

Research Article

Stearoyl-CoA desaturase activity in bovine cumulus cells protects the oocyte against saturated fatty acid stress

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Abstract

Metabolic rich and poor conditions are both characterized by elevated free fatty acid levels and have been associated with impaired female fertility. In particular, saturated free fatty acids have a dose-dependent negative impact on oocyte developmental competence, while monounsaturated free fatty acids appear less harmful. Cumulus cells seem to protect the oocyte against free fatty acids, and the aim of this study was to determine the mechanism behind this protection. In particular, the role of the enzyme stearoyl-CoA desaturase (SCD) that converts saturated into monounsaturated fatty acids was investigated. SCD gene and protein were abundantly expressed in cumulus cells, but expression was low in oocytes. The level of SCD protein expression in cumulus cells did not change when COCs were exposed to saturated stearic acid during maturation. SCD inhibition in the presence of stearic acid significantly reduced the developmental competence of oocytes and increased the incidence of apoptosis in cumulus cells. The esterified oleic/stearic acid ratio of the neutral lipid fraction in cumulus cells decreased in the presence of SCD inhibitors when COCs were exposed to saturated free fatty acids during maturation, indicating the SCD-specific conversion of saturated fatty acids under noninhibiting conditions. The observation that cumulus cells can desaturate the potentially toxic stearic acid into oleic acid via SCD activity provides a mechanistic insight into how the cumulus cells protect the oocyte against toxicity by saturated fatty acid.

Summary Sentence

Stearoyl-CoA desaturase in bovine cumulus cells converts saturated into monounsaturated fatty acid and protects the oocyte against fatty acid-induced lipotoxicity.

Key words: cumulus, oocyte, fatty acid, embryo, stearoyl-CoA desaturase.

Introduction

Metabolic rich and poor conditions, like obesity and negative energy balance, are characterized by elevated levels of free fatty acids in blood due to increased mobilization of body fat reserves. Both conditions are associated with impaired female fertility, which may be

caused by a reduced quality of the cumulus-oocyte complex (COC). Follicular fluid reflects the metabolic condition of the female and mirrors the free fatty acid levels in blood [1–3]. To this end the adverse effects of elevated free fatty acid levels on the COC have gained a broad interest in the reproduction field.

The oocyte appears to be rather sensitive to high concentrations of free fatty acids. In particular, saturated palmitic and stearic acid, which are the dominating fatty acids in blood and follicular fluid, have a negative impact on oocyte developmental competence [1, 4–7]. In contrast, monounsaturated oleic acid, which is equally abundant in follicular fluid, is harmless even at high concentrations [5]. Within COCs, cumulus cells nourish the oocyte metabolically and are connected with the oocyte via gap junctional contact [5, 8–10]. Cumulus cells appear to regulate the uptake of fatty acids by the oocyte, as in the absence of cumulus cells the incorporation of free fatty acid by the oocyte is increased significantly during in vitro maturation [11]. Fatty acids are valuable nutrient sources that are efficiently stored in lipid droplets in cells and take part in the formation of cellular membranes. Indeed, lipid metabolism is essential for COCs and fatty acid breakdown is critical for proper oocyte maturation [12].

Despite the importance of lipids for cellular function, increased concentrations of saturated free fatty acids can damage organelles including the endoplasmic reticulum (ER) and mitochondria in COCs, as demonstrated by elevated levels of ER stress markers and reduced mitochondrial membrane potential [7, 13, 14]. Furthermore, saturated free fatty acids induce apoptosis in granulosa and cumulus cells and reduce the developmental competence of oocytes dose-dependently [1, 5, 11, 15]. These adverse effects of saturated free fatty acids on cumulus cells and the oocyte are in line with studies on various somatic cell types [16–21]. Interestingly, the negative impact of saturated free fatty acids on oocyte developmental competence can be counteracted by oleic acid [5]. In somatic cells, monounsaturated free fatty acids such as oleic (C18:1) and palmitoleic acid (C16:1) appear to stimulate distribution of fatty acids toward lipid droplets and β -oxidation in mitochondria, away from apoptotic pathways [17, 19, 20]. This indicates that the impact of elevated free fatty acid concentrations on the COC and the competence of the oocyte is dictated by the ratio of saturated versus monounsaturated fatty acid.

The follicular fluid of metabolically stressed cows contains relatively low levels of saturated stearic acid and high levels of monounsaturated oleic acid in comparison to blood [3]. Interestingly, when COCs were exposed to elevated levels of free fatty acids in follicular fluid, this resulted in an accumulation of these fatty acids in esterified form (neutral lipids) in lipid droplets of the cumulus cells, whereas the competence of the oocytes to develop to blastocysts after fertilization was not affected [3]. Cumulus cells might therefore protect the oocyte against fatty acid exposure by storing the fatty acids as neutral lipids, a process that is facilitated in the presence of monounsaturated fatty acid.

A potential mechanism to avoid lipotoxicity of stearic (C18:0) and palmitic acid (C16:0) is conversion into monounsaturated forms of oleic and palmitoleic acid. The enzyme stearoyl-CoA desaturase (SCD) catalyzes this conversion for both stearic and palmitic acid by the introduction of a cis-carbon-carbon atom double bond at the delta-9 position of the hydrogencarbon chain. SCD, also known as $\Delta 9$ desaturase, is a rate-limiting enzyme localized at the ER membrane. Depending on the species, different genes code for different types of SCD with different tissue distribution, but a similar desaturase function. The Muridae genomes harbor four genes coding for SCDs: *Scd1*, *Scd2*, *Scd3*, and *Scd4* [22, 23], while the human and bovine genomes only contain two SCD genes: *SCD1* and *SCD5* [24, 25].

The aim of the current study is to determine how cumulus cells protect the oocyte against free fatty acids.

Materials and methods

Chemicals

Unless stated otherwise, all chemicals used were obtained from Sigma Chemical Co (St. Louis, MO, USA) and were of the highest purity available. Solvents (acetone, acetonitrile, chloroform, methanol, and hexane) were of high-performance liquid chromatography (HPLC) grade (Labscan, Dublin, Ireland).

