Elevated non-esterified fatty acid concentrations during in vitro murine follicle growth alter follicular physiology and reduce oocyte developmental competence

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Objective: To study how long-term elevated non-esterified fatty acid (NEFA) concentrations, typical in metabolic disorders such as obesity or type 2 diabetes, affect murine follicular development, follicle quality, and subsequent oocyte developmental competence in vitro.
Design: Experimental study.
Setting: In vitro culture setting.
Animal(s): Female and male 13-day old, B6CBAF1 mice of proven fertility were sacrificed for harvesting ovaries and epididymal sperm, respectively.
Intervention(s): Early secondary murine follicles were cultured in vitro in the presence of NEFAs until the antral stage (12 days). Treatments consisted of one or a mixture of NEFAs (stearic acid [SA], palmitic acid [PA], oleic acid [OA]) in physiological (basal) or pathological (high SA, high OA, high NEFA) concentrations.
Main Outcome Measure(s): Follicular development; follicle and oocyte diameters; secretion of progesterone, estradiol, and inhibin B; and luteinized granulosa cell gene expression patterns were investigated. Oocytes from NEFA-exposed follicles were fertilized in vitro, and presumptive zygotes were cultured until the blastocyst stage.
Result(s): Exposure to high SA reduced follicle diameters and day-12 antrum formation. Elevated NEFA concentrations changed luteinized granulosa cell messenger–ribonucleic acid abundance of genes related to energy/fatty acid/steroid metabolism, apoptosis, and oxidative stress. High NEFA and high SA treatments increased progesterone synthesis, compared with high OA follicles. Oocyte developmental competence was substantially reduced in oocytes retrieved from high OA–, high SA–, and high NEFA–exposed follicles compared with basal–treated follicles.
Conclusion(s): This study showed, for the first time, that lipolysis-linked, elevated NEFA concentrations can potentially impair fertility, by altering follicular physiology and reducing oocyte developmental competence. (Fertil Steril® 2014;102:1769–76. ©2014 by American Society for Reproductive Medicine.)
Key Words: Metabolic disorder, obesity, NEFA, folliculogenesis, oocyte developmental competence

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Metabolic disorders, such as obesity and type 2 diabetes, are characterized by an increased mobilization of body fats, which is associated with elevated serum non-esterified fatty acid (NEFA) concentrations (1, 2). These lipolysis-linked elevated NEFA concentrations have been shown to be cytotoxic for several cell types, such as pancreatic β-cells and Leydig cells (3, 4), suggesting a potentially hazardous effect on somatic cell survival and function. High NEFA concentrations are also relevant in the pathogenesis of obesity and type 2 diabetes, by altering, for example, insulin signaling (5).

Additionally, they have been proposed as an important link between maternal metabolic disorders and subfertility. We previously showed that elevated serum NEFA concentrations were reflected in bovine (6) and human (7, 8) follicular fluid from the pre-ovulatory follicle. Such elevated NEFA concentrations affect the proliferation and steroidogenesis of both granulosa and theca cells of the bovine ovarian follicle (9, 10) and induce apoptosis in human granulosa cells (11). In fact, elevated NEFA concentrations in human follicular fluid have been associated with inferior cumulus-oocyte complex morphology (12). In addition, we and others (13–16) have shown that elevated NEFA concentrations during the final maturation phase of bovine oocytes in vitro were detrimental for the oocyte's developmental competence and subsequent embryo quality.

To date, it has never been investigated if or to what extent folliculogenesis, follicle quality, and the developmental competence of the enclosed oocyte are affected by continuous exposure to elevated NEFA concentrations during follicle growth in vitro. Such long-term follicular NEFA exposure more closely resembles the in vivo situation in, for example, obese women, compared with the previously described short-term exposure studies. Therefore, we hypothesized that long-term elevated NEFA concentrations alter follicular physiology, ultimately leading to an impaired oocyte developmental competence, caused by either direct effects on the cumulus-oocyte complex or indirect effects through altered granulosa cell function, or both. Hence, the aim of this study was to investigate the effect of long-term NEFA exposure on follicular growth, follicle quality, and the developmental competence of the enclosed oocyte, by using a well-established murine follicle culture model (17, 18).

