BIOLOGY OF REPRODUCTION **85,** 62–69 (2011)
Published online before print 10 February 2011.
DOI 10.1095/biolreprod.110.088815

Oleic Acid Prevents Detrimental Effects of Saturated Fatty Acids on Bovine Oocyte Developmental Competence[1]

Hilde Aardema,[4] Peter L.A.M. Vos,[4] Francesca Lolicato,[5] Bernard A.J. Roelen,[4] Hiemke M. Knijn,[3,4] Arie B. Vaandrager,[5] J. Bernd Helms,[5] and Bart M. Gadella[2,4,5]

Departments of Farm Animal Health[4] and Biochemistry and Cell Biology,[5] Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

ABSTRACT

Mobilization of fatty acids from adipose tissue during metabolic stress will increase the amount of free fatty acids in blood and follicular fluid and, thus, may affect oocyte quality. In this in vitro study, the three predominant fatty acids in follicular fluid (saturated palmitic and stearic acid and unsaturated oleic acid) were presented to maturing oocytes to test whether fatty acids can affect lipid storage of the oocyte and developmental competence postfertilization. Palmitic and stearic acid had a dose-dependent inhibitory effect on the amount of fat stored in lipid droplets and a concomitant detrimental effect on oocyte developmental competence. Oleic acid, in contrast, had the opposite effect, causing an increase of lipid storage in lipid droplets and an improvement of oocyte developmental competence. Remarkably, the adverse effects of palmitic and stearic acid could be counteracted by oleic acid. These results suggest that the ratio and amount of saturated and unsaturated fatty acid is relevant for lipid storage in the maturing oocyte and that this relates to the developmental competence of maturing oocytes.

blastocyst, fatty acid, fertilization, gamete biology, in vitro fertilization, lipid storage, oocyte development, oocyte maturation

INTRODUCTION

High-yielding dairy cows face metabolic stress during the early postpartum period. This results in a negative energy balance (NEB) due to energy loss by milk production that cannot be compensated by energy intake [1–3]. The NEB is believed to impair the fertility of these cows [2–5]. During periods of metabolic stress, massive body fat mobilization increases the free fatty acid concentration (fatty acid complexed to albumin) in both blood and follicular fluid [5–11]. The increase in and changed composition of free fatty acids may impair fertility by affecting oocyte quality due to transport of fatty acid into the oocyte [9–11].

After fatty acids are taken up by somatic cells, they are esterified into triacylglycerols (TAG) and cholesterol-esters and stored as neutral lipids in lipid droplets. In the oocyte it has been suggested that neutral lipids fulfil an important function in supplying energy and in biosynthesis of membranes during early embryonic development [12–16]. The esterification of fatty acids and storage into lipid droplets may also protect the oocyte against fatty acid-induced lipotoxicity [17]. In line with this, accumulation of neutral storage lipids in oocytes has been related to improved developmental competence [18, 19]. The fatty acid composition of esterified lipids in porcine, cow, and sheep oocytes is dominated by palmitic, oleic, and stearic acid and mirrors the composition of free fatty acids present in blood and follicular fluid [8, 14, 20]. Furthermore, these fatty acid compositions are comparable with those of the adipose tissue from which they are liberated, suggesting a dynamic fatty acid exchange [21]. The fatty acid composition of high- and low-quality oocytes differs: high-quality oocytes contain more oleic, linoleic, and arachidonic acid [15]. This indicates that the fatty acid composition of oocytes and their environment influences development competence. Moreover, in vitro studies have demonstrated differences in the tolerance somatic cells have of different fatty acids, saturated fatty acids being toxic and unsaturated fatty acids being relatively harmless [17, 22–25]. To examine the impact of saturated and unsaturated fatty acids on bovine oocytes, they were exposed to saturated palmitic or stearic acid and unsaturated oleic acid during in vitro maturation. After 23 h of maturation, the lipid droplets and postfertilization developmental competence of oocytes were examined. Exposure to saturated palmitic or stearic acid was compared with exposure to unsaturated oleic acid, since these fatty acids are the most prominent fatty acids in follicular fluid of early postpartum cows [8].

MATERIALS AND METHODS

Reagents and Antibodies

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Collection of Oocytes

Bovine ovaries were collected from a local abattoir and transported to the laboratory within 2 h after withdrawal. Ovaries were washed in physiological saline (0.9% NaCl) and kept in physiological saline with 0.1% (v/v) penicillin-streptomycin (Gibco BRL, Paisley, U.K.) at a temperature of 30°C. Follicles ranging from 3 to 8 mm were aspirated under low vacuum by a suction pump with a 19-gauge needle and allocated to a 50-ml conical tube. Cumulus oocyte complexes (COCs) with a minimum of three layers of cumulus were selected and first washed in HEPES-buffered M199 (Gibco BRL) and subsequently washed and cultured in M199 maturation medium (Gibco BRL) supplemented with 2.2 mg/ml $NaHCO_3$.

Selected COCs were cultured in four-well culture plates (Nunc A/S, Roskilde, Denmark) containing maturation medium (M199 supplemented with 0.02 IU/ml follicle-stimulating hormone [Sioux Biochemical Inc., Sioux Center, IA]), 0.02 IU/ml luteinizing hormone (Sioux Biochemical Inc.), 7.71 μg/ml cysteamine, 10 ng/ml epidermal growth factor in 0.1% (w/v) fatty acid-free bovine serum albumin (BSA) and 1% (v/v) penicillin-streptomycin (Gibco BRL). The oocytes were matured in groups of 35 COCs in 500 μl and incubated under a humidified atmosphere of 5% CO_2 in air for 23 h at 39°C.

[1]Supported by Pfizer Animal Health.
[2]Correspondence: FAX: 31 30 2535492; e-mail: b.m.gadella@uu.nl
[3]Current address: CRV BV, 6800 AL Arnhem, The Netherlands.

Received: 24 September 2010.
First decision: 8 November 2010.
Accepted: 4 February 2011.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

In Vitro Maturation Media with Palmitic, Stearic, and/or Oleic Acid

COCs were exposed to palmitic, stearic, or oleic acid in a concentration of 0 (control group), 100, 250, or 500 μM fatty acid during the entire maturation period of 23 h. Two maturation groups of COCs were exposed to either a combination of 250 μM palmitic and 250 μM oleic acid or 250 μM stearic and 250 μM oleic acid. Fatty acid-free BSA was prepared after charcoal treatment according to Chen [26] to liberate all fatty acids (>99.9%; own data not shown) as well as contaminating metabolic components such as remnant citrate, pyruvate, and lactate [27]. The resulting fatty acid-free BSA (2.35 mM) was complexed to 12 mM fatty acid in 20% KOH to obtain a 10.7-mM solution of fatty acid (albumin has five binding pockets for fatty acid, and the conditions were set to saturate albumin with the fatty acid of choice). A concentration of either 10 mM palmitic, stearic, or oleic acid was bound to 10% (w/v) fatty acid-free BSA (fatty acid:BSA ratio of 5:1). The used concentrations of fatty acid in this study were based on in vivo-measured individual and total fatty acid concentrations in follicular fluid at Day 16 after parturition, during the NEB of the cow [8].