Collection and maturation of cumulus-oocyte complexes

Bovine ovaries were collected at a slaughterhouse and transported to the laboratory within 2 h of slaughter. Approval of an independent ethical committee was not needed as ovaries were a rest product of the regular slaughter process in the slaughterhouse. Antral follicles between 2 and 8 mm in diameter were aspirated by means of a suction pump under low vacuum. The follicular aspirates were pooled in a conical tube and allowed to settle for 15 min. Oocytes with a multilayered cumulus investment were selected from the follicular fluid, washed three times in HEPES-buffered M199 (Gibco BRL, Paisley, UK), and randomly allocated in groups of 35–70 COCs per well in four-well culture plates (Nunc A/S, Roskilde, Denmark). In vitro maturation (IVM) was carried out for 23 h according to our standard protocol [5] in maturation medium consisting of 500 μ l M199 per well, and no coverage of oil, supplemented with 26.2 mM NaHCO_3 , 0.02 IU/ml FSH (Sioux Biochemical Inc., Sioux Center IA, USA), 0.02 IU/ml LH (Sioux Biochemical Inc.), 7.7 μ g/ml cysteamine, 10 ng/ml epidermal growth factor, and 1% (v/v) penicillin-streptomycin (Gibco BRL) at 39°C in a humidified atmosphere of 5% CO_2 in air.

Fertilization and embryo culture

After 23 h of maturation, in vitro fertilization was performed with 0.5×10^6 /ml sperm from a bull with proven fertility. At 18–22 h after sperm addition, the cumulus cells and adhering sperm cells were removed by vortexing the presumed zygotes for 3 min in the experiment with SCD inhibition. Subsequently, the zygotes were transferred in groups of 35–70 to wells with 500 μ l pre-equilibrated synthetic oviductal fluid (SOF, [26]). Fertilization and embryo culture were performed, according to our standard procedure [5], in a humidified incubator at 38.5°C with 5% CO_2 and respectively 20% and 7% O_2 . At day 5 of culture, cleaved embryos were transferred to fresh SOF and cultured until day 8 on which embryonic development was assessed.

In vitro maturation with free fatty acids and stearoyl-CoA desaturase 1 inhibitor

The free fatty acids used in the maturation experiments were processed according to our standard protocol [5] and bound to 100% delipidified bovine serum albumin resulting custom-tailored lipidified BSA, and were stored in stock at a concentration of 10 mM bound to 10% (w/v) fatty acid-free BSA (fatty acid:BSA stoichiometry of 5:1).

For the experiments assessing the importance of cumulus cells in protecting oocytes against free fatty acids, the cumulus cells of

Table 1. Details of primers used for PCR.

Code	Gene	Primer sequence (5' ==> 3')	Direction	Position	Annealing temp (°C)	GenBank accession no.
SCD1	Stearoyl-CoA desaturase 1	TCCTGATCATTGGCAACACCA TGGGCAGCACTATTCACCAG	S as	554-574 931-950	60	NM.173959.4
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	AGGCCATCACCATCTTCCAG GGCGTGGACAGTGGTCATAA	S as	179-198 485-504	61	AJ000039
Actin	Actin	TTACAACGAGCTGCGTGTGG TGGCAGGAGTGTGAACGTC	s as	42-61 147-166	59	AH001130.2
PGK	Phosphoglycerokinase	CTGGACAAGCTGGATGTGAA AACAGCAGCCTTGATCCTCT	s as	72-91 160-179	61	BT021601

oocytes were removed after a maturation period of 8 h by vortexing COCs for 3 min in HEPES-buffered M199 [27]. After removal of the cumulus cells, the denuded oocytes were placed back in their original wells containing standard maturation medium without or with 250 μ M stearic acid. As a control, groups of intact COCs were matured in maturation medium without or with 250 μ M stearic acid. For the experiment where cumulus cells were removed at 8 h, cumulus cells from the control group with maturation as intact COCs were removed before fertilization by vortexing for 3 min, which is a modification from our standard procedure. Oocytes were fertilized and cultured according to our standard protocol (see above). In total 440–469 COCs were used per experimental group in four independent experimental runs.

For the experiments assessing the function of SCD1 in cumulus cells, COCs were matured with or without free fatty acids in the presence or absence of 1 μ M SCD1 inhibitor (#1716, BioVision, Milpitas CA, USA; CAY10566, Cayman Europe, Tallinn, Estonia; [28]). A dose–response relationship with 0, 0.5, 1.0, 2.0, and 5.0 μ M of the SCD1 inhibitor was established based on previous experiments [28], and a concentration of 1 μ M was used in further experiments. Two independent SCD1 inhibitors were used. The different concentrations of fatty acid BSA were as follows: 100 μ M stearic and 150 μ M palmitic acid and 250 μ M stearic acid or 250 μ M palmitic acid in experiments wherein the fatty acids were tested individually. After maturation, COCs were fertilized and cultured according to our standard protocol (see above). The total numbers of COCs used were 430 and 275 respectively in four and three experimental runs. At day 5 of culture the total number of cleaved zygotes and at day 8 the number of blastocysts was scored, as a parameter for oocyte developmental competence. The calculation of the cleavage and blastocyst rates was based on the total number of oocytes.

Collection of cumulus cells for quantitative real-time PCR and lipid analysis

The SCD1 gene and protein expression was assessed in cumulus cells from COCs collected before maturation and after 23 h of maturation without free fatty acids or with either 100 μ M stearic acid and 150 μ M palmitic acid or a combination of stearic, palmitic, and 200 μ M oleic acid. The levels of administered fatty acids complexed to BSA were based on the in vivo fasting heifer concentrations in follicular fluid [3]. For lipid analysis, COCs were matured for 23 h in control medium or in the presence of 250 μ M stearic acid complexed to BSA with and without SCD inhibitor (1 μ M; BioVision). Per group the cumulus cells of 10 (lipid analysis) or 50 COCs (quantitative real-time PCR) were separated from oocytes using a narrow-bore Pasteur pipette. The remaining oocytes with adhering remnants of cumulus investment were vortexed for 3 min in HEPES-buffered M199. After collection of the denuded oocytes, the supernatant was centrifuged

(5 min, 4000 g) and the pellet was combined with the cumulus cells that were collected by use of the narrow-bore pipette. After centrifugation (5 min, 4000 g), the pellet was washed in 1 ml PBS, where after the supernatant was removed and the pellet containing the cumulus cells was stored in 300 μ l RLT buffer at -80° C until RNA extraction and lipid analysis. Four replicates per experimental group were used for qRT-PCR analysis; three to four replicates per experimental group were used for lipid analysis.