MATERIALS AND METHODS

Animals, Follicle Isolation, and Culture

Animals were housed and bred according to procedures indicated by national law in Belgium, and experiments were performed with ethical approval (concession number LA12302070, EC-11-01). All products were purchased from Sigma-Aldrich, unless otherwise stated. For each experiment, 4 B6CBAF1 mice, age 13 days, were sacrificed, after which the ovaries were removed and washed in L15 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Greiner bio-one) and 50 IU/ml penicillin G sodium salt. Follicles were mechanically isolated from the ovaries and cultured individually in 75-μl minimal essential medium (MEM) (Life Technologies) supplemented with 5% FBS, 5 μg/ml–5 μg/ml–5 ng/ml insulin–transferrin–selenium, and 0.75% bovine serum albumin (BSA) (MEM*) in a 96-well plate. The same batch of FBS was used in every experiment (NEFA concentration of 110 μM, thus accounting for 5.5 μM in each medium sample originating from 5% FBS addition). Throughout the culture period, follicles were incubated at 37°C, 6% CO_2, and maximal humidity.

On day 1, only early pre-antral follicles with a diameter between 100 and 130 μm, with visible theca cells present over more than one third of the circumference of the follicle and a good connection between the oocyte and the granulosa cells were selected for culture and transferred to 75-μl MEM* supplemented with 10 mIU/ml follicle-stimulating hormone (FSH; Merck-Serono) and 10 mIU/ml luteinizing hormone (LH; Merck-Serono). On days 4 and 8 of culture, 40% of the medium was renewed with fresh MEM* supplemented with 10 mIU/ml FSH. On day 12 of culture, 40% of the medium was refreshed with MEM* supplemented with 10 mIU/ml FSH, 3.75 IU/ml human chorionic gonadotrophin (hCG, Merck-Serono), and 25 ng/ml epidermal growth factor for ovulation induction and oocyte in vitro maturation. On days 4, 8, and 12 of culture, follicular development was evaluated as previously described (19), leading to a classification of follicles into follicular (F), follicular–diffuse (F/D), diffuse (D), diffuse–antral (D/A), and antral (A) stages. A follicle was considered to be of good quality (binary outcome measure) when it reached the F/D, D, D/A, or A stage on day 4; the D, D/A, or A stage on day 8; and the D/A or A stage on day 12 of culture.

Follicle and Oocyte Diameters

Follicle diameters (μm) were assessed by measuring the distance between 2 sides of the follicle (excluding theca cells), straight through the center of the oocyte with a ColorView camera (Olympus Soft Imaging Solutions GmbH) positioned on an inverted microscope in real time. Oocyte diameters included zona-pellucida thickness.

Steroid and Inhibin B Concentrations in Spent Medium before and after the Ovulatory Stimulus

Culture-medium samples were taken from all antral follicles (D/A and A) on days 12 and 13 at the time of medium renewal and oocyte retrieval, respectively, and pooled per plate. Day-12 medium samples were frozen directly at −80°C. Day-13 medium samples were frozen after pelleting of luteinized granulosa cells for gene expression analyses. Progesterone (P) and estradiol (E_2) measurements were conducted based on a competitive immunoassay format according to the manufacturer's instructions (Meso Scale Discovery). Mean within- and between-assay variations were 3.9% and 13.8% for E_2, and 4.5% and 17.1% for P measurements, respectively. Absolute P rise (day-13 P − day-12 P); E_2 reduction ([day-13 E_2 − day-12 E_2] · − 1); and relative E_2 reduction (absolute E_2 reduction · 100/day-12 E_2) were calculated. Day-12 inhibin B concentrations were measured using the OBI MCA1312KZZ Inhibin B Enzyme Linked Immuno Sorbent Assay (DSL), according to the manufacturer's instructions (mean within- and

between-assay variations were 2.7% and 4.6%, respectively). Relative P rise corrected for inhibin B concentrations, as a measure for granulosa cell number, was calculated as: (day-13 P • 100/day-12 P)/inhibin B concentrations.

Gene Expression Analyses

After the isolation of the cumulus-oocyte complexes for fertilization, the remaining luteinized granulosa cells from those antral follicles were transferred to a sterile, RNase free 1.5-ml microcentrifuge tube, by means of a micropipette and a 200-μl tip. A minimal contamination with theca cells from the monolayer formed below the follicle is to be assumed. Luteinized granulosa cells were pooled per plate and centrifuged (5 minutes, 375 g); the pelleted granulosa cells were snap frozen, by putting the microcentrifuge tube into liquid nitrogen. Samples were analyzed by means of quantitative real-time polymerase chain reaction, as described by Van Hoeck et al. (20), with minor modifications. Results were normalized to the endogenous control histone H2AFZ. The choice of genes analyzed in this study was based on the previously obtained knowledge of genes affected by short-term NEFA exposure in bovine oocytes, cumulus cells, and embryos (21). We furthermore opted to implement several genes specifically related to follicle quality. Therefore, we implemented genes related to energy metabolism, protein metabolism, fatty acid metabolism, steroid metabolism, apoptosis, deoxyribonucleic acid damage and repair, oxidative stress, and follicle quality. Details of the genes investigated, the primers used, and the approximate size of the amplified fragments are presented in Supplemental Table 1 (available online).