Determination of Uptake and Incorporation of Radio-Labeled Palmitic or Oleic Acid

COCs were matured in 500 μl maturation medium and exposed to a concentration of 100 μM palmitic and 2.5 μCi [9, 10 (n) - ^{3}H] palmitic (60 Ci/mmol; NEN, Boston, MA) or 100 μM oleic acid and 2.5 μCi [9, 10 (n) - ^{3}H] oleic acid (7 Ci/mmol; Amersham Pharmacia Biotech, Little Chalfont, U.K.) for 23 h. In this study a total of 180 COCs was used in two independent runs. After maturation COCs were denuded via vortexing and washed four times in PBS. Lipid extraction was performed on 45 oocytes per sample. Lipids were extracted with chloroform-methanol according to the method of Bligh and Dyer [28]. Briefly, chloroform and methanol (2:1, v/v) were added to the oocytes with 0.8 ml PBS and mixed. After separation of the alcoholic phase by centrifugation (5 min, 3000 × g), the chloroform phase was collected. This procedure was repeated three times, and the chloroform phase was evaporated. The lipid extract was redissolved in chloroform and methanol (2:1, v/v) for thin-layer chromatography. Thin-layer chromatography was performed on prefab silica plates (HPTLC grade; Merck, Darmstadt, Germany) in a solvent system of hexane/diethyl ether/acetic acid 80:20:2 (v/v/v) at room temperature for characterization of neutral lipids. References of TAG, cholesterol-ester, diacylglycerol, and cholesterol were run in the same analysis to determine the exact position of the different fractions on the plate. Appropriate non-labeled pure lipid extract was added to the oocyte lipids to provide enough material for detection on the thin-layer plates. [^{3}H] containing spots were scraped off, and radioactivity in the spots was measured by liquid scintillation counting.

Lipid Droplet Staining of Oocytes

After maturation, oocytes were fixed and stained with a specific neutral lipid stain [29] for lipid droplet analysis for each oocyte. Matured COCs were washed twice in PBS and denuded by vortexing in PBS with 0.05 mg/ml fatty acid-free BSA for 4 min. Denuded oocytes were then washed again in PBS and fixed in 4% (v/v) paraformaldehyde (PF; Electron Microscopy Sciences, Hatfield, PA) at 37°C for 1 h and stored in 1% (v/v) PF at 4°C for a maximum of 1 wk. Oocytes were washed twice in PBS with 0.3% (w/v) polyvinylpyrrolidone (PVP), permeabilized for 30 min in PBS with 0.1% (w/v) saponin (PBS-S; Riedel-de Haën, Seelze, Germany) and 0.1 M glycin (Merck) and washed in PBS-S. To determine the maturational stage, oocytes were stained with 10 μg/ml TO-PRO-3 (Molecular Probes, Eugene, OR) for 20 min and subsequently three times washed in PBS-S. After this, neutral lipids in lipid droplets were stained according to a modified protocol of Chinese hamster ovary cells [30]. Lipid droplets were stained with the specific neutral lipid stain BODIPY 493/503 (Molecular Probes) in PBS (20 μg/ml, 1 h), and oocytes were washed three times in PBS with 3 mg/ml PVP. Oocytes were then mounted in a 0.12-mm eight-well Secure-Seal Spacer (Molecular Probes) on a glass slide (Superfrost Plus; Menzel, Braunschweig, Germany), covered in Vectashield (Vector Laboratories, Burlingame, CA), and sealed with a microscope slide (Superfrost Plus). In this study a total of 1340 COCs was used for lipid droplet analysis in at least three independent runs.

Immunofluorescent Staining for Adipose Differentiation-Related Protein

Immunolabeling was performed on oocytes as previously described [31]. Rabbit polyclonal antibodies against adipose differentiation-related protein (ADRP) were purchased from Abcam (Ab52355; Cambridge, U.K.). Both primary and secondary antibodies were diluted in blocking buffer and centrifuged at 100 000 × g for 1 h before use to prevent inclusion of aggregated antibodies in the immunostaining procedure. Oocytes were incubated with primary antibody overnight at 4°C. As negative controls, purified mouse or rabbit IgG (BD Biosciences, San Jose, CA) matching the host species of primary antibodies was used, and the dilutions of negative controls were identical to the dilutions of the primary antibodies used in the same experiment. Oocytes were rinsed three times in PBS after primary antibody incubation. ADRP-labeled oocytes were subsequently incubated with Alexa-568-conjugated goat anti-rabbit IgG (Molecular Probes) for 1 h at room temperature, followed by the above-described neutral lipid and DNA staining.

Fluorescent Imaging of Oocytes

Confocal microscopy was performed by using a Bio-Rad Radiance 2100 MP setup (Zeiss/Bio-Rad, Hertfordshire, U.K.) attached to a Nikon Eclipse TE300 inverted microscope (Nikon, Badhoevedorp, The Netherlands) at a magnification of 40× (1.25 NA). BODIPY 493/503, TO-PRO-3, and conjugated Alexa-568 were sequentially excited by argon laser (488 nm), red-helium neon diode laser (637 nm), and green 568-nm line. Images were acquired using LaserSharp 2000 software (Zeiss/Bio-Rad). Nuclear stage of oocytes was determined and oocytes were classified as germinal vesicle, metaphase-I (from germinal vesicle breakdown up to metaphase-I plate), or metaphase-II (from anaphase-I up to metaphase-II plate). The middle part of the oocyte was determined by defining the top and bottom position of the oocyte with LaserSharp 2000 software measurements, and three defined slices of the oocyte (middle part included) were measured. The middle part was investigated to ensure comparable images for each oocyte, representing the middle region of the oocyte. The three slices at a distance of 10 μm were highly correlated for lipid droplet number and mean lipid droplet size (in μm^2), with a stack of images at a distance of 5 μm through the whole oocyte ($R \geq 0.8$), secured to give a reliable picture of the whole oocyte.

Lipid Droplet Analysis

The 8-bit grayscale images of the three slices per oocyte were imaged with ImageJ (NIH; http://rsb.info.nih.gov/ij/) software. From the matured groups only metaphase II stage oocytes were analyzed. Lipid droplets were analyzed from a size of 0.3 μm^2 (equal to four pixels); this minimal threshold was set to overcome false positive counting from background pixels. From this the number of lipid droplets and the size per individual droplet (in μm^2) from the three slices per oocyte could be calculated. Metaphase-II oocytes from the control medium of the experiments with oleic and/or palmitic acid differed in the number of lipid droplets from metaphase-II oocytes, compared to the control medium of the experiments with stearic acid and with the combination of stearic and oleic acid.