Quantitative real-time PCR

Total RNA isolation was performed using the RNeasy micro kit (Qiagen, Valencia CA, USA) according to the manufacturer's instructions; RNA was eluted with 18 μ l RNase-free water. Reverse transcription was performed in a total volume of 20 μ l containing 10 μ l RNA sample, 4 μ l 5x reverse transcriptase buffer (Gibco BRL), 0.036 units/ μ l random primers (Thermo Fisher Scientific, Waltham MA, USA), 8 units/ μ l RNA-sin (Promega Benelux, Leiden, The Netherlands) and 150 units Superscript III reverse transcriptase (Thermo Fisher Scientific), and final concentrations 10 mM dithiothreitol (DTT) and 0.5 mM of each deoxynucleotide (dNTP; Promega). The mixtures were incubated for 45 min at 50° C, for 10 min at 80° C, and stored at -20° C. The primers used for analysis of the relative mRNA expression of SCD1 and of the reference genes GAPDH, PGK, and ACTIN are summarized in Table 1.

Each primer of a pair was located on a separate exon. Quantitative real-time PCR (qPCR) was performed in duplo on a 96-well using a real-time PCR detection system (CFX Connect Real-Time PCR Detection System; Bio-Rad Laboratories, Hercules, CA, USA). Standard curves were fourfold serial dilutions of pooled cDNA from all samples. The reaction mixture (20 μ l) contained 0.5 μ l cDNA, 0.5 μ M of each primer (Eurogentec, Seraing, Belgium), and 10 μ l of iQ SYBR green supermix (Bio-Rad Laboratories). After an initial denaturation step at 95° C for 3 min, 40 cycles were carried out each consisting of 95° C for 15 s, the primer-specific annealing temperature (Table 1) for 15 s and 72° C for 20 s. Melting curves were plotted to determine the purity of the product; in addition, all PCR products were sequenced. Standard curves were produced by plotting the log of the starting amount versus the threshold cycle. The standard curves were subsequently used to calculate the relative starting quantity for each experimental sample by interpolation. Data normalization was performed using the geometric mean of the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerokinase (PGK), and Actin.

Western blotting

Cells were lysed in RIPA buffer (Thermo Fisher Scientific), containing 1% halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) at 4° C for 30 min. Lysates contained 50 denuded oocytes

Table 2. Details of antibodies used for western blotting.

Antibody	Protein	Type antibody	Host species	Target species protein	Company name	Dilution
SCD1	Stearoyl-CoA-desaturase 1	Custom	Rabbit	Bovine	Pacific Immunology	1:1000
β -Actin	β -Actin	Polyclonal	Goat	Human	Santa Cruz Biotechnology	1:1000
IgG	Immunoglobulin G	Polyclonal	Goat	Rabbit	Pierce Biotechnology	1:10000
IgG	Immunoglobulin G	Polyclonal	Rabbit	Goat	Santa Cruz Biotechnology	1:10000

or cumulus cells corresponding to 50 COCs per 10 μ l. Lysates were diluted 3:1 with 4x Laemmli sample buffer (Bio-Rad Laboratories), boiled for 5 min, and stored at -20°C until use.

Samples and precision plus protein standards (Bio-Rad Laboratories) were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Trans-blot, Bio-Rad Laboratories). The membranes were rinsed in TBS-Tween [(TBS with 0.05% Tween-20 (ICN, Aurora OH, USA)] and blocked with 5% nonfat dry milk in TBS-Tween (blocking buffer) for 1 h and probed with primary SCD1 antibody (custom rabbit anti-bovine SCD1, Pacific Immunology, Ramona CA, USA; a kind gift of Dr. Benjamin Corl, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA) or with a polyclonal antibody against β -actin (sc-1616, Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:1000 in blocking buffer at 4°C overnight. Membranes were then washed in washing buffer and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (31460; Pierce Biotechnology, Rockford IL, USA) or with HRP-conjugated rabbit anti-goat IgG (SC-2768; Santa Cruz Biotechnology) diluted 1:10000 in blocking buffer. Membranes were then washed again, and proteins were detected using SuperSignal[®] West Dura Extended Duration Substrate kit and exposure to X-ray film (both from Thermo Fisher Scientific). The antibodies used for western blotting are summarized in Table 2.

Extraction of lipids and saponification of total fatty acids

The total lipid fraction from cumulus cells originating from 10 COCs was extracted as described before [3], with small modifications. The total lipid fraction including neutral and phospholipids was extracted according to Bligh and Dyer [29]. Fifty microliters of aqueous phase (0.3 M KOH solution in methanol) was added to the dried lipid extract and heated for 1 h at 75°C in 200 μ l screw-capped glass vials. Deuterated palmitic acid [7,7,8,8- $^2\text{H}_4$] (Cambridge Isotopes Laboratories Inc., Cambridge, MA, USA) was added as an internal standard to the samples (10 nmol/sample) at the beginning of the lipid extraction procedure to allow quantification. Isolation of the total fatty acid fraction was essentially performed as described by Kates [30]. Nonsaponifiable lipids were extracted using three portions of 150 μ l hexane (subsequent upper phases were removed). The resulting lower phase was acidified with 50 μ l 6 N HCl (in distilled water) and the free fatty acids were extracted using three portions of 150 μ l hexane. The hexane from these three combined upper phases was evaporated under a constant stream of nitrogen at 40°C and stored in an atmosphere of 100% nitrogen at -20°C until analysis.

Analysis of fatty acids by high-performance liquid chromatography–mass spectrometry

Fatty acids were dissolved in 100 μ l methanol:acetonitrile:chloroform:water (46:20:17:17, v/v/v/v), and 40 μ l was injected onto

a Halo C18 (150 \times 3.0 mm; particle size of 2.7 μm) HPLC column (Advanced Material Technology Inc., Wilmington, DE, USA) at 40°C . Lipids were eluted using a linear gradient, from acetonitrile:methanol:water (6:9:5, v/v/v) 2.5 mM ammonium acetate to acetone:methanol (4:6, v/v) 2.5 mM ammonium acetate for 15 min, followed by isocratic elution with the latter solvent for 10 min and regeneration of the column for 5 min, all at a flow rate of 0.6 ml/min. Mass spectrometry of free fatty acids was performed using electrospray ionization on a 2000 qTRAP system (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). Source temperature was set to 450°C , and nitrogen was used as curtain gas. The declustering potential was set to -40 V. Full scans were performed in negative mode in the mass:charge (m/z) range from 225 to 400 amu. Peaks were identified by comparison of retention time and mass spectra with authentic standards, and calibration curves were generated to correct for differences in response factors.