Oocyte Isolation, In Vitro Fertilization, and Embryo Culture

For each replicate, 2 male B6CBAF1 mice of proven fertility were sacrificed, after which sperm was released from the epididymis in M16 medium supplemented with 3% BSA. After visual evaluation of sperm motility and initial sperm count (Bürker counting chamber), the sperm suspension was diluted to a concentration of 10,000 motile spermatozoa/μl. Sperm capacitation continued for 2.5 hours in total.

On day 13 of follicle culture, 20 hours after the day-12 ovulatory stimulus, cumulus-oocyte complexes from all antral (D/A and A) follicles were isolated with a small glass capillary and fertilized in groups of 10 in droplets of 30-μl M16 medium with 3% BSA with mineral oil overlay by adding 10 μl of sperm suspension (10,000 motile spermatozoa/μl). After 3 hours of fertilization, presumptive zygotes were manually denuded with a small glass capillary in M16 medium with 300-μg/ml hyaluronidase (422 IU/ml), washed twice, and cultured in groups of 10 in 75 μl of M16 medium in a 96-well plate. Plates were incubated at 37°C, 6% CO_2, and maximal humidity. Embryo cleavage was assessed on day 14 (day 1 after insemination); embryo development was scored on day 18 (day 5 after insemination) of culture. Cleavage rate (number of 2-cell embryos/number of oocytes); blastocyst development (number of blastocysts/number of oocytes); blastocyst development from cleaved zygotes and hatching rate (number of hatching embryos/number of blastocysts) were calculated.

Experimental Design

Follicles were exposed to one or a mixture of NEFAs in concentrations mimicking physiological and pathological serum NEFA concentrations, based on both bovine (14) and human (7, 8) data. Three predominant NEFAs present in serum were used (12, 14): stearic acid (SA), palmitic acid (PA), and oleic acid (OA). These were dissolved in 100% ethanol to prepare the following NEFA stocks: 28 mM, 140 mM, and 112 mM SA; 21 mM, 105 mM, and 210 mM OA; and 23 mM, 115 mM, and 230 mM PA for the basal, moderate, and high NEFA treatment groups, respectively. These stock solutions were added in a volume of 25 μl (112 mM SA stock) or 10 μl (other stock solutions) to 10 ml MEM*, to obtain the final concentrations described below. Media were placed in a sonication bath for 4 hours under continuous temperature control at 30–35°C, vortex-mixed for 3 minutes, and filtered (0.2 μm). All medium samples were analyzed for total NEFA concentrations using RX Daytona (Randox Laboratories, United Kingdom). Preliminary experiments were set up to investigate the effect of 0.45% solvent (ethanol).

Experiment 1: effect on follicular development. Experiment 1 was conducted by randomly assigning selected follicles from 4 mice per replicate (4 replicates) to experimental treatments from day 1 until day 13 of culture: [1] solvent control (n = 153; 0.45% ethanol), [2] basal NEFA (n = 159; 28-μM SA + 21-μM OA + 23-μM PA = 72-μM NEFA), [3] moderate NEFA (n = 160; 140-μM SA + 105-μM OA + 115-μM PA = 360-μM NEFA), and [4] high NEFA (n = 154; 280-μM SA + 210-μM OA + 230-μM PA = 720-μM NEFA). Follicular development was assessed as previously described on days 4, 8, and 12 of culture.

Experiment 2: effect on follicular development, steroidogenesis, granulosa cell gene expression patterns, and oocyte developmental competence. In experiment 2, a high SA and high OA treatment group was implemented, because of the importance of individual FAs and FA ratios (13, 15). Selected follicles from 4 mice per replicate (4 replicates) were randomly assigned to experimental treatments from day 1 until day 13 of culture: [1] basal NEFA (n = 154; see experiment 1), [2] high SA (n = 160; 280-μM SA), [3] high OA (n = 159; 210-μM OA), and [4] high NEFA (n = 156; see experiment 1). Follicular development was assessed on days 4, 8, and 12 of culture; follicle and oocyte diameters; P, E_2 and inhibin B concentrations in the culture medium; and Day-13 luteinized granulosa cell gene expression patterns were investigated as described. Mature oocytes were fertilized, and early embryo development was studied.