Immunoblotting

Cumulus-free oocytes were obtained as previously described and directly lysed in an appropriate amount of lithium dodecyl dulfate loading buffer (Invitrogen, Carlsbad, CA) in the presence of 0.1 M dithiothreitol. For ADRP protein detection, 50 oocytes were used per sample (corresponding to approximately 6 μg of total protein [32]). The sample was heated for 5 min at 100°C prior to immunoblotting. Proteins were separated in a 4% stacking and 12% running SDS-PAGE gel and wet blotted onto polyvinylidene difluoride membranes (GE Healthcare, Buckinghamshire, U.K.). After blocking for 1 h with ReliaBLOT (Bethyl Laboratories, Inc., Montgomery, TX) at room temperature, blots were incubated with primary antibodies diluted in PBS with 0.2 % v/v Tween-20 (PBS-T) and 1% BSA overnight at 4°C. After washing the blot in PBS-T, secondary antibodies were added for 1 h. After rinsing with PBS-T, protein was visualized using chemiluminescence (ECL-detection kit; Supersignal West Pico; Pierce, Rockford, IL). The ADRP antibody was raised against a human peptide fragment with full amino acid sequence homology. Monoclonal mouse α-tubulin antibody (clone DM1A) was obtained from Sigma Chemical Co.

In Vitro Embryo Production

Following maturation, COCs were fertilized in vitro in groups of 35. Procedures for in vitro fertilization were performed as described by Parrish et al. [33] with minor modifications [34]. Briefly, sperm cells were added to the fertilization medium (modified Tyrode's medium also called Fert-TALP [33], without glucose and 1% (v/v) penicillin-streptomycin instead of gentamycin [34]) to a final concentration of 0.25×10^6 sperm cells/ml in the presence of 10 μg/ml heparin, 20 μM d-penicillamine, 10 μM hypotaurine, and 1 μM epinephrine ($t = 0$). After 20 h of incubation, cumulus cells of presumptive zygotes were removed by vortexing for 3 min and groups of 35 presumptive zygotes were transferred to 500 μl synthetic oviductal fluid medium

TABLE 1. Fatty acids taken up by maturing bovine oocytes are nearly completely metabolized.

Oocyte lipid fraction	Radioactivity (%)[a]	
	100 μM + [^{3}H] palmitic acid	100 μM + [^{3}H] oleic acid
Cholesterol-esters	2 ± 0.7	2 ± 0.5
Triacylglycerol	50 ± 0.4	36 ± 0.4
Free fatty acid	5 ± 0.6	4 ± 0.8
Diacylglycerol	12 ± 1.6	14 ± 1.1
Phospholipids and oxidation products of fatty acids	31 ± 4.3	45 ± 1.0

[a] Distribution of radioactivity in different lipid fractions from oocytes after 23 h of maturation with 100 μM unlabeled C9, 10 (n) – [^{3}H] palmitic acid or 100 μM unlabeled C9, 10 (n) – [^{3}H] oleic acid; results are presented as mean ± standard deviation.

supplemented with essential and nonessential amino acids and 0.1% BSA (w/v) (SOF medium; [35]) at 39°C in a humidified atmosphere of 5% CO_2 and 7% O_2 in air. At 5 days postfertilization, all cleavage stages were transferred to fresh SOF medium, and the proportion of cleavage stages was scored per group. At 8 days postfertilization, the number of blastocysts was scored. Note that the whole culture was performed in the absence of fetal calf serum [35]. In total, 5300 COCs were used for the culture experiments in at least three independent runs.

Scoring of Oocyte Developmental Competence after In Vitro Maturation with Palmitic, Stearic, and/or Oleic Acid

At 5 days postfertilization, the number of cleaved embryos, and embryos with 8 or more cells (≥8-cell embryos) were scored. At 8 days postfertilization, the number of blastocysts was determined. Results presented here are described as the percentage rates of cleavages, ≥8-cell embryos, or blastocysts from the original metaphase-II oocytes used for in vitro embryo production.

Statistical Analysis

Statistical analysis was performed in SPSS version 16.0 (SPSS Inc., Chicago, IL) with condition and experimental run as fixed factors. Analysis of lipid droplet size and number was done by general linear modeling with Bonferroni correction. The experiments with stearic acid were analyzed apart from oleic and palmitic acid since lipid droplets in the pool of oocytes differed. Lipid droplet size was analyzed after transformation to the natural logarithm to achieve a normal distribution. Analysis of oocyte developmental competence (maturation rate, cleavage, ≥8-cell embryos and number of blastocysts) was performed with logistic regression for grouped data. $P < 0.05$ was considered statistically significant.

RESULTS

Maturing Oocytes Actively Take Up and Metabolize Fatty Acids

In order to determine the capacity of oocytes to incorporate fatty acids in their lipid droplets, maturing oocytes were exposed to radio-labeled palmitic or oleic acid (total fatty acid concentration was 100 μM). After lipid extraction and thin-layer chromatographic separation, the radioactivity was measured in the isolated lipid fractions of the oocytes. Under both conditions almost all radioactivity was detected in TAG (storage lipid) as well as in phospholipids/fatty acid oxidation products, showing active uptake and metabolism of the fatty acids by the oocytes (Table 1). Only <5% of the incorporated radioactivity was recovered in the free fatty acid fraction, whereas >95% of the fatty acids were metabolized (Table 1). The oocytes took up a smaller amount of [^{3}H] palmitic acid than [^{3}H] oleic acid, with a difference of 30 ± 5%.

