A novel approach to quantifying ovarian cell lipid content and lipid accumulation in vitro by confocal microscopy in lean women undergoing ovarian stimulation for in vitro fertilization (IVF)

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Abstract

Purpose To quantify intracellular lipid levels in cumulus cells (CCs) and mural granulosa cells (MGCs) of lean women undergoing gonadotropin therapy for in vitro fertilization (IVF), based upon different cell preparation methods.

Methods CCs and MGCs from 16 lean women undergoing ovarian stimulation for IVF were studied. Cells were pooled by cell type, with each type of cell separated into two groups for determination of initial lipid content (Method 1) and subsequent lipid accumulation in vitro (Method 2). Cells for initial lipid content were immediately fixed at the time of the oocyte retrieval with 4 % paraformaldehyde in suspension, while

those for subsequent lipid accumulation in vitro were cultured for 4 h with 5 % fetal calf serum and then fixed. Cells were treated with lipid fluorescent dye BODIPY® FL C₁₆ and nuclear marker DAPI. Intracellular lipid was quantified by confocal microscopy, using ImageJ software analysis.

Results There was no significant effect of cell type ($P=0.2$) or cell type-cell preparation method interaction ($P=0.8$) on cell area (Method 1: CC 99.7 ± 5.1 , MGC 132.8 ± 5.8 ; Method 2: CC 221.9 ± 30.4 , MGC $265.1\pm 48.5\ \mu\text{m}^2$). The mean area of all cells combined was significantly less for cells prepared by Method 1 ($116.2\pm 4.9\ \mu\text{m}^2$) vs. Method 2 ($243.5\pm 22.5\ \mu\text{m}^2$, $P<0.00005$). Intracellular lipid level, however, was significantly altered by cell preparation method ($P<0.05$; cell preparation method-cell type interaction, $P<0.00001$). Initial lipid content was significantly lower in CC (74.5 ± 9.3) than MGC (136.3 ± 16.7 fluorescence/cell area, $P<0.00005$), while subsequent lipid accumulation in vitro was significantly higher in CC (154.0 ± 9.1) than MGC (104.6 ± 9.9 fluorescence/cell area, $P<0.00001$). The relatively diminished initial CC lipid content compared to subsequent CC lipid accumulation in vitro ($P<0.00001$), and the opposite pattern for MGC ($P<0.05$), significantly lowered the CC/MGC lipid ratio in Method 1 (0.55 ± 0.04) vs. Method 2 (1.58 ± 0.10 , $P<0.00001$).

Conclusions Differential uptake or utilization of lipid by CC and MGC occurs during oocyte maturation and steroidogenesis, respectively, with the amount of lipid present in ovarian cells a function of both the follicular microenvironment at the time of the oocyte retrieval and the capacity of these cells to accumulate lipid in vitro over time.

Capsule Ovarian cell lipid content and lipid accumulation in vitro can be quantified by confocal microscopy in lean women undergoing ovarian stimulation for in vitro fertilization (IVF).

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Introduction

Carbohydrate and lipid metabolism are inextricably linked during normal ovarian folliculogenesis, with the combination of glucose utilization and fatty acid oxidation by ovarian cells providing energy substrates for oocyte development and steroidogenesis [5, 22, 23, 32]. As part of this process, fatty acids are normally stored as lipid droplets in ovarian cells [41] and used as an energy source during oocyte maturation [5]. As an example, carnitine palmitoyl transferase I (CPT1B), the rate limiting enzyme in fatty acid oxidation, increases in murine cumulus oocyte complexes (COCs) during gonadotropin therapy followed by human chorionic gonadotropin (hCG) administration to normally enhance mitochondrial free fatty acid oxidation as a fuel source for oocyte meiotic resumption and early embryogenesis [9, 29].