Necrosis and apoptotic cell death detection by enzyme-linked immunosorbent assay

Necrosis and apoptosis were detected using a cell death detection enzyme-linked immunosorbent assay (ELISA)^{PLUS} kit (Roche, Basel, Switzerland). Groups of 15 COCs were cultured in 100 μ l maturation medium either with or without 250 μM stearic acid in the presence or absence of 1 μM SCD inhibitor. After 20 h of culture, the COCs were washed two times in PBS containing 0.05% w/v PVA and lysed, after which the histone-associated DNA fragments were quantified in the lysate (apoptosis) and in the culture medium (necrosis) according to the manufacturer's instructions. A DNA–histone complex provided with the kit was used to test the functionality of the assay. The ELISAs were conducted twice for each group with four replicates per group.

Lipid droplet staining of cumulus-oocyte complexes after in vitro maturation

The neutral lipid present in lipid droplets of COCs was stained with BODIPY493/503 (Molecular Probes, Eugene, OR, USA), and DNA was stained with To-Pro-3 (Molecular Probes) according to our standard protocol [5]. Confocal laser scanning microscopy was performed by using a model TCS SPE-II setup (Leica Microsystems GmbH, Wetzlar, Germany) attached to an inverted semi-automated DMI4000 microscope (Leica) at $\times 20$ magnification (0.6 NA). BODIPY 493/503 and TO-PRO-3 were sequentially excited by an Ar laser (488 nm) and HeNe diode laser (635 nm). Prism filter sliders were set for emission 501–547 nm (BODIPY) and 644–689 nm (TO-PRO-3). Image reconstruction was done with LAS.AF software (Leica). Fluorescence in the cumulus cell layer was quantified after digital removal of the oocyte region of the images using ImageJ (<https://imagej.nih.gov/ij/>). Imaris 8.2.0 software (Bitplane AG, Zurich, Switzerland) was applied to determine the number of nuclei

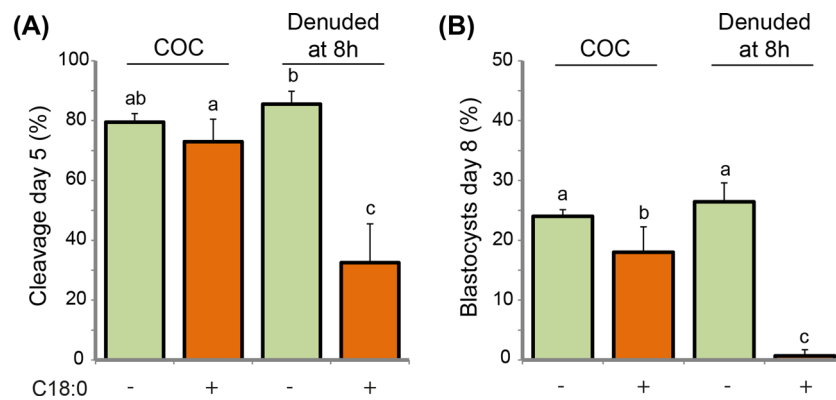


Figure 1. Graphs representing cleavage rate (A) and blastocyst development (B), at respectively day 5 and day 8 of culture, from oocytes matured in control medium (green bars) or exposed to 250 μ M stearic acid (C18:0; orange bars) with cumulus cells (as COC) or without cumulus cells from 8 h of maturation. Error bars represent standard deviation. Results are presented as mean \pm SD; data with different letters differ significantly ($P < 0.05$).

and to measure the number and sizes of the lipid droplets in each cumulus cell layer. Objects were isolated using the spot creation wizard. Initial object size for isolation was set to 0.5 μ m for lipid droplets and 4 μ m for nuclei. Settings were adjusted in representative images by visual inspection to reliably detect the objects. These parameters were used for object isolation of the entire dataset. In total, 243 COCs, on average 40 COCs per group, were individually analyzed in two experimental runs.

Statistical analysis

Statistical analysis was performed in SPSS version 24.0 (SPSS Inc., Chicago, IL, USA) with condition and experimental run as fixed factors. Analysis of oocyte developmental competence (cleavage and number of blastocysts) was performed with logistic regression for grouped data. Analysis of the gene expression data and the comparison of the levels of apoptosis and necrosis in cumulus cells in the different experimental groups was performed by one-way ANOVA with Bonferroni correction. The univariate analysis of variance was used to analyze the data concerning the lipid analysis. Except for the culture data, all statistics were performed on \ln (ln) transformed data to achieve a normal distribution. All measures are reported as means \pm SD. A P value of <0.05 was considered statistically significant.

Results

Cumulus cells protect the oocyte against saturated stearic acid

To determine the importance of cumulus cells in protecting oocytes against fatty acids, oocytes were matured as COCs during the entire maturation period in the presence of stearic acid for 23 h, or cumulus cells were removed from oocytes at 8 h maturation followed by a 15 h maturation period in the absence of cumulus cells. After maturation, the oocytes, which were all denuded at the end of maturation, were fertilized and embryos were cultured for 8 days in standard culture medium. Oocytes with (COCs) and without cumulus cells that had matured in control medium exhibited comparable developmental competence, shown by cleavage ($79.5 \pm 2.9\%$; and $85.5 \pm 4.3\%$) and blastocyst rates ($24.0 \pm 1.1\%$ and $26.4 \pm 3.1\%$) (Figure 1). Furthermore, oocytes from COCs exposed to stearic acid had similar cleavage and blastocyst rates ($73.0 \pm 7.5\%$ and $18.0 \pm 4.2\%$) as oocytes from the group of COCs exposed to the

control medium (Figure 1). In contrast, in the absence of cumulus cells the developmental competence of oocytes exposed to stearic acid was decreased shown by significantly lower cleavage and blastocyst rate of ($32.5 \pm 13.0\%$; $P < 0.001$ and $0.7 \pm 1.0\%$; $P < 0.001$) compared to COCs exposed to stearic acid during maturation. These data indicate that cumulus cells are a prerequisite for protection of the oocyte against saturated free fatty acids.