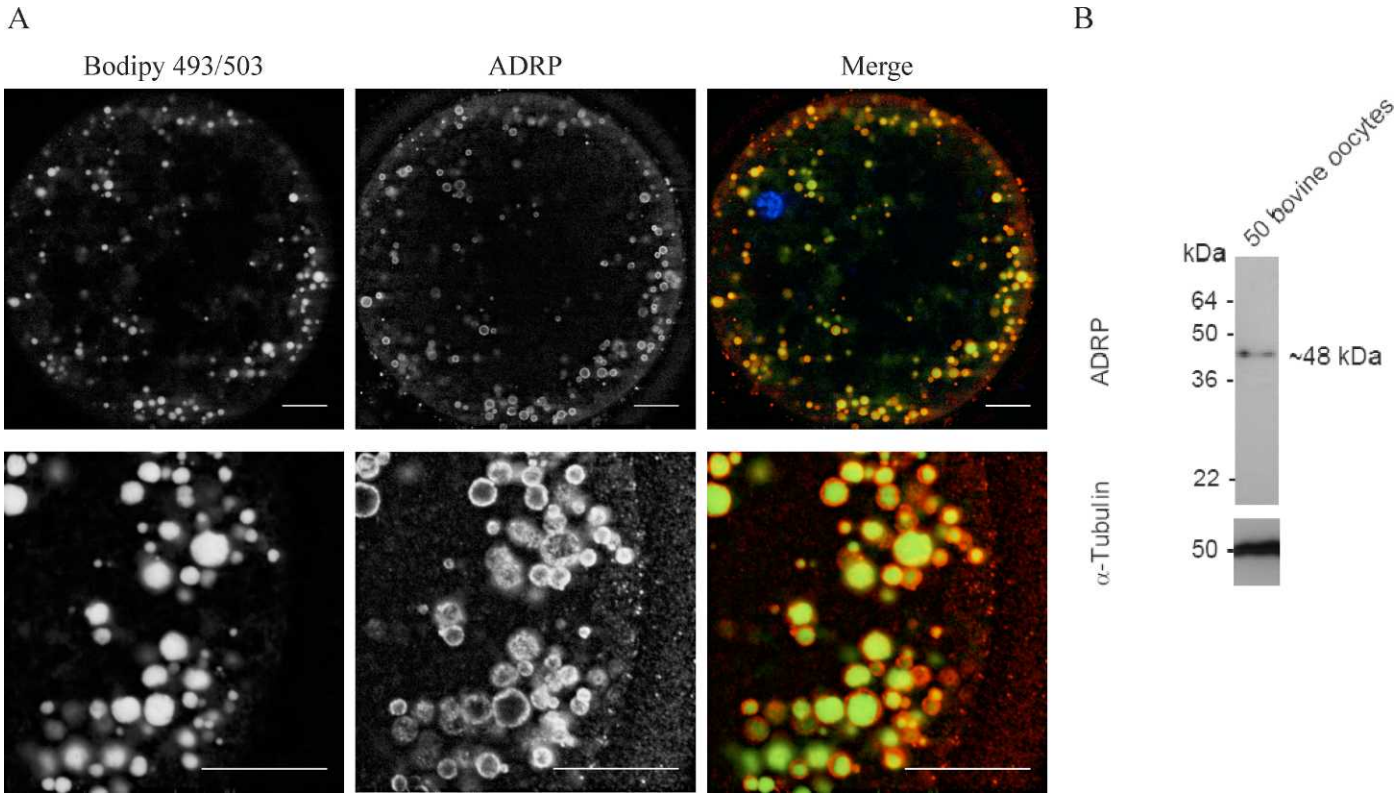

FIG. 1. Confocal images of mature oocytes at metaphase II stage after maturation in control medium (**A**). Merges show lipid droplets in green, ADRP in red, and DNA in blue. Bar = 20 μm. ADRP coats lipid droplets in bovine oocytes and colocalizes with BODIPY 493/503 labeling. Western blot (**B**). Fifty cumulus-free oocytes were used per sample loading lane. The ADRP observed band at ~48 kDa corresponds to the predicted size according to the manufacturer. α-Tubulin was used as loading control.

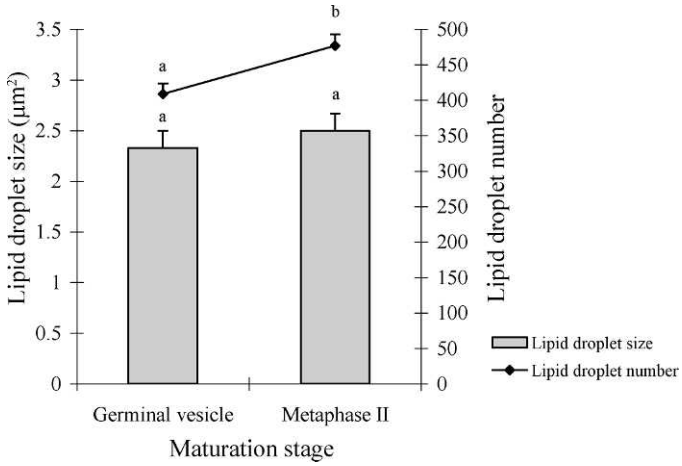

FIG. 2. Lipid droplet size (μm^2) and total number of lipid droplets per immature and mature oocyte (metaphase-II stage) after maturation in control medium. The number of lipid droplets increased slightly but significantly during maturation in control medium, whereas size of lipid droplets was unaffected. Results are presented as mean ± SEM. Data with different letters differ significantly ($P \leq 0.05$).

Lipid Droplets Are Identified with a Specific Neutral Lipid Stain

Lipid droplets, the storage reservoir of esterified fatty acids (stored either as TAG or as cholesterol esters) in cells, were identified with a neutral lipid stain. To determine the specificity of the neutral lipid stain for lipid droplets, oocytes were co-immunolabeled with an antibody against the lipid droplet-specific protein ADRP [36]. Figure 1 shows the presence of lipid droplets in a metaphase II oocyte. The merges depict that the BODIPY-neutral, lipid-stained structures clearly show red immunolabeling on their surfaces for ADRP; this validates that these structures are lipid droplets (Fig. 1A). The ability of the ADRP antibody to recognize bovine ADRP was demonstrated by immunoblotting (Fig. 1B).

High Levels of Palmitic and Stearic Acid Induce a Reduction of Lipid Storage in Maturing Oocytes

Given the fact that maturing oocytes took up fatty acids from their environment and that they actively metabolized these fatty acids, we have investigated whether exposure to low, middle, and high fatty acid levels (100, 250, and 500 μM, respectively) affected oocytes' lipid storage and developmental capacity. Oocytes were matured in vitro, and lipid droplets were identified with the above-described specific neutral lipid stain while a DNA stain was used to determine the stage of oocyte maturation. In control matured oocytes, the number of lipid droplets was slightly but significantly increased in metaphase II-stage oocytes compared to the germinal vesicle stage, but the size of the lipid droplets remained unchanged (Fig. 2). Additional exposure to oleic acid (500 μM) resulted in a significantly increased number and size of lipid droplets in oocytes after maturation (Figs. 3 and 4, A and D). In contrast, the number and size of lipid droplets in oocytes that were exposed to either palmitic or stearic acid during maturation were significantly reduced or tended ($P = 0.059$ and $P = 0.056$)