Therefore, fatty acid storage in ovarian cells of women undergoing in vitro fertilization (IVF) likely represents uptake and utilization of lipid intermediates as energy for these cells, which in turn might affect acquisition of human oocyte developmental competence, defined as the ability of the oocyte to complete meiosis and undergo fertilization, embryogenesis and term development [8, 29]. Furthermore, amounts of lipid in cumulus and mural granulosa cells may differ between cell types to tightly regulate utilization of lipid intermediates essential for oocyte meiotic resumption and steroidogenesis, respectively.

Beyond these physiological implications, obesity [1] adversely affects IVF-related pregnancy outcome by increasing amounts of gonadotropins administered [3, 4, 10, 39], decreasing numbers of oocytes retrieved [10, 39, 40], and impairing oocyte quality as well as pregnancy outcomes in some [15, 27], but not all [3, 4, 18, 28, 36] studies. These findings have led to the concept that increased adiposity can promote excess lipid accumulation in nonadipose tissues (lipotoxicity) [38], which in ovarian cells could induce oxidative stress, mitochondrial dysfunction and cellular apoptosis, thereby impairing oocyte developmental competence [41, 43].

The present study describes the initial lipid content and lipid accumulation in vitro of cumulus and mural granulosa cells from lean women undergoing gonadotropin therapy for IVF, using two cell preparation methods. Initial lipid content (Method 1) and lipid accumulation in vitro (Method 2) were measured by confocal microscopy in ovarian cells following immediate removal from follicles at the time of oocyte retrieval or after short-term (4-h) exposure to cell culture conditions, respectively. Lean IVF patients were specifically studied to eliminate the confounding adverse effects of obesity on ovarian cell lipid content and oocyte developmental competence.

Our study demonstrates that initial lipid content and lipid accumulation in vitro of ovarian cells from lean IVF patients vary by cell type and cell preparation method, and establish normative data whereby future studies can explore the adverse effects of altered ovarian cell lipid metabolism on oocyte developmental competence in obese women undergoing IVF.

Materials and methods

Subjects

Following approval by the UCLA Institutional Review Board, 16 lean women undergoing gonadotropin therapy for IVF were recruited. These women were undergoing IVF for primary diagnoses of endometriosis ($N=1$), advanced maternal age ($N=5$), unexplained infertility ($N=3$), exclusive male factor infertility ($N=3$), hypogonadotropic hypogonadism ($N=1$) and oocyte donation ($N=3$). All women signed informed consent before study participation. All study participants were between the ages of 25 and 44 years and had normal serum prolactin levels and thyroid function studies. No woman had galactorrhea, endometriomas, or ovarian cysts greater than 18 mm in diameter.

Gonadotropin stimulation for IVF and oocyte retrieval

Women received either a gonadotropin-releasing hormone (GnRH) antagonist (Ganirelix, Merck & Co. Inc., WhiteHouse Station, NJ) [30], luteal phase leuprolide acetate (Lupron, TAP Pharmaceuticals, Deerfield, IL,) [7] or microdose leuprolide acetate [31] ovarian stimulation protocol. Recombinant human or urinary gonadotropins were initiated at a starting dose of 225–450 IU sc daily for the first 3 days and then increased or decreased thereafter as clinically indicated. Serial estradiol (E_2) levels and two-dimensional transvaginal ultrasound (TVUS) follicle measurements were performed until at least two dominant follicles reached ≥ 17 mm in diameter and serum E_2 levels reached approximately 300 pg/mL/dominant follicle. Human chorionic gonadotropin (10,000 IU, intramuscularly), choriogonadotropin alfa (500 ug sc, Ovidrel, EMD Serono, Inc., Rockland, MA) or leuprolide acetate (4 mg sc every 12 h for 2 doses) was then administered followed by transvaginal oocyte retrieval 35.5 h later.