Statistical Analyses

Statistical analyses were performed with PASW statistics 18 (for Windows; SPSS Inc.). Differences in diameters (follicle and oocyte) and concentrations of P, E_2, and inhibin B between groups were investigated with a mixed-model ANOVA

with post hoc Sheffé tests, where treatment was entered as a fixed factor, and replicate as a random factor. The interaction between replicate and treatment was first included in the model but then omitted from the final model because it was not significant. A binary logistic regression approach was used to study differences in follicular development (binary outcome: good- or bad-quality scoring on days 4, 8, and 12 of culture as described); cleavage rate; blastocyst formation; blastocyst formation from cleaved zygotes; and hatching rate. The interaction between replicate and treatment was first included in the model but then omitted when found to be not significant. Relative transcript abundance was analyzed with SigmaStat software (Jandel Scientific), using a one-way ANOVA with multiple pairwise comparisons, and the Student-Newman-Keuls test as a post-hoc method. Data are presented as means ± SD or SEM. Statistical significance was set at $P<.05$.

RESULTS

Experiment 1

There was no significant difference in development among follicle groups on day 4. Fewer basal NEFA (77.4%) and moderate NEFA (76.9%) follicles had "good-quality" scores on day 8, compared with solvent control follicles (87.6%, $P=.02$). However, on day 12, fewer high NEFA follicles (69.5%) reached the antral stage compared with solvent control follicles (79.1%, $P=.07$).

The preliminary experiments showed no significant difference between solvent control (76.6%) and basal NEFA (73.5%) antrum formation, compared with our lab control without ethanol or NEFAs (79.7%). For this reason, and to render the experimental design as biologically relevant as possible, we implemented the basal NEFA treatment, which represents normal physiological NEFA concentrations, instead of the solvent control treatment, as an experimental control in experiment 2.

Experiment 2

Follicular development scores of "good quality" on day 4 of the culture were significantly lower for high SA (85%) compared with high NEFA follicles (93%, $P=.02$). On day 8, follicular development was reduced in high SA (81%) and high OA (79%) follicles, compared with high NEFA follicles (88%, $P=.05$ and $P=.03$, respectively). On day 12, fewer high SA follicles (68%) had reached the antral stage, compared with basal (79%) and high NEFA follicles (83%, $P=.03$ and $P<.01$, respectively). Also, high OA (72%) follicles displayed reduced antrum formation compared with high NEFA follicles ($P=.04$). For all percentages of follicular development, the SE of the proportion was 3%–4%.

Follicle and oocyte diameters were measured. Mean day-4 oocyte diameters were lower in basal NEFA, compared with high SA follicles (Table 1, $P=.035$). Furthermore, day-8 follicle diameters were higher for high OA follicles, compared with high SA follicles (Table 1, $P<.01$). Day-12 follicle diameters were significantly lower for high SA follicles, compared with basal NEFA, high OA, and high NEFA follicles (Table 1; $P=.045$, $P=.010$, and $P=.027$, respectively), but oocyte diameters were similar among groups.

TABLE 1

Follicular and oocyte diameters (μm) on days 4, 8, and 12 of culture for basal NEFA–, high SA–, high OA–, and high NEFA–treated follicles.

Day	Diameter (μm)	Basal NEFA	High SA	High OA	High NEFA
4	Follicle	189 ± 68	194 ± 83	219 ± 93	210 ± 78
	Oocyte	64 ± 6[a]	66 ± 7[b]	66 ± 7[a,b]	65 ± 6[a,b]
8	Follicle	541 ± 235[a,b]	472 ± 223[a]	601 ± 262[b]	549 ± 225[a,b]
	Oocyte	71 ± 8	70 ± 7	74 ± 8	73 ± 9
12	Follicle	815 ± 241[b]	726 ± 254[a]	834 ± 274[b]	820 ± 253[b]
	Oocyte	80 ± 8	79 ± 9	82 ± 9	80 ± 7

Note: Data are presented as mean ± SD.
[a,b] Different superscripts within a row indicate a statistically significant difference between those treatments, $P<.05$.