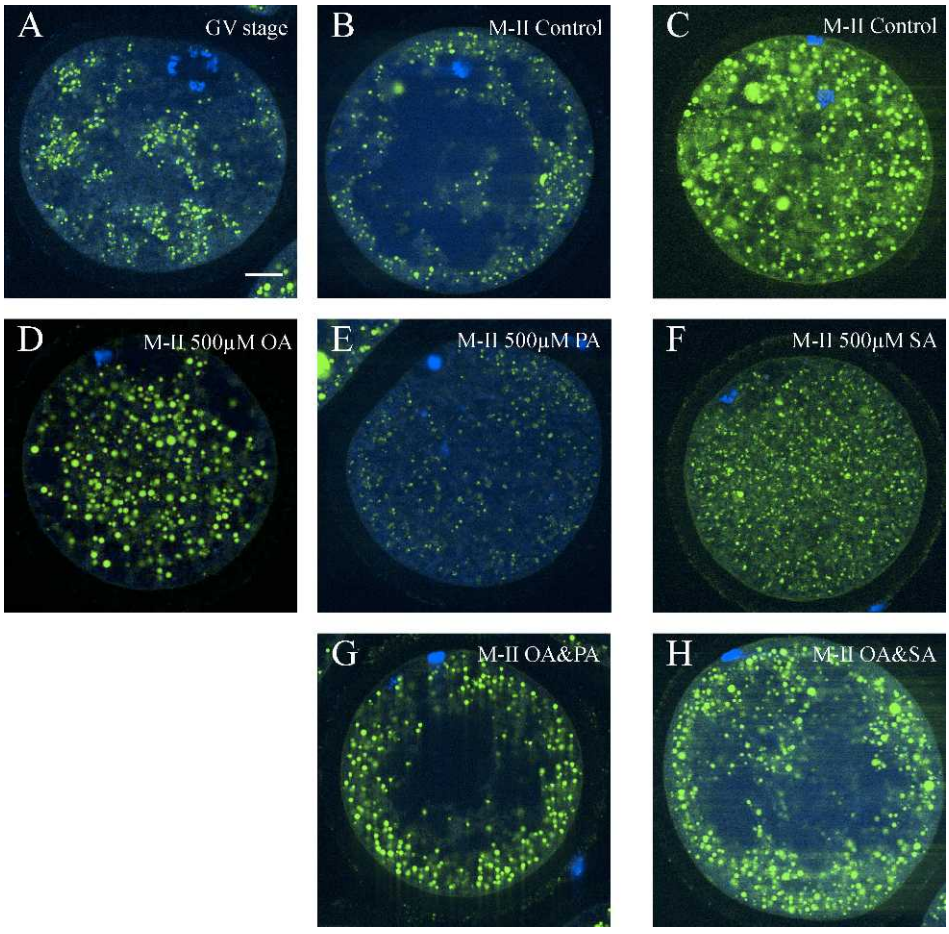

FIG. 3. Confocal images of immature oocytes at the germinal vesicle stage (**A**) and mature oocytes at the metaphase II stage after maturation in control medium during experiments of oleic and palmitic acid (**B**) and maturation in control medium during experiments with stearic acid (**C**). Mature oocytes at metaphase II stage after exposure to 500 μM oleic (**D**), 500 μM palmitic (**E**), 500 μM stearic acid (**F**), a combination of 250 μM palmitic and oleic acid (**G**), or a combination of 250 μM oleic and stearic acid during maturation (**H**). Merges show lipid droplets in green and DNA in blue. Bar = 20 μm. Note the reduced amount of BODIPY 493/503 labeling in **E** and the increased labeling in **D**. PA, palmitic acid; SA, stearic; OA, oleic acid; MII, metaphase-II oocytes.

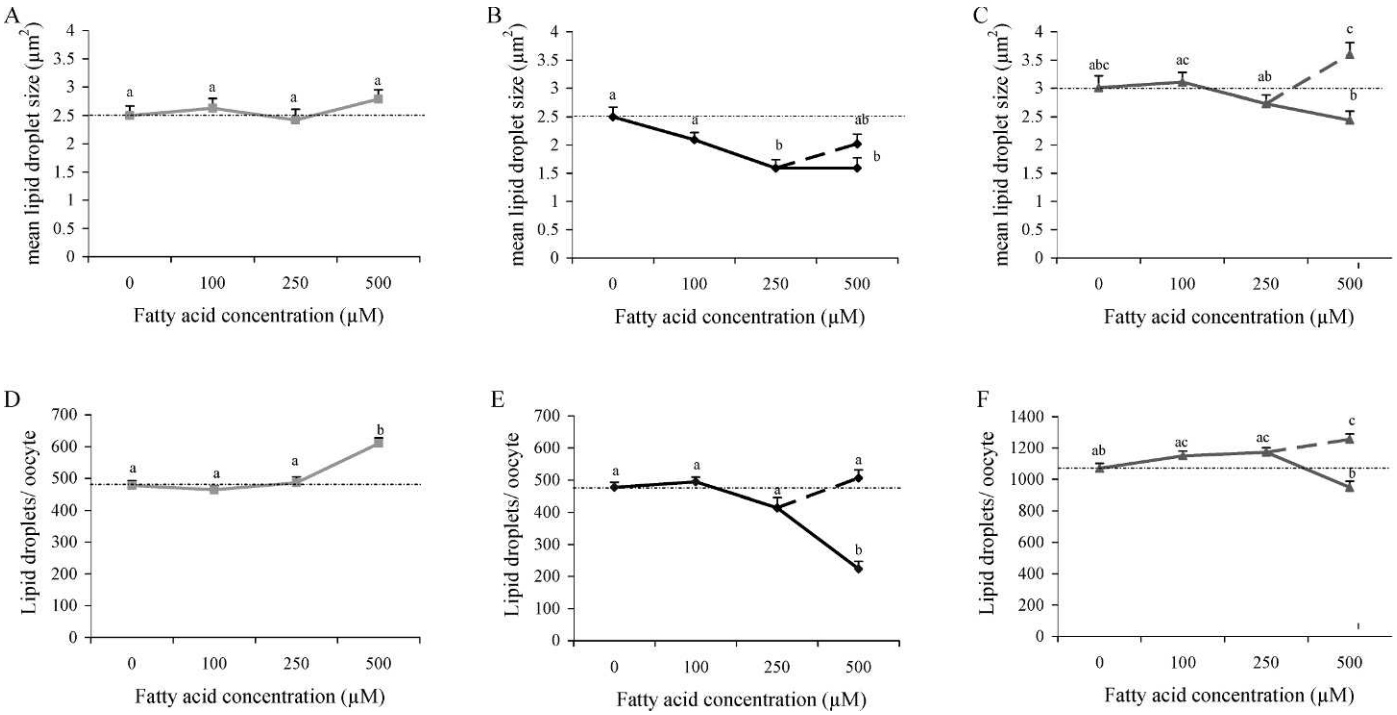

FIG. 4. The mean size (**A**–**C**) and number of lipid droplets (**D**–**F**) in oocytes after exposure to different concentrations of oleic acid (**A** and **D**), palmitic acid (**B** and **E**), a combination of palmitic and oleic acid (dashed line in **B** and **E**) and stearic acid (**C** and **F**), or a combination of stearic and oleic acid (dashed line in **C** and **F**) during maturation. Note the decrease in both number and size of lipid droplets after palmitic acid or stearic acid exposure ($P = 0.056$ and $P = 0.059$) and the compensation of this effect by combining palmitic or stearic acid with oleic acid. Unexpectedly, the number of lipid droplets in the batch of oocytes (including control oocytes) used for the stearic acid experiments was higher than the batch used for palmitic and oleic acid experiments (**F**). Although we cannot explain these differences, they can be attributed to a number of environmental influences (such as season). Results are presented as mean ± SEM. Data with different letters differ significantly ($P \leq 0.05$).