Stearoyl-CoA desaturase expression in cumulus cells

A potential route to avoid saturated free fatty acid stress by cumulus cells is the conversion of stearic acid into harmless monounsaturated oleic acid by SCD. Therefore, the expression of *SCD1* mRNA and protein was examined by qRT-PCR and immunoblotting, respectively. In cumulus cells, *SCD1* mRNA was detected and its expression was significantly upregulated during maturation, which was independent of the presence of free fatty acids (Figure 2A). *SCD1* protein was also detected in cumulus cells, but no increase in expression during maturation was observed. However, in comparison to the control condition the expression level did not change when COCs had been matured in the presence of stearic acid (Figure 2B). Expression of *SCD1* in oocytes was hardly detectable, and no difference was observed after maturation with or without stearic acid (Figure 2B). These data indicate that SCD is of minor importance in the oocyte, but is present in cumulus cells.

Stearoyl-CoA desaturase activity in cumulus cells protects the oocyte against saturated free fatty acid

COCs were matured in the presence of specific SCD inhibitors to investigate the function of SCD1 in cumulus cells. Initially, two distinct SCD1 inhibitors were used. After maturation, COCs were fertilized and the potential zygotes were cultured to the blastocyst stage in standard medium; in Figure 3, the cleavage and blastocyst rates of the oocytes after maturation in the different conditions are presented. The oocytes from COCs exposed to saturated fatty acids without SCD1 inhibition demonstrated blastocyst rates comparable with oocytes from the control medium ($33.3 \pm 6.8\%$) (Figure 3). When COCs were exposed to stearic acid while SCD1 activity was inhibited, a significant and for both SCD1 inhibitors comparable reduction was observed in the developmental competence of the oocytes to develop into a blastocyst ($9.5 \pm 4.7\%$ and $11.4 \pm 3.6\%$, for Biovision and Cayman, respectively) in relation to the condition with stearic acid when SCD1 was active ($25.0 \pm 7.5\%$; $P < 0.01$)

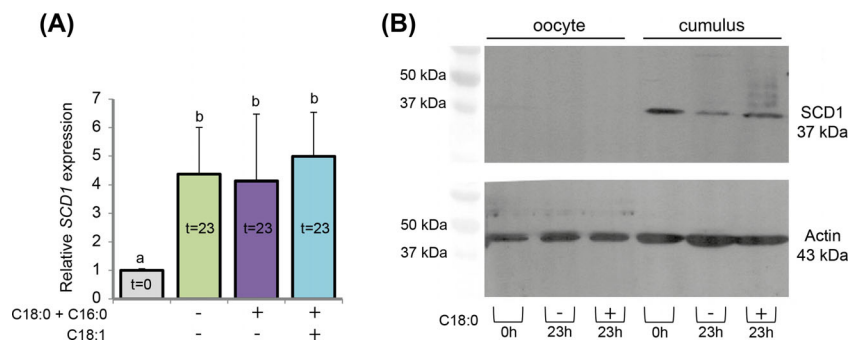


Figure 2. *SCD1* gene and protein expression in cumulus cells and oocytes. (A) The increase of *SCD1* mRNA expression in cumulus cells of COCs at 23 h of maturation in comparison to 0 h of maturation, maturation in control medium (green bar), and after exposure to stearic and palmitic acid (100 and 150 μ M; purple bar), and stearic, palmitic, and oleic acid (100, 150 and 200 μ M; blue bar). Results are presented as mean \pm SD; data with different letters differ significantly ($P \leq 0.01$). (B) Representative immunoblot showing expression of SCD1 and actin in oocyte and cumulus cells. SCD1 protein expression in oocytes and cumulus cells is shown at 0 h and after 23 h of maturation in control medium and in the presence of 250 μ M stearic acid.

(Figure 3B). In the presence of palmitic acid, the reduction of the blastocyst rate when SCD activity was inhibited ($17.9 \pm 10.4\%$ and $14.0 \pm 6.3\%$, for Biovision and Cayman, respectively), was not significant compared to the palmitic acid condition when SCD was active ($22.1 \pm 1.1\%$) (Figure 3D). Since both SCD1 inhibitors resulted in comparable results, follow-up experiments were performed with only one of the SCD1 inhibitors. When oocytes were exposed to a combination of stearic and palmitic acid, inhibition of SCD1 resulted in a significant reduction in the blastocyst rate ($12.5 \pm 7.2\%$ versus $29.0 \pm 8.7\%$; $P < 0.001$) (Figure 3F). SCD1 inhibition in control medium resulted in comparable cleavage and blastocyst rates after in vitro fertilization (Figure 3). Combined, these data indicate that SCD activity in cumulus cells protects the oocyte against saturated free fatty acid stress.

Inhibition of stearoyl-CoA desaturase in presence of stearic acid results in apoptosis in cumulus cells

Since saturated free fatty acids can induce apoptotic cascades [16–20], we examined the occurrence of apoptosis and necrosis in cumulus cells of COCs. After maturation in control medium with or without a SCD1 inhibitor, few cumulus cells of COCs were apoptotic or necrotic (Figure 4). However, SCD1 inhibition in the presence of stearic acid resulted in a significant induction of apoptosis in cumulus cells of matured COCs (Figure 4). There was no significant difference in the level of necrosis among the different groups (Figure 4). Combined, these data show that SCD1 in cumulus cells protects against lipotoxicity in COCs.

Stearoyl-CoA desaturase in cumulus cells converts saturated into monounsaturated fatty acids

Next, we investigated the levels of stearic and oleic acid in the lipid of cumulus cells by using mass spectrometry. After culture in standard maturation medium, the concentration of oleic acid in cumulus cells was approximately threefold that of stearic acid, and was not changed after SCD1 inhibition (Figure 5). Of note is that SCD1 inhibition did not affect the levels of palmitic acid and its desaturated form, palmitoleic acid (ratio was approximately 900:1 in all conditions measured, independent of the presence or absence of SCD1 inhibitors; data not shown). Exposure to stearic acid decreased the ratio of oleic/stearic acid, in comparison to the control condition ($P = 0.001$), indicating the uptake of stearic acid. Under the same

condition but with SCD1 inhibition, the ratio of oleic/stearic acid further decreased ($P = 0.002$) (Figure 5). These data indicate active conversion of stearic acid into monounsaturated oleic acid by SCD1 in cumulus cells when COCs are exposed to stearic acid.