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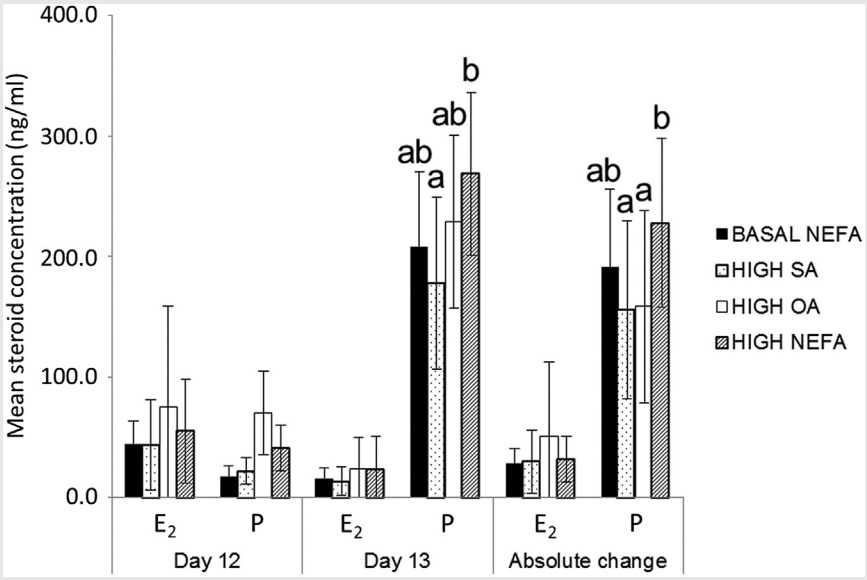
Steroidogenesis before and after the ovulatory stimulus was evaluated. Day-12 and day-13 E_2 concentrations, day-12 P concentrations, and absolute E_2 reduction were not affected by treatment. Day-13 P levels were higher in high NEFA follicles compared with high SA follicles ($P=.002$; Fig. 1). In addition, P rise was significantly higher in high NEFA follicles compared with both high SA and high OA follicles ($P=.012$ and $P=.017$, respectively; Fig. 1). However, relative P rise that was corrected for inhibin B concentrations was higher for high SA and high NEFA follicles compared with high OA follicles ($P=.045$, $P<.01$, respectively). Furthermore, high OA follicles had a higher relative E_2 reduction compared with high NEFA follicles (70.15% ± 8.91% vs. 62.33% ± 8.64%, $P=.026$).

Day-13 granulosa cell gene expression was evaluated. The messenger ribonucleic acid (mRNA) transcript abundance (Fig. 2) of *Tp53*, *Bax,* and the ratio *Bax/Bcl2* was elevated in basal and high NEFA compared with high OA luteinized granulosa cells. *Gadd45b* expression was higher in high OA granulosa cells compared with basal and high NEFA. *Gapdh* expression was higher in basal and high NEFA compared with high SA luteinized granulosa cells. *Slc2a1* expression was higher in high NEFA compared with high OA and high SA granulosa cells. *Rgs2* expression was higher in high NEFA compared with high OA and basal granulosa cells. *Acaca* was overexpressed in basal compared with high OA cells; *Hsl* expression was higher in high NEFA and high OA compared with basal luteinized granulosa cells. *Lhcgr* expression was higher in high NEFA compared with high OA cells; *Cyp19a1* expression was elevated in high SA compared with high NEFA granulosa cells. *Sod1* expression was higher in basal and high NEFA compared with high OA. Expression of *Gpx1* and *Clu* was higher in high SA compared with basal granulosa cells.

Cleavage and embryo development data are presented in Table 2. The cleavage rate (day 1 after insemination) was significantly reduced for high NEFA compared to basal NEFA oocytes ($P<.01$). Blastocyst development was reduced for high SA, high OA, and high NEFA treatment compared with basal NEFA ($P<.01$ for all 3 comparisons). Blastocyst

FIGURE 1



Follicle steroidogenesis. Estradiol and P concentrations in the culture medium of day-12 and day-13 follicles cultured in basal NEFA, high SA, high OA, or high NEFA conditions. Absolute E_2 reduction and P rise between day 12 and day 13 are also shown. Data are presented as mean ± SD. [a,b] Different superscripts on a bar indicate a statistically significant difference between treatments ($P<.05$).

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formation from cleaved zygotes was reduced for high SA embryos compared with both basal and high NEFA embryos ($P<.05$). In addition, high OA embryos had a lower blastocyst formation rate from cleaved zygotes compared with basal embryos. Hatching rate was lower for high OA embryos compared with basal NEFA, high SA, and high NEFA embryos ($P<.05$).

DISCUSSION

Elevated NEFA concentrations, typical in metabolic disorders such as obesity and type 2 diabetes (5), have been proposed as a key factor linking maternal metabolic disorders and disappointing fertility results. In this study, we investigated how the biological unit of the growing follicle and the resulting oocyte quality are affected by long-term exposure to elevated NEFA concentrations. We showed that long-term elevated NEFA concentrations only moderately affect follicular growth and antrum formation, with the most pronounced effect induced by the high SA treatment. Elevated NEFA concentrations altered gene expression patterns in day-13 luteinized granulosa cells, revealing that NEFA exposure mainly affected pathways involved in apoptosis, lipid metabolism, oxidative stress, and steroidogenesis, which was also evidenced by P, E_2, and inhibin B analyses in spent medium. Most importantly, the oocytes originating from the NEFA-exposed follicles displayed a significantly reduced developmental competence (blastocyst formation).

