *CYP19A1*, *STAR* and *CCND2*. BSA and OA (400 µM) indicate vehicle control and tested fatty acid respectively. Transcript abundance was normalized to the reference gene *RPLPO*. The data are shown as means ± S.E.M. Different letters indicate significantly different means (unpaired *t* test; $n = 3$, $P < 0.05$).

the follicular fluid recovered from OA injected follicles might be responsible for the observed lower ovulation rate. A similar decrement of E2 concentrations by OA was also observed in the *in vitro* experiments both in independent as well as in combination treatments. Also, further investigation revealed that gene expression of *CD36* was significantly up-regulated while both *CYP19A1* and *STAR* were significantly down-regulated in GC recovered from OA injected follicles. These results evidently indicate that following intrafollicular injection, OA was actively transported into the GC, with further down-regulation of both *CYP19A1* and *STAR*. Since *CYP19A1* in bovine is the key enzyme for E2 synthesis, the resulting lower concentration of E2 in follicular fluid is clearly in line with suppressed *CYP19A1* expression. Thus, considering the *in vivo* data we anticipate that OA effects might dominate among the mixed fatty acids that are mobilized during the period of NEB in postpartum dairy cattle.

Conclusion

In the present study, we showed that PA and SA in contrast to OA induced the expression of key genes of steroidogenesis and E2 production. These differential effects might be attributed to differential saturation or stereo metric positions of double bonds in fatty acids. Combined effects of fatty acids are largely dominated by mostly negative effect of OA on both hormone production and transcript abundance. Suggestively, this might mirror the *in vivo* situation, where OA is the most up-regulated fatty acid during NEB in the follicular fluid. Additionally, lower *CYP19A1* and *STAR* expression followed by lower E2 production after intrafollicular OA injection confirms the assertion that this fatty acid impedes steroidogenesis both *in vitro* and *in vivo* in bovine GC. Collectively, these effects of NEFA might therefore be responsible for mechanism through which the NEB influences the reproductive performance of high-yielding dairy cows during the early postpartum period. Finally, we suggest that these findings may partly be also applicable to obese women with increased serum concentrations of free fatty acids suffering from impaired fertility.

Supplementary data
This is linked to the online version of the paper at https://doi.org/10.1530/EC-19-0011.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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