Stearoyl-CoA desaturase 1 activity is related with increased lipid storage in cumulus cells

An important difference between stearic and palmitic acid and their monounsaturated counterparts is that unsaturated fatty acids are more easily distributed toward lipid droplets, away from apoptotic pathways [17, 19]. The effect of SCD1 inhibition on lipid storage was determined in cumulus cells from COCs exposed to free fatty acids in order to examine whether cumulus cells use this process to prevent lipotoxicity. In COCs matured in control medium, large numbers of lipid droplets were already present, as had been observed previously [5]. Cumulus cells of COCs exposed to oleic acid contained most and the largest lipid droplets (Figure 6). Inhibition of SCD1 in the control or oleic acid group did not significantly affect the number and size of lipid droplets (Figure 6). When COCs were exposed to stearic acid, an increase in the amount of lipid droplets was observed while in the simultaneous presence of a SCD1 inhibitor the number of lipid droplets was drastically reduced ($P < 0.001$) (Figure 6). These data suggest that conversion of stearic into oleic acid by SCD1 in cumulus cells promotes the distribution of fatty acid toward lipid droplets.

Discussion

This study shows that SCD activity in cumulus cells effectively protects the oocyte against lipid-induced damage by conversion of saturated into monounsaturated fatty acids.

The cumulus cell layer that surrounds the oocyte appears to be of fundamental importance to protect the oocyte against saturated free fatty acids residing in the environment of the COC. It has already been established that cumulus cells nurture the oocyte and are essential for proper maturation of the oocyte [31]. Here we establish that cumulus cells are able to desaturate the potentially toxic stearic acid into oleic acid via SCD activity and, by doing so, effectively protect the oocyte against lipotoxicity.

Both *SCD1* and *SCD5* (data not shown) were detected in bovine cumulus cells, and the *SCD1* mRNA expression significantly increased during maturation. Human cumulus cells also express *SCD1* and *SCD5* [32]. The human and bovine genomes code for two SCD

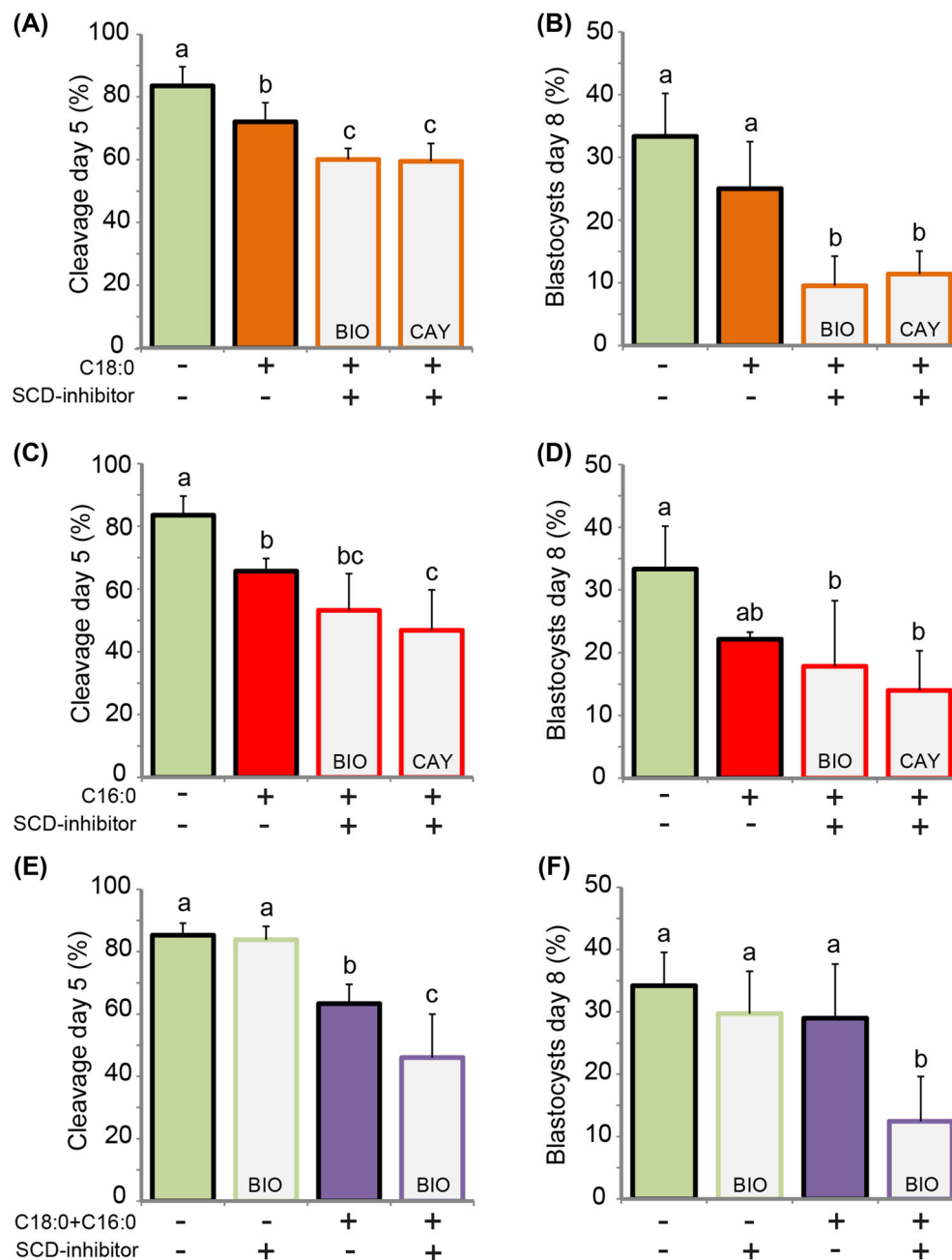


Figure 3. SCD activity in cumulus cells prevents negative impact of saturated free fatty acid on oocyte developmental competence. Percentages of cleavage (A, C, E) and blastocyst formation (B, D, F) of oocytes from COCs matured in control medium (green bars) and in the presence of (A, B) saturated stearic acid (250 μ M; orange bar), (C, D) palmitic acid (250 μ M; red bar), or (E and F) saturated stearic and palmitic acid (100 and 150 μ M; purple bar) and with SCD inhibition (white bars with outline in color of experimental condition). The SCD1 inhibitors were from Biovision (BIO) or Cayman (CAY). Results are presented as mean \pm SD; data with different letters differ significantly ($P < 0.05$).

genes, *SCD1* and *SCD5*, while the Muridae genomes harbor four SCD homologs *Scd1–4* [23, 25, 33]. Human *SCD1* is highly homologous to *Scd1* of Muridae and is ubiquitously expressed, while human *SCD5* is predominantly expressed in brain and pancreas [22, 24, 25, 32, 34]. In this study, we focused on SCD1 due to the abundant expression of this SCD type in cumulus cells. Two different SCD1 inhibitors were used and gave similar results, indicating that the results were not due to off-target events.