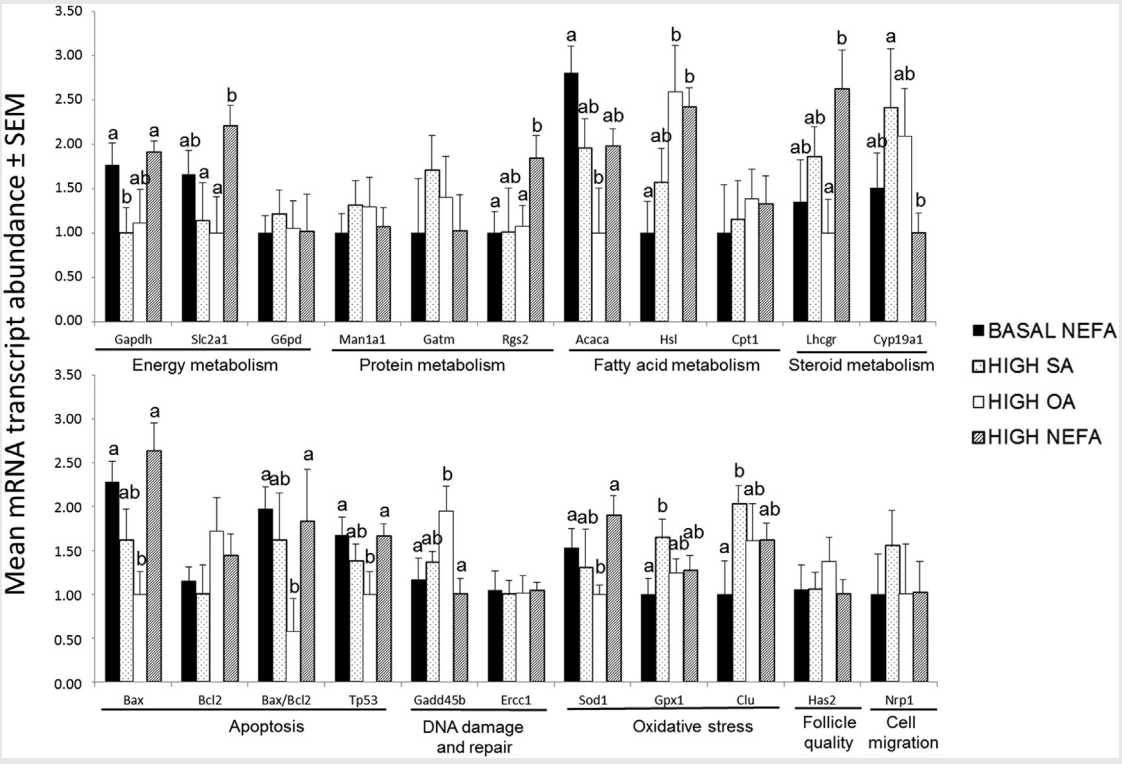
Follicular Development and Quality

Our results showed that the high SA treatment caused the most pronounced effect on follicular development, by reducing day-12 antrum formation and follicular diameters. Interestingly, the same SA concentration, but in a mixture of NEFAs (high NEFA treatment) did not affect follicular growth. This finding is in agreement with the observation that mono- and poly-unsaturated FAs can prevent the pro-apoptotic effect of PA in pancreatic β-cells (3, 22). Oleic acid also prevented the negative effect of PA and SA during in vitro bovine oocyte maturation on oocyte developmental competence (15), possibly through an increased shuttling of PA and SA into neutral lipid droplets. In addition, treatment with high OA seemed to reduce apoptosis in day-13 luteinized granulosa cells, as indicated by *Tp53* and *Bax* mRNA transcript abundance and the ratio *Bax/Bcl2*, which is ultimately responsible for the release of cytochrome c from the mitochondrion (23). The observation that *Gadd45b* expression was upregulated in high OA granulosa cells substantiates this, because it is a cell cycle checkpoint regulator that is proposed to be involved in the prevention of apoptosis or DNA damage in granulosa cells of bovine dominant follicles (24).