Patient and IVF cycle characteristics

Sixteen lean IVF patients (mean body mass index (BMI), 21.8 ± 0.6 [SEM] kg/m^2 ; ages 25–44 years) with basal serum follicle stimulating hormone (FSH) and E_2 levels of ≤ 19 mIU/mL and ≤ 68 pg/mL, respectively, received either a GnRH antagonist ($N=9$), microdose leuprolide acetate ($N=5$), or luteal leuprolide acetate ($N=2$) protocol. Total

amounts of gonadotropins used were 1,350–4,500 IU, with peak serum E2 levels between 1,030–7,478 pg/mL. For oocyte maturation, patients received human chorionic gonadotropin ($N=6$), choriogonadotropin alfa ($N=7$), or leuprolide acetate ($N=3$). The total number of oocytes retrieved per patient ranged from 5 to 30.

Preparation of cumulus and mural granulosa cells

Cumulus and mural granulosa cells from pooled follicular fluid were collected during oocyte retrieval and transferred separately to culture dishes. Cumulus cells were mechanically isolated from COCs, while sheets of mural granulosa cells were removed from the pooled, aspirated follicular fluid of each patient, as previously described [11, 44]. Cells were washed several times in 5 mL of MOPS (4-morpholinepropanesulfonic acid) buffered medium (G-MOPS™, VitroLife, Englewood, CO) containing 10 % serum substitute supplement (Irvine Scientific, Santa Ana, CA). Cells were then resuspended in 100 μ L of recombinant human hyaluronidase (40–120 U/ml) (ICSI Cumulase®, Malov, Denmark) and pipetted up and down for 1 min before being placed in the MOPS buffered medium of their respective dishes. Pooled cumulus and mural granulosa cells were transferred in separate conical tubes and centrifuged at 1,600 rpm for 5 min at 24 °C. Within the next 1–2 h, cumulus and mural granulosa cells were transported on ice to the research laboratory where cells were resuspended in phosphate buffered saline (PBS) and centrifuged for 5 min at 800 rpm at 20 °C.

Cumulus and mural granulosa cells were pooled by cell type, with each type of cell sample separated into two groups for measurement of initial lipid content (Method 1) and lipid accumulation in vitro (Method 2). For initial lipid content, isolated cumulus and mural granulosa cells were immediately fixed at the time of oocyte retrieval with 4 % paraformaldehyde in suspension for 20 min. Initial lipid content of cumulus and mural granulosa cells was determined using the fluorescence probe BODIPY® FL C₁₆ (0.8 μ g/mL) (1 h in the dark at room temperature) (Invitrogen, Grand Island, NY), including 5 min incubation with the nuclei staining 4',6-diamidino-2-phenylindole (DAPI) (0.5 μ g/mL) (Invitrogen, Grand Island, NY). Cells were resuspended in 30 μ L of PBS and 3 μ L of polyvinylpyrrolidone (PVP) (Irvine Scientific, Santa Ana, CA) before being placed on glass slides for imaging.

For lipid accumulation in vitro, isolated cumulus and mural granulosa cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Inc., Manassas, VA) and DMEM/Ham's F12 (1:1) (Mediatech, Inc., Manassas, VA) respectively; supplemented with 5 % fetal calf serum (Hyclone, ThermoScientific, South Logan, UT) and

a antibiotic-antimycotic solution of penicillin (100 IU/mL), streptomycin (100 μ g/mL), and amphotericin (0.25 μ g/mL) (Mediatech, Inc. Manassas, VA); and plated on 8 well culture slides (BD Falcon). Cells were then incubated for 4 h at 37 °C with 5 % CO₂ (i.e., the least amount of time required for attachment of cells to slides) and then fixed in 4 % paraformaldehyde for 10 min and kept at 4 °C. After staining as described above, cell culture slides were disassembled and mounted with rectangular coverslips in 10 μ L of PBS per well of fixed cells.

Confocal microscopy and analysis

For both methods, images were captured using the Leica TCS-SP2-AOBS confocal microscope with x63 oil objective under different gain settings. The 488-line argon laser was used to capture the BODIPY® FL C₁₆ lipid stain, and the diode 405 nm laser was used to capture DAPI nuclear stain. Image acquisition was performed using Leica Confocal Software (LCS) version 2.61 Build 1,537. Fluorescent images of cumulus and mural granulosa cell lipid incorporation were quantified using ImageJ (<http://rsbweb.nih.gov/ij/>) to determine mean fluorescence (fluorescence/unit area) and cell area (μ m²) (5–20 cumulus and mural granulosa cells per patient).