to reduce (Figs. 3 and 4, B, C, E, and F). Remarkably, oocytes matured in the presence of a combination of palmitic (250 μM) and oleic acid (250 μM) tended to have a higher number of lipid droplets ($P = 0.074$) compared to palmitic acid alone (250 μM; Fig. 4E). Oocytes matured in the presence of a combination of stearic and oleic acid (250 μM) had a significantly increased lipid droplet size compared to stearic acid alone (250 μM; Fig. 4C).

Palmitic and Stearic Acid Exposure Impairs Postfertilization Developmental Competence

Oocytes were exposed to the above-mentioned fatty acid conditions during maturation. After this exposure, the progress of oocyte nuclear maturation and the post-fertilization developmental competence was studied. In all conditions a similar percentage of oocytes reached the metaphase-II stage (approximately 80%), indicating that the fatty acid exposures did not affect oocyte nuclear maturation (Fig. 5, A–C). The post-fertilization development, however, was significantly and dose-dependently reduced after exposure to palmitic or stearic acid during oocyte maturation (Fig. 5, E, F, H, and I). In contrast to palmitic acid and stearic acid, oleic acid did not adversely affect the post-fertilization development and at highest dose even showed a moderate increase in the number of blastocysts at Day 8 of culture (Fig. 5, D and G). Remarkably, the negative effects of exposure to palmitic or stearic acid were completely counteracted by simultaneous exposure to equimolar levels of oleic acid during maturation (both at a concentration of 250 μM) (Fig. 5, E, F, H, and I).

DISCUSSION

The concentration of free fatty acids in blood and follicular fluid increases in cows during the NEB in the early postpartum period due to the mobilization of storage fat in adipose tissue [5–11]. The increased fatty acid levels may cause the observed decrease in fertility. It has been suggested that the changes in fatty acid content of follicular fluid will affect oocyte quality, possibly by influencing its lipid metabolism [9–11]. In line with this we here show that mammalian oocytes indeed efficiently incorporate and metabolize external fatty acids. It has been observed that embryos are capable of taking up fatty acids from the environment [37–39], but our data show that lipid uptake occurs already at an earlier stage. Unfertilized and even immature oocytes are capable of incorporating fatty acids in their neutral lipid and phospholipid fractions. Therefore, it is possible that the differential NEB-associated fatty acid exposure directly influences the oocyte rather than the reported indirect effects on granulosa and cumulus cells [8, 40].

The amount and size of lipid droplets in oocytes in our experiment were changed after exposure to fatty acids during maturation. This also suggests that fatty acids from the medium directly affect the oocyte. However, the effects on lipid droplets in oocytes largely depended on the type and concentration of the fatty acids to which the oocytes were exposed during maturation. Our study shows that the adverse effects of palmitic and stearic acid as well as the compensatory effects of oleic acid to both saturated fatty acids were comparable in both batches. Exposure to higher concentrations of palmitic or stearic acid during maturation resulted in smaller droplets and at 500 μM to a significantly reduced, or a tendency for a reduced, number of lipid droplets, whereas 500 μM oleic acid