Follicle Metabolism

Treatment with high SA seemed to reduce glucose uptake and consumption (glycolysis) by mural luteinized granulosa cells (decreased expression levels of *Slc2a1* and *Gapdh*) compared with basal- and/or high NEFA-treated follicles. Furthermore, the increased mRNA abundance of *Sod1* in high NEFA and *Gpx1* in high SA exposed follicles compared with high OA and basal follicles, respectively, suggests increased exposure to oxidative stress. Elevated NEFA concentrations may indeed alter glucose metabolism by inducing insulin resistance, and

FIGURE 2



Granulosa cell gene expression patterns. Comparison of relative transcript abundance in murine day-13 granulosa cells. Follicles were cultured for 12 days in basal NEFA, high SA, high OA, or high NEFA conditions (4 replicates), and granulosa cells were obtained on day 13, at 20 hours after a final maturation stimulus. [a,b] Different superscripts on a bar indicate a statistically significant difference between treatments ($P<.05$). Data are presented as relative transcript abundance ± SEM.

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increase fatty acid beta-oxidation, leading to increased reactive oxygen species production (5).

Follicle Steroidogenic Profile

Progesterone plays a key role in the acquisition of oocyte developmental competence (25). The P data, corrected for the number of granulosa cells by taking inhibin B concentrations into account (26, 27), suggest that high SA and high NEFA follicles had a stronger reaction to the day-12 ovulatory stimulus compared with high OA follicles. It was previously suggested that apoptotic bovine granulosa cells can maintain steroidogenesis as long as the steroidogenic organelles remain intact (9, 28) and that these organelles cluster during the process of apoptosis (9, 29), which could create the notion of a higher steroidogenic capacity, when in fact it is a consequence of apoptosis.

High OA follicles showed a high day-12 P concentration, which could be indicative of premature luteinization (rise in P levels on the day of hCG administration [30]), which could potentially lower the sensitivity of the granulosa cells to the subsequent ovulatory stimulus. This possibility is substantiated by the decreased *Lhcgr* mRNA transcript abundance in high OA follicles compared with high NEFA follicles. To our knowledge, we have shown for the first time, by studying the follicle as a whole in a culture model, that altered NEFA conditions may affect follicular steroid synthesis and responsiveness to an ovulatory stimulus. This may explain the higher incidence of cycle and ovulatory disorders, which are often seen in women suffering from metabolic disorders (31).

Oocyte Developmental Competence

Elevated NEFAs during the final maturation phase of bovine oocytes have been shown to increase oocyte NEFA uptake and metabolism (15), which has been associated with hampered oocyte maturation, increased oxidative stress, and impaired developmental competence of the oocyte as well as an altered physiology and metabolism of the resultant embryo (13, 14, 20, 21). In agreement with these findings, we showed that oocyte developmental competence was substantially reduced in oocytes originating from NEFA-exposed follicles. Interestingly, hatching rate was reduced for high OA embryos. Alterations in the composition of the zona pellucida, and altered embryonic ability to crack the zona pellucida, have been proposed as mechanisms that could explain differences in hatching rate between in vivo– and in vitro–derived embryos at different diameters (32, 33).

In addition, numerous factors, such as proteases, growth factors, and transcription factors, may affect the process of

TABLE 2

Details on cleavage rate (day 1 after insemination) and embryo development (day 5 after insemination) from oocytes originating from basal NEFA–, high SA–, high OA– and high NEFA–treated follicles.

Rate (%)	Basal NEFA (n = 118)	High SA (n = 88)	High OA (n = 97)	High NEFA (n = 124)
Cleavage	69.5[a]	59.1[a,b]	56.7[a,b]	52.8[b]
Blastocyst	59.3[a]	30.7[b]	36.1[b]	42.3[b]
Blastocyst from cleaved zygotes	85.4[a]	51.9[b]	63.6[b,c]	80.0[a,c]
Hatching from total n of blastocyst	40.0[a]	48.1[a]	14.3[b]	44.2[a]

[a,b,c] Different superscripts within a row indicate a statistically significant difference between those treatments, $P < .05$.

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blastocyst hatching (34). Interestingly, we showed that oxidative stress was reduced in high OA luteinized granulosa cells (reduced *Sod1* expression compared with basal follicles), which may have influenced the oxidative status of the resultant embryo. The finding that an oxyradical burst causes hatching in mouse blastocysts (35) might explain the reduced hatching rate in high OA embryos. However, to strengthen this hypothesis, research is needed on the exact mechanism by which elevated OA concentrations during follicular development affect hatching rate.