Images taken as single channel images were subsequently converted to overlay images and all images were saved in TIFF format. Single channel images of BODIPY® FL C₁₆ and DAPI were used to create an overlay image, with single channel BODIPY® FL C₁₆ used for lipid quantification in ImageJ. Each image was calibrated for scale before performing cell area and fluorescence measurements. Background staining was accounted for by using five negatively stained regions per cell, which were subtracted from the total mean fluorescence. To compare mean fluorescence differences between the two cell preparation methods, fluorescent images of 5 different cumulus and mural granulosa cell samples from Method 1 also were measured using gain settings for Method 2. A conversion factor (1.67) was determined and used to correct for differences in brightness due to different gain settings between the two cell preparation methods.

Statistical analysis

Cell fluorescence and area were compared by two-way analysis of variance (ANOVA) using cell type and cell preparation method as factors to determine the independent effects of these variables and their possible interaction. When statistically significant interactions were present by ANOVA, *post hoc* univariate analysis was performed on the variables. The amount of lipid in cumulus cells relative to mural granulosa cells (i.e.,

Fig. 1 Overlap images of BODIPY® FL C₁₆ and DAPI. Method 1: Initial lipid content of **a** cumulus cells and **b** mural granulosa cells; Method 2: Lipid accumulation in vitro of **c** cumulus cells and **d** mural granulosa cells. Overlap and single channel images were taken with a confocal x63 oil objective, and quantification was performed on single channel BODIPY® FL C₁₆ images

the cumulus to mural granulosa cell lipid ratio) was compared between cell preparation methods by paired *T*-test. Linear regression was used to correlate ovarian

cell lipid and cell area with patient BMI and age as well as amount of gonadotropin administered. Values are represented as means $\pm$ standard errors.

Table 1 Patient data regarding lipid content and lipid accumulation in vitro: cumulus cell (CC) and mural granulosa cell (MGC) characteristics

Patient	BMI (kg/m ²)	Age (years)	Lipid content				Lipid accumulation in vitro			
			CC Area (μm ²)	MGC Area (μm ²)	CC Lipid (fluorescence/cell area)	MGC Lipid (fluorescence/cell area)	CC Area (μm ²)	MGC Area (μm ²)	CC Lipid (fluorescence/cell area)	MGC Lipid (fluorescence/cell area)
1	20	43	123	175	25.1	81.8	326	400	98	54
2	21	25	103	140	38.7	64.5	519	324	114	54
3	24	37	81	123	23.4	65.1	245	155	144	62
4	23	38	114	170	25.1	40.1	159	365	150	82
5	25	32	153	144	85.8	122	115	292	141	67
6	21	33	81	130	41.2	81.3	424	123	147	120
7	24	27	85	96	54.8	28	71	330	125	108
8	19	40	119	133	79.5	102	119	242	185	105
9	18	30	96	97	127	191	146	201	129	82
10	20	43	97	128	106	300	315	64	151	120
11	23	36	72	125	52.6	117	148	93	150	133
12	25	44	96	135	117	207	221	122	239	207
13	24	41	110	128	101	177	297	393	195	149
14	23	41	103	172	84.7	167	115	632	210	105
15	18	40	71	118	109	186	212	362	132	120
16	23	31	91	110	121	152	119	144	154	107

Results

Ovarian cell lipid and area characteristics

Lipid appeared as a homogenous pattern of fluorescence within the cytoplasm of both cell types, regardless of cell preparation method (Fig. 1a, b, c and d). The amount of ovarian cell lipid, however, was significantly affected by the method of cell preparation (cell preparation method effect, $P<0.05$; cell preparation method-cell type interaction, $P<0.00001$). In Method 1, initial lipid content was significantly lower in cumulus (74.5±9.3) than mural granulosa cells (136.3±16.7 fluorescence/cell area, $P<0.00005$) (Table 1, Fig. 2a). In a reciprocal manner, lipid accumulation in vitro by Method 2 was significantly higher in cumulus