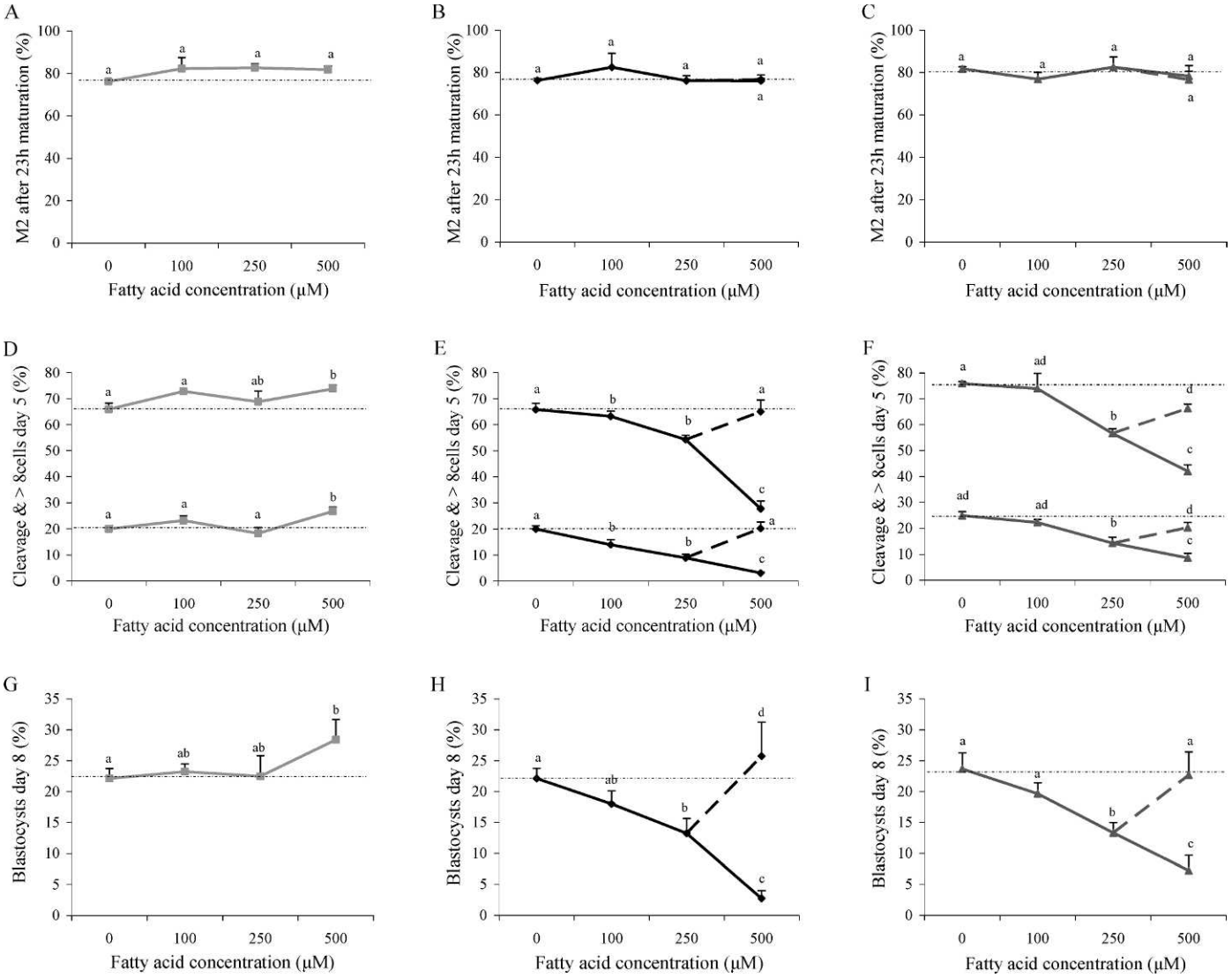

FIG. 5. Percentage of metaphase II stage oocytes (**A**–**C**), cleavage, and ≥8 cells at Day 5 of culture (**D**–**F**), and blastocysts at Day 8 of culture (**G**–**I**) from oocytes exposed to 100, 250, or 500 μM oleic acid (**A**, **D**, and **G**), palmitic acid (**B**, **E**, and **H**), 250 μM palmitic and oleic acid (dashed line, **B**, **E**, and **H**) and stearic acid (**C**, **F**, and **G**), or 250 μM stearic and oleic acid (dashed line in **C**, **F**, and **G**) during 23 h of maturation. The reduction of developmental competence of oocytes by palmitic or stearic acid exposure and compensation of this adverse effect by oleic acid coincides with the effects noted on lipid droplets (Fig. 4). Results are presented as mean ± SEM. Data with different letters differ significantly ($P \leq 0.05$).

resulted in more and larger lipid droplets. Interestingly, the reduction of stored neutral lipids after palmitic or stearic acid exposure was accompanied by a severely impaired postfertilization development of oocytes. Poor incorporation of saturated palmitic acid and efficient incorporation of mono-unsaturated oleic acid in TAG has been described for somatic cell types and may explain the observed difference in lipotoxicity between the saturated and unsaturated fatty acids [17, 22–24]. The ability to efficiently store esterified fatty acids in lipid droplets might deter a rise in lipotoxic effects that could be derived from fatty acids in the cell. This is in line with our observation that a high concentration of oleic acid resulted in an increased storage of neutral lipids and did not affect the developmental competence of exposed oocytes. Accordingly, lipid-rich oocytes were indeed shown to possess better developmental competence [18, 19].

The incorporation of fatty acid in triglycerides can be a method to store energy for the preimplantation development [37, 41–43]. The use of lipids from endogenous reserves during early embryonic development has been indicated by a) a decrease in triglyceride, b) the necessity of β oxidation of fatty acids by mitochondria during development, and c) the potential to develop in the complete absence of exogenous nutrients [13, 15, 16, 37–39, 41, 44–46].

Interestingly, the negative effect of palmitic and stearic acid on oocyte developmental competence was completely counteracted by oleic acid and, moreover, this combination of fatty acids tended to increase the number of lipid droplets and the size of lipid droplets in comparison to only palmitic or stearic acid. The observed increase in neutral lipids was not due to an increase of the fatty acid concentration, since 500 μM of palmitic or stearic acid alone did not increase neutral lipid storage. In addition to the possibility that oleic acid uptake by the maturing oocyte on its own restored neutral lipid storage, it is also possible that coexposure to the fatty acids caused an oleic acid-dependent metabolic channeling of palmitic and stearic acid into the lipid droplets. The incorporation of palmitic acid in lipid droplets in the presence of oleic acid has

been shown in Chinese hamster ovary cells and could explain the improved development of the oocytes, since palmitic acid is channeled away from palmitic acid-induced cell-dependent apoptotic pathways [17, 22, 24, 25, 40, 47]. Mono-unsaturated fatty acids have been reported to prevent palmitate-induced apoptosis by the induction of Bcl-2 and the prevention of mitochondrial release of cytochrome c, or by the competition between fatty acids for transport into the cell or cell metabolism [17, 22–24, 47]. Further studies should clarify the precise mode of action by which palmitic and stearic acid mediate their deleterious effect during maturation of oocytes and the mechanism by which oleic acid prevents the postfertilization lipotoxicity.

In conclusion, we have shown that exposure to external fatty acids modulates lipid storage in maturing oocytes. Palmitic and stearic acid caused a decrease in lipid storage and reduced postfertilization developmental competence. Oleic acid had no adverse effect at high dosage but caused a slight increase in lipid storage and postfertilization development. Oleic acid was also capable of compensating for the adverse effects of palmitic and stearic acid. In accordance, this implies that not only the concentration, but more importantly the ratio of saturated and unsaturated fatty acid in follicular fluid affects the developmental competence of the oocyte.

ACKNOWLEDGMENTS

The authors would like to acknowledge the technical assistance of Dr. M. Houweling, C.H.Y. Oei, and Dr. H.T.A van Tol during the experiments. We would like to thank A. de Graaff and Dr. R. Wubbolts at the Centre for Cellular Imaging for their technical assistance. The help of statistician J. van den Broek with the statistical analysis was greatly appreciated.