Because of the pathophysiological and biological relevance of exposing follicles as a functional unit, for a longer period of time, we specifically chose to use the murine follicle culture model. This model has been proven suitable for in vitro testing of ovarian function (36) and supports follicular growth, steroid production, antrum formation, and the acquisition of oocyte developmental competence, with high resemblance to the in vivo situation (17, 18). But, even though we were able to implement a NEFA exposure for a longer period of time, the culture model used presents the limitation that it still does not cover the whole period of folliculogenesis and oogenesis. Future research should continue to focus on optimizing in vitro culture systems that allow the growth of follicles from the primordial stage up until the antral stage, with the development of a competent oocyte in vitro, in order to study how, for example, elevated NEFA concentrations may affect follicular growth starting at the primordial stage.

In conclusion, we studied the effect of long-term NEFA exposure on oogenesis, folliculogenesis, follicle quality, and subsequent oocyte developmental competence. Our data show for the first time that such long-term exposure to elevated NEFA concentrations only moderately affects follicular growth and antrum formation but substantially alters pre-ovulatory follicular steroid synthesis, the responsiveness to the ovulatory stimulus, and luteinized granulosa cell gene expression patterns. These changes ultimately resulted in severely impaired oocyte developmental competence. From a clinical point of view, our data help to illuminate the pathways by which lipolysis-linked elevated NEFA concentrations may contribute (by altering follicular physiology and oocyte developmental competence) to subfertility in metabolically compromised women. Ongoing research focuses on the physiology and metabolism of the resultant and surviving pre-implantation embryos and the potential consequences for postnatal health.

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SUPPLEMENTAL TABLE 1

Details of primers used for quantitative real-time polymerase chain reaction analyses.

Gene	Primer sequence (forward/reverse primer)	Fragment size (base pairs)	GenBank accession no.
Gapdh	ACCCAGAAGACTGTGGATGG/AYGCCTGCTTCACCACCTTC	247	BC102589
Slc2a1	CTGATCCTGGGTCGCTTCAT/ACGTACATGGGCACAAAACCA	68	NM_174602.2
G6pd	CGCTGGGACGGGGTGCCCTTC/ATCCGCCAGGCCTCCCGCAGTTCATCA	347	XM_583628.4
Acaca	AAACCAGCACTCCCGATTCAT/GGCCAAACCATCCTGTAAGC	175	FN185963.1
Hsl	GGGAGCTCCAGTCGGAAGA/GCTGTGTGCACCAAACTACG	287	NM_001039507.2
Cpt1a	CTGCCCGCCTGGGAAATGCTGTCAGTCTCTCCTCCCCGGGCTGG	328	NM_001034349.2
Lhcgr	GCCAACCCATTTCTGTACGC/CAGCCTGGGAAGGCTTACTT	178	NM_013582.2
Cyp19a1	TCCACACTGTTGTGGGTGAC/AGGGAAGTACTCGAGCCTGT	218	NM_007810.3
Bax	CTACTTTGCCAGCAAACTGGTCCCAAAGTAGGAGAGGA	158	NM_173894.1
Bcl2	GTCCCGCCTCTTCACCTTTCAG/GATTCTGGTGTTTCCCCGTTGG	147	NM_009741.4
Tp53	CTCAGTCCTCTGCCATACTAGGATCCAGGATAAGGTGAGC	364	NM_174201.2
Gadd45b	CTTCTGGTCGCACGGGAAGG/GCTCCACCGCGGCAGTCACC	277	AF441860.1
Ercc1	GTGCTGCTGGTTCAAGTGGA/GCAGTCAGCCAAGATGCACAT	80	NM_001127324.1
Sod1	GTGCAAGGCACCATCCACTTCG/CACCATCGTGCGGCCAATGATG	309	NM_174615
Gpx1	GCAACCAGTTTGGGCATCACTCGCACTTTTCGAAGAGCATA	116	NM_174076.3
Has2	TCAGCGAAGTTATGGGCAGG/GATGAGGCAGGGTCAAGCAT	265	NM_008216.3
Man1a1	GTCACTGCAGAGTGAACGGA/AAGGATGAGCCTCGGTGTTG	183	NM_008548.4
Gatm	GTGGGCAGAGCTGAAAATGC/GGCACCACGATGGAAGTAGT	387	NM_025961.5
Rsg2	TCCTGTCACTTACCAACCGC/CACTGCGGAGAGGAACCATT	216	NM_009061.4
Clu	AGCCGTGCGGAATGAGATAG/TTCTTCCCGAGAGCAGCAAG	196	NM_013492.2
Nrp1	AGCATCCAATCAAGCCGACA/CCGAAGCTCAGGTGTGTCAT	313	NM_008737.2

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