The SCD expression in oocytes appeared to be at a low level which is in line with previous findings in the rat ovary [35]. There was a significant expression of SCD (both at the transcriptional and translational level) in bovine cumulus cells, similar to the rat ovary where *Scd1* and *Scd2* are abundantly expressed in granulosa and cumulus cells [35], suggesting that cumulus has the capacity to desaturate fatty acids. Moreover, the low expression in the oocyte indicates that the oocyte itself has a very limited capacity to

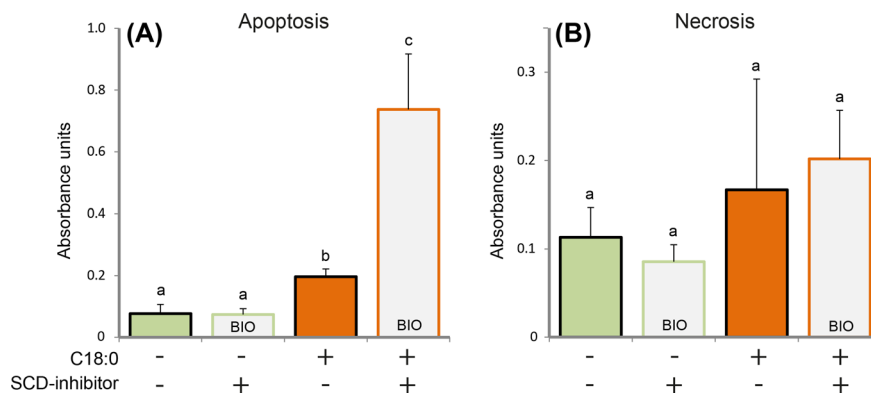


Figure 4. Apoptosis and necrosis rates in COCs after exposure to stearic acid with and without SCD1 activity. SCD1 activity was inhibited (white bars with outline in the color of experimental condition) in the control group (green bars) and in the presence of saturated stearic acid (250 μ M; orange bar). In panel A, the apoptosis levels in cumulus cells from the different conditions are presented; panel B presents the necrosis levels. Results are presented as mean \pm SD. Data with different letters differ significantly ($P < 0.05$). BIO = SCD1 inhibitor from Biovision.

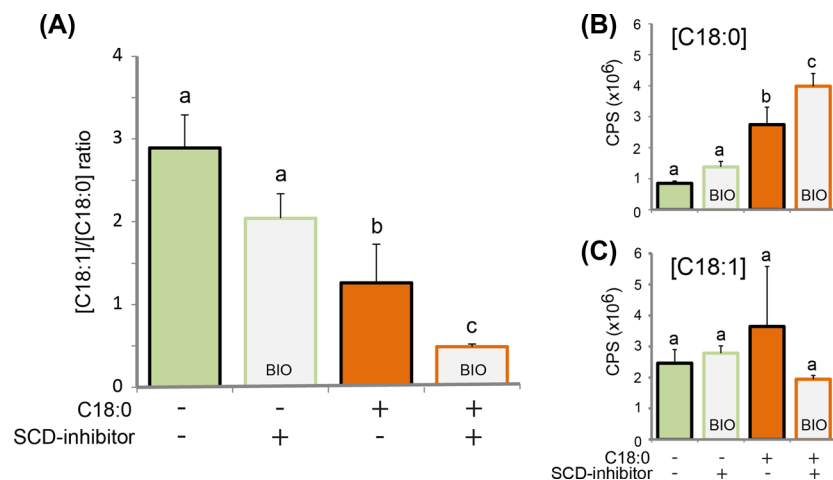


Figure 5. The molar ratio of C18:1/C18:0 based on the levels in matured cumulus cells. SCD1 activity was inhibited (white bars with outline in the color of experimental condition) in the control group (green bars) and in the presence of saturated stearic acid (250 μ M; orange bars) during maturation. Panel A presents the C18:1/C18:0 ratio based on the levels of C18:0 presented in panel B and C18:1 in panel C in the lipid of cumulus cells from matured COCs. Results are presented as mean \pm SD. Data with different letters differ significantly ($P < 0.05$). BIO = SCD1 inhibitor from Biovision. CPS = ion counts per second.

desaturate saturated fatty acids, although we cannot exclude a function of SCD1 in oocytes. The presence of stearic acid in the maturation medium resulted in a slightly higher expression of SCD1 protein in cumulus cells in comparison to the control condition. This result has also been found in other cell types in which SCD expression could be stimulated by several metabolites, including saturated and monounsaturated free fatty acids [24, 36]. Interestingly, the observed higher oleic acid to stearic acid ratio in follicular fluid when compared to the levels in blood [1, 3, 37] may be explained by follicular SCD activity in granulosa and cumulus cells.

The current data show that cumulus cells and SCD activity are crucial in protecting the oocyte against saturated fatty acids. Oocytes matured in the absence of cumulus cells and oocytes from COCs matured in the presence of SCD inhibitors demonstrated strongly reduced developmental competence when exposed to stearic acid. Saturated fatty acids can result in lipotoxicity by the induction of ceramide formation, mitochondrial release of cytochrome-c, and caspase activation [16–20]. Indeed, stearic and palmitic acid have a

dose-dependent negative impact on oocyte developmental competence, due to COC damage by the induction of elevated ceramide formation, ER stress, and apoptotic events in cumulus cells and reduced mitochondrial membrane potential and increased formation of ROS in oocytes [1, 5, 7, 11, 13]. This response of COCs to exogenous saturated fatty acids is highly comparable with responses observed in COCs exposed to metabolic stress in obese mice, which suggests involvement of free fatty acids [38].