(154.0±9.1) than mural granulosa cells (104.6±9.9 fluorescence/cell area, $P<0.00001$). For cumulus cells, therefore, initial lipid content by Method 1 was lower than lipid accumulation in vitro by Method 2 ($P<0.00001$), while the opposite pattern was true for mural granulosa cells ($P<0.05$). Consequently, the cumulus to mural granulosa cell lipid ratio was lower in Method 1 (0.55±0.04) compared to Method 2 (1.58±0.10, $P<0.00001$).

There were no significant effects of cell type ($P=0.2$) or interactions between cell type and cell preparation method ($P=0.8$) on cell area (Method 1: cumulus cells 99.7±5.1, mural granulosa cells 132.8±5.8; Method 2: cumulus cells 221.9±30.4, mural granulosa cells 265.1±48.5 μm^2). Therefore data for cell area were pooled by cell type for analysis of cell preparation method. The mean cell area of all cells combined was significantly less for cells prepared by Method 1 (116.2±4.9 μm^2) compared to Method 2 (243.5±22.5 μm^2 , $P<0.00005$) (Table 1, Fig. 2b).

Ovarian cell area, initial lipid content and lipid accumulation in vitro did not correlate with patient BMI or amount of gonadotropin administered, given the ranges of BMI and doses of gonadotropin administered to these lean IVF patients. Neither initial lipid content nor lipid accumulation in vitro of either ovarian cell type significantly correlated with patient age.

Discussion

Folliculogenesis is a complex process, whereby multiple endocrine and intraovarian paracrine interactions create a changing intrafollicular microenvironment for appropriate oocyte development. Within this microenvironment, droplets of stored lipid within ovarian cells provide lipid intermediates that are important for cumulus cell-oocyte signaling and steroidogenesis during oocyte development [42]. During follicle differentiation, for example, CPT1B gene expression, the rate-limiting enzyme in mitochondrial fatty acid oxidation, increases with oocyte maturation in gonadotropin-primed murine COCs [9]. A parallel increase in gene expression of sterol regulatory element-binding protein (SREBP)-1c, a key transcriptional regulator of lipid biosynthesis in human luteinized granulosa cells, also occurs [23]. Our novel use of confocal microscopy to quantify initial lipid content (Method 1) and lipid accumulation in vitro (Method 2) in cumulus and mural granulosa cells from lean IVF patients agrees with the proposed role of lipid metabolism in COC signaling and steroidogenesis during normal human oocyte development. We hypothesize that the initial lipid content determined in freshly isolated ovarian cells represents the in vivo interactions of the follicular microenvironment at the time of the oocyte retrieval (static process), while lipid accumulation in vitro indicates the

Fig. 2 **a** Mean ovarian cell fluorescence and **b** ovarian cell area based upon cell preparation method. The amount of ovarian cell lipid was significantly altered by cell type and cell preparation method. Initial lipid content by Method 1 was significantly lower in cumulus than mural granulosa cells; lipid accumulation in vitro by Method 2 was significantly higher in cumulus than mural granulosa cells (cell type effect). For cumulus cells, initial lipid content by Method 1 was lower than lipid accumulation in vitro by Method 2, while the opposite pattern was true for mural granulosa cells (cell preparation method effect). Mean cell area of cumulus and mural granulosa cells combined was significantly less for all cells prepared by Method 1 (initial lipid content) compared to Method 2 (lipid accumulation in vitro). *, $P<0.00005$; **, $P<0.00001$ cumulus vs. mural granulosa cell; †, $P<0.05$; ††, $P<0.00005$; †††, $P<0.00001$ Method 1 vs. Method 2

capacity of these cells to accumulate lipids over time (dynamic process).