REFERENCES

1. Rukkwamsuk T, Kruip TA, Wensing T. Relationship between overfeeding and overconditioning in the dry period and the problems of high producing dairy cows during the postparturient period. Vet Q 1999; 21:71–77.
2. Walters AH, Bailey TL, Pearson RE, Gwazdauskas FC. Parity-related changes in bovine follicle and oocyte populations, oocyte quality, and hormones to 90 days postpartum. J Dairy Sci 2002; 85:824–832.
3. van Knegsel AT, van den Brand H, Dijkstra J, Tamminga S, Kemp B. Effect of dietary energy source on energy balance, production, metabolic disorders and reproduction in lactating dairy cattle. Reprod Nutr Dev 2005; 45:665–688.
4. Britt JH. Impacts of early postpartum metabolism on follicular development and fertility. Bov Proc 1992; 24:39–43.
5. Butler WR. Energy balance relationships with follicular development, ovulation and fertility in postpartum dairy cows. Livestock Prod Sci 2003; 83:211–218.
6. van den Top AM, Wensing T, Geelen MJ, Wentink GH, van't Klooster AT, Beynen AC. Time trends of plasma lipids and enzymes synthesizing hepatic triacylglycerol during postpartum development of fatty liver in dairy cows. J Dairy Sci 1995; 78:2208–2220.
7. Jorritsma R, de Groot MW, Vos PL, Kruip TA, Wensing T, Noordhuizen JP. Acute fasting in heifers as a model for assessing the relationship between plasma and follicular fluid NEFA concentrations. Theriogenology 2003; 60:151–161.
8. Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A. 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.
9. Roche JF. The effect of nutritional management of the dairy cow on reproductive efficiency. Anim Reprod Sci 2006; 96:282–296.
10. Fouladi-Nashta AA, Gutierrez CG, Gong JG, Garnsworthy PC, Webb R. Impact of dietary fatty acids on oocyte quality and development in lactating dairy cows. Biol Reprod 2007; 9–17.
11. Leroy JL, Van Soom A, Opsomer G, Goovaerts IG, Bols PE. 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.
12. Kruip TAM, Cran DG, Beneden TH, Dieleman SJ. Structural changes in bovine oocytes during final maturation in vivo. Gamete Res 1983; 8:29–47.
13. Ferguson EM, Leese HJ. Triglyceride content of bovine oocytes and early embryos. J Reprod Fertil 1999; 116:373–378.
14. McEvoy TG, Coull GD, Broadbent PJ, Hutchinson JS, Speake BK. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. J Reprod Fertil 2000; 118:163–170.
15. Kim JY, Kinoshita M, Ohnishi M, Fukui Y. Lipid and fatty acid analysis of fresh and frozen-thawed immature and in vitro matured bovine oocytes. Reproduction 2001; 122:131–138.
16. Sturmey RG, O'Toole PJ, Leese HJ. Fluorescence resonance energy transfer analysis of mitochondrial:lipid association in the porcine oocyte. Reproduction 2006; 132:829–837.
17. Listenberger LL, Han X, Lewis SE, Cases S, Farese RV Jr, Ory DS, Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci U S A 2003; 100:3077–3082.
18. Nagano M, Katagiri S, Takahashi Y. Relationship between bovine oocyte morphology and in vitro developmental potential. Zygote 2006; 14:53–61.
19. Jeong WJ, Cho SJ, Lee HS, Deb GK, Lee YS, Kwon TH, Kong IK. Effect of cytoplasmic lipid content on in vitro developmental efficiency of bovine IVP embryos. Theriogenology 2009; 72:584–589.
20. Homa ST, Racowsky C, McGaughey RW. Lipid analysis of immature pig oocytes. J Reprod Fertil 1986; 77:425–434.
21. Rukkwamsuk T, Geelen MJ, Kruip TA, Wensing T. Interrelation of fatty acid composition in adipose tissue, serum, and liver of dairy cows during the development of fatty liver postpartum. J Dairy Sci 2000; 83:52–59.
22. Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes 2001; 50:1771–1777.
23. Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY. Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. Diabetes 2001; 50:69–76.
24. Mishra R, Simonson MS. Saturated free fatty acids and apoptosis in microvascular mesangial cells: palmitate activates pro-apoptotic signaling involving caspase 9 and mitochondrial release of endonuclease G. Cardiovasc Diabetol 2005; 4:2.
25. Listenberger LL, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J Biol Chem 2001; 276:14890–14895.
26. Chen RF. Removal of fatty acids from serum albumin by charcoal treatment. J Biol Chem 1967; 242:173–181.
27. Hanson RW, Ballard FJ. Citrate, pyruvate, and lactate contaminants of commercial serum albumin. J Lipid Res 1968; 9:667–668.
28. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959; 37:911–917.
29. Gocze PM, Freeman DA. Factors underlying the variability of lipid droplet fluorescence in MA-10 Leydig tumor cells. Cytometry 1994; 17:151–158.
30. Testerink N, van der Sanden MH, Houweling M, Helms JB, Vaandrager AB. Depletion of phosphatidylcholine affects endoplasmic reticulum morphology and protein traffic at the Golgi complex. J Lipid Res 2009; 50:2182–2192.
31. Holzenspies JJ, Stoorvogel W, Colenbrander B, Roelen BA, Gutknecht DR, van Haeften T. CDC2/SPDY transiently associates with endoplasmic reticulum exit sites during oocyte maturation. BMC Dev Biol 2009; 9:8.
32. Grealy M, Diskin MG, Sreenan JM. Protein content of cattle oocytes and embryos from the two-cell to the elongated blastocyst stage at day 16. J Reprod Fertil 1996; 107:229–233.
33. Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988; 38:1171–1180.
34. Izadyar F, Zeinstra E, Colenbrander B, Vanderstichele HM, Bevers MM. In vitro maturation of bovine oocytes in the presence of bovine activin A does not affect the number of embryos. Anim Reprod Sci 1996; 45:37–45.
35. van Tol HT, van Eerdenburg FJ, Colenbrander B, Roelen BA. Enhancement of bovine oocyte maturation by leptin is accompanied by an upregulation in mRNA expression of leptin receptor isoforms in cumulus cells. Mol Reprod Dev 2008; 75:578–587.
36. Listenberger LL, Brown DA. Fluorescent detection of lipid droplets and associated proteins. Curr Protoc Cell Biol 2007; Chapter 24:Unit 24.2.
37. Hillman N, Flynn TJ. The metabolism of exogenous fatty acids by preimplantation mouse embryos developing in vitro. J Embryol Exp Morphol 1980; 56:157–168.
38. Khandoker MAMY, Tsujii H. Metabolism of exogenous fatty acids by preimplantation rabbit embryos. Jpn J Fertil Steril 1998; 43:195–201.
39. Khandoker MAMY, Tsujii H. The metabolism of exogenous fatty acid by preimplantation rat embryos. Asian-Aus J Anim Sci 1999; 12:1181–1187.
40. Mu YM, Yanase T, Nishi Y, Tanaka A, Saito M, Jin CH, Mukasa C, Okabe T, Nomura M, Goto K, Nawata H. Saturated FFAs, palmitic acid

and stearic acid induce apoptosis in human granulosa cells. Endocrinology 2001; 142:3590–3597.
41. Ferguson EM, Leese HJ. A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development. Mol Reprod Dev 2006; 73:1195–1201.
42. Sturmey RG, Hawkhead JA, Barker EA, Leese HJ. DNA damage and metabolic activity in the preimplantation embryo. Hum Reprod 2009; 24:81–91.
43. Sturmey RG, Reis A, Leese HJ, McEvoy TG. Role of fatty acids in energy provision during oocyte maturation and early embryo development. Reprod Domest Anim 2009; 44(suppl 3):50–58.
44. Kane MT. Minimal nutrient requirements for culture of one-cell rabbit embryos. Biol Reprod 1987; 37:775–778.
45. Hewitson LC, Martin KL, Leese HJ. Effects of metabolic inhibitors on mouse preimplantation embryo development and the energy metabolism of isolated inner cell masses. Mol Reprod Dev 1996; 43:323–330.
46. Sturmey RG, Leese HJ. Energy metabolism in pig oocytes and early embryos. Reproduction 2003; 126:197–204.
47. Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. Diabetes 2003; 52:726–733.