Compared to saturated fatty acids, there is a mild cellular response to monounsaturated fatty acids that has been contributed to their distinct intracellular routing in the cell. Monounsaturated fatty acids are more rapidly routed towards lipid droplets for storage as neutral lipids after esterification or to mitochondria for fatty acid breakdown [17, 19, 20]. Previously, we demonstrated that exogenous oleic acid could counteract the negative impact of saturated fatty acids on oocyte developmental competence [5]. The neutralizing impact of oleic acid on saturated fatty acids has also been found in other cell types and the effect is reported to rely on the

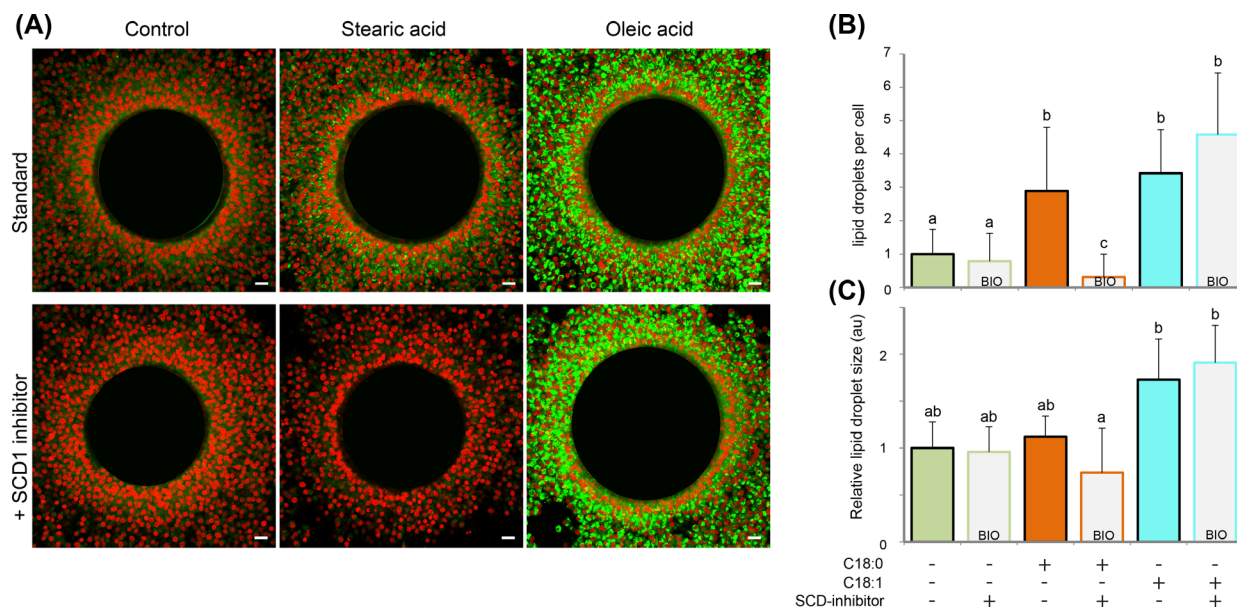


Figure 6. Lipid droplet formation in cumulus cells after exposure to stearic and oleic acid without and with SCD1 inhibition. Panel A shows the confocal images of lipid droplets (green) and cumulus cell nuclei (red) for the different maturation groups; control, stearic, and oleic acid without and with a SCD1 inhibitor; (B) Graph representing the number of lipid droplets in cumulus cells after maturation in control medium (green bar), and in the presence of stearic acid (orange bar) or oleic acid (blue bar) during 23 h of maturation without and with inhibition of SCD1 activity (white bars with outline in the color of experimental condition); (C) Graph representing the mean size of lipid droplets, data are normalized on the basis of the control group without SCD1 inhibition. The average diameter of lipid droplets in the control group was $0.99 \pm 0.14 \mu\text{m}$. Results are presented as mean \pm SD. Data with different letters differ significantly ($P < 0.05$). BIO = SCD1 inhibitor from Biovision. Scale bar represents $20 \mu\text{m}$.

stimulation of lipid storage and β -oxidation and thus the prevention of fatty acids to induce apoptosis [17, 19, 20]. SCD inhibition in the presence of stearic acid resulted in a decreased desaturation index of C18:1/C18:0 and a concomitant increased level of apoptosis in the cumulus cells in comparison to the stearic acid group where SCD was not inhibited. Interestingly, hepatic cells of *Scd1*^{-/-} mice showed reduced apoptosis when monounsaturated fatty acids were present in the culture medium [39] which further demonstrates the importance of SCD activity in maintaining proper saturated and monounsaturated fatty acid ratios in cells.

Cumulus cells also seem to regulate and prevent fatty acid transfer to the oocyte. A first indication that cumulus cells may effectively protect the oocyte against high levels of fatty acid was the observation that cumulus showed abundant lipid storage after exposure to elevated free fatty acid levels in follicular fluid during metabolic stress. The oocytes did not show this response and remained functionally competent, while removal of the cumulus caused dramatic lipid accumulation [3, 11]. In addition, cumulus cells can significantly reduce the lipid transfer of exogenous fatty acids toward the oocyte [11]. In the current study, we demonstrated that denuded oocytes in the presence of stearic acid lose functional competence. Here we also observed increased lipid storage in cumulus cells after exposure to stearic acid in contrast to the group with inhibited SCD function. SCD activity in the presence of stearic acid led to increased C18:1/C18:0 ratios in the cumulus cells and together with the higher amount of lipid droplets, this indicates that the formation of oleic acid in cumulus cells facilitates the storage of neutral lipids in the cumulus cells. Interestingly, in a study with human adipocytes the cells that were intolerant to saturated palmitic acid demonstrated lower *SCD1* mRNA expression and lower lipid accumulation than adipocytes tolerant to palmitic acid [36]. Furthermore, also in cows

there appears to be a broad variation in phenotypic SCD activity, at least in the mammary glands, due to genetic origin [40]. To this end, the impact of elevated free fatty acid concentrations on an individual may strongly depend on the potential to desaturate saturated fatty acids. Apparently, SCD activity in cumulus cells and the consequent conversion of saturated fatty acid results in increased safe storage of fatty acids in lipid droplets in cumulus cells.

Due to SCD's central role in lipid storage, SCD1 inhibition has been considered as a potential therapy against obesity and other metabolic disorders, such as diabetes [39, 41]. Indeed, mice genetically deficient for *Scd1* are resistant to diet-induced weight gain and hepatic steatosis and demonstrated increased insulin sensitivity [42, 43]. However, the importance of SCD activity in cumulus cells in maintaining the developmental competence of oocytes certainly deserves attention when SCD inhibition therapy is considered.

In conclusion, SCD activity in cumulus cells appears to be a prerequisite for a functional and protective barrier of cumulus cells to prevent a negative impact of saturated fatty acids on the developmental competence of the oocyte. The endogenous oleic acid formed after the desaturation of the potentially toxic stearic acid allows the maintenance of a healthy ratio of saturated and monounsaturated fatty acids and stimulates storage of fatty acids as neutral lipids in lipid droplets of cumulus cells. The presence and need for SCD activity in cumulus cells enrolls an important new function in addition to the nurturing role that the cumulus cells fulfill for the oocyte.

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