In the present study, the relative amount of lipid in cumulus cells varied by the method of cell preparation. In cumulus cells, initial lipid content was about 50 % lower than lipid accumulation *in vitro*, perhaps because of differential uptake or utilization of lipid intermediates, based upon proximity of cumulus cells to the oocyte at the time of lipid determination. In this regard, paracrine interactions within the COC are essential for the unique function of each cell type [13, 14, 34], with cumulus cells providing energy substrate for oocyte utilization through up-regulation of cholesterol synthetic pathways [33]. Therefore, low initial lipid content relative to lipid accumulation *in vitro* might occur if cumulus cells recently exposed to oocytes exhibited increased lipid utilization, while similar cumulus cells exposed to lipid-rich fetal calf serum demonstrated enhanced lipid uptake, as previously shown in cultured bovine oocytes [17]. Conversely, greater initial lipid content than lipid accumulation *in vitro* in mural granulosa cells also might occur if this type of cell was recently exposed to lipid intermediates in follicular fluid and showed enhanced lipid uptake [43].

Our study further demonstrates that measurement of ovarian cell area also is influenced by method of cell preparation. As expected, ovarian cells used to measure initial lipid content (Method 1), which were fixed immediately at the time of oocyte retrieval in paraformaldehyde suspension, maintained a spherical shape. These same cells, when cultured *in vitro* for 4 h to determine lipid accumulation (Method 2), were able to adhere to culture slides and flatten into a broader shape.

Further emphasizing how ovarian cell characteristics vary by patient characteristics, imaging techniques and cell preparation methods, the amount of ovarian cell lipid in our study was not statistically correlated with patient BMI, age or amount of gonadotropin administered, within the ranges examined in our lean IVF patients. Our new findings differ from a previous transmission electron microscopy study, in which age in IVF patients of unspecified BMI inversely correlated with lipid droplets in mural granulosa cells cultured *in vitro* for 24 h, rather than 4 h (as in our study) under different culture conditions [35]. The mechanisms responsible for these differences in ovarian cell lipid levels, based upon patient characteristics and study design, remain to be determined.

Our study of lean women undergoing IVF sets the stage to further address the adverse effects of increased adiposity in IVF patients on oocyte quality [34], as mediated through oxidative stress [37]. Elevated triglyceride and insulin levels in follicles of obese IVF patients accompany granulosa cell abnormalities of gene expression involving cholesterol trafficking [26] and disordered steroidogenesis [6, 23].

Furthermore, elevated intrafollicular fatty acid levels in women, adjusting for BMI, predict poor COC morphology, decreased embryo development and reduced embryo implantation [16, 26]. In support of this, human lipid-rich follicular fluid disrupts murine COC functioning *in vitro*, causing endoplasmic reticulum stress and impaired oocyte maturation [43], consistent with COCs of obese mice displaying increased lipid, endoplasmic reticulum stress, apoptosis and decreased oocyte quality [41]. As shown in animal models, therefore, ovarian cell lipotoxicity likely alters ovarian cell lipid content and harms the oocyte through increased endoplasmic reticulum stress linked with insulin resistance and inflammation [12, 21, 27, 43]. This phenomenon may explain the reduced IVF pregnancy outcome with increased adiposity reported in some, but not all studies [2, 4, 19, 20, 24, 25, 28, 36, 40].

In conclusion, our novel approach to quantifying ovarian cell lipid content and lipid accumulation *in vitro* by confocal microscopy in lean IVF patients shows that differential uptake or utilization of lipid by cumulus and mural granulosa cells occurs during oocyte maturation and steroidogenesis, respectively [13, 23, 34]. Therefore, the amount of lipid present in these ovarian cells is likely to be a function of both the follicular microenvironment at the time of the oocyte retrieval and the capacity of these cells to accumulate lipids *in vitro* over time. These fundamental and technically important observations regarding ovarian cell lipid metabolism in lean IVF patients are crucial to consider in future studies that explore the adverse effects of altered ovarian cell lipid metabolism through lipotoxicity on oocyte developmental competence in obese women undergoing IVF.

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The authors declare that they have no conflict of interest.

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