Cholesterol uptake or trafficking, steroid biosynthesis, and gonadotropin responsiveness are defective in young poor responders

Gamze Bildik, Ph.D.,[a,b] Yashar Esmaeilian, Ph.D.,[c] Francesko Hela, M.Sc.,[b] Nazli Akin, Ph.D.,[d] Ece İltumur, M.D.,[b] Sevgi Yusufoglu, M.Sc.,[b] Ceren Sultan Yildiz, B.Sc.,[b] Kayhan Yakin, M.D., Ph.D.,[c,e] and Ozgur Oktem, M.D.[b,c,e]

[a] Department of Experimental Therapeutics, the University of Texas MD Anderson Cancer Center, Houston, Texas; [b] The Graduate School of Health Sciences, Koç University, Istanbul, Turkey; [c] Research Center for Translational Medicine, Koç University, Istanbul, Turkey; [d] Follicle Biology Laboratory (FOBL), Department of Pathology/Molecular and Cellular Medicine, Vrije Universiteit, Brussels, Belgium; and [e] Department of Obstetrics and Gynecology, Koç University School of Medicine, Istanbul, Turkey

Objective: To investigate whether poor ovarian response in young patients undergoing in vitro fertilization simply involves lesser follicle growth due to diminished ovarian reserve or whether there are intrinsic perturbations in the ovary.
Design: A translational research study.
Setting: University Hospital Translational Research Center.
Patient(s): A total of 40 patients undergoing in vitro fertilization (20 normal and 20 poor responders) with ovarian stimulation using a gonadotropin-releasing hormone antagonist and recombinant follicle-stimulating hormone were included in the study.
Intervention(s): None.
Main Outcome Measure(s): Luteal granulosa cells obtained during oocyte retrieval procedures were used for the experiments. Cell culture, quantitative real-time polymerase chain reaction, immunoblotting, confocal time-lapse live-cell imaging, and hormone assays were used.
Result(s): We tracked the steroidogenic pathway starting from the very initial step of cholesterol uptake to the final step of estradiol and progesterone production in luteal granulosa cells and identified some previously unknown intrinsic defects in the poor responders. Most notably, the expression of low-density lipoprotein receptors was significantly down-regulated and the uptake of cholesterol and its cytoplasmic accumulation and transportation to mitochondria were substantially delayed and reduced in the poor responders. Further, the expression of the steroidogenic enzymes steroidogenic acute regulatory protein, 3β-hydroxysteroid dehydrogenase, and aromatase as well as gonadotropin receptors was defective, and the response of the cells to exogenous follicle-stimulating hormone and human chorionic gonadotropin was blunted, leading to compromised basal and gonadotropin-stimulated estradiol and progesterone production in the poor responders.
Conclusion(s): This study demonstrates that poor ovarian response in young individuals should not simply be regarded as lesser follicle growth due to diminished ovarian reserve because the underlying pathogenetic mechanisms appear to be much more complex. (Fertil Steril® 2022;117:1069–80. ©2022 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Poor ovarian response, luteal granulosa cells, steroidogenesis, cholesterol uptake, confocal microscopy

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Correspondence: Ozgur Oktem M.D., Department of Obstetrics and Gynecology, School of Medicine, Koc University, Davutpasa, 34010 Topkapi, Istanbul, Turkey (E-mail: ooktem@ku.edu.tr).

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Poor ovarian response (POR) to stimulation is one of the greatest challenges for clinicians performing in vitro fertilization (IVF) and is characterized by a suboptimal response to gonadotropin stimulation, which results in lesser follicle growth, reduced peak estradiol (E_2) levels, and lower oocyte yield and live birth rates. The definition of poor responders is vague, and the patient population is heterogeneous. As such, 41 different definitions have been used to define patients with POR according to a systematic review of 47 randomized studies (1).

To reduce this heterogeneity, the European Society of Human Reproduction and Embryology introduced the Bologna criteria to define poor responders (2). According to these criteria, at least 2 of the following 3 features must be present to define poor response in patients undergoing IVF: advanced maternal age (≥40 years) or any other risk factor for POR, previous history of POR (≤3 oocytes with a conventional stimulation protocol), and an abnormal ovarian reserve test result (i.e., antral follicle count [AFC] = 5–7 follicles or antimüllerian hormone [AMH] level = 0.5–1.1 ng/mL). Additionally, 2 episodes of POR after maximal stimulation are sufficient to define a patient as a poor responder in the absence of advanced maternal age or an abnormal ovarian reserve test result (2). The Bologna criteria were criticized by some on the grounds that they did not take into account oocyte quality and clearly define the risk factors that can be associated with the development of a poor response. Later on, the Patient-Oriented Strategies Encompassing IndividualizeD Oocyte Number (POSEIDON) criteria were introduced to overcome the shortcomings of the Bologna criteria and allow better stratification of patients with a poor prognosis (3). These criteria suggest 4 distinct subgroups based on quantitative and qualitative parameters such as age and expected aneuploidy rate, ovarian reserve biomarkers (AFC and/or AMH), and ovarian response. Group 1 included patients aged <35 years with sufficient prestimulation ovarian reserve parameters (AFC≥5 and AMH≥1.2 ng/mL). Group 2 included patients aged ≥35 years with sufficient prestimulation ovarian reserve parameters (AFC≥5 and AMH≥1.2 ng/mL). Group 3 included patients aged <35 years with poor ovarian reserve prestimulation parameters (AFC<5 and AMH<1.2 ng/mL). Group 4 included patients aged ≥35 years with poor ovarian reserve prestimulation parameters (AFC<5 and AMH<1.2 ng/mL) (3). Two main categories appeared based on this classification system, namely, "expected" (groups 3 and 4) and "unexpected" (groups 1 and 2) PORs (3).

Our motivation in designing and conducting this study was to address the fundamental question of whether POR in young patients aged ≤35 years with low ovarian reserve undergoing IVF is simply a state of lesser follicle growth as a result of diminished ovarian reserve (DOR) or whether subtle intrinsic defects in gonadotropin responsiveness and steroidogenesis are the underlying molecular pathogenetic mechanism. Unfortunately, this question is yet to be answered, and most of the studies published so far have investigated molecular aberrations in a heterogeneous population of patients with DOR, advanced age, or elevated follicle-stimulating hormone (FSH) levels rather than in documented poor-responding patients undergoing IVF (4–14). Therefore, our study population consisted of normal- and poor-responding patients aged ≤35 years undergoing IVF. The index control group had good ovarian reserve biomarkers (AFC≥5 and AMH≥1.2 ng/mL) and underwent ovarian stimulation for different nonovarian factor infertility etiologies. Poor responders were identified based on the documentation of ≤3 oocytes in the current IVF cycle in which luteal granulosa cells (GCs) were harvested and used for the experiments and an abnormal ovarian reserve test result (AFC<5 and AMH<1.2 ng/mL) preceding the IVF attempt.

To address the study question, we designed a translational research study consisting of several different experimental methodologies, as illustrated in Supplemental Figure 1 (available online), using which the molecular characteristics of the different steps of steroidogenesis and gonadotropin response were analyzed in the luteal GCs of normal and poor responders.

MATERIAL AND METHODS

This study was approved by the institutional review board of Koc University (IRB #2019.299.IRB2.092).

Patients

A total of 40 patients undergoing IVF (age≤35 years) with ovarian stimulation using antagonist protocols with a gonadotropin-releasing hormone antagonist (cetrorelix acetate) and recombinant FSH (Gonal-F) were included in the study. Of these patients, 20 were control patients who had documented a normal response to ovarian stimulation (8–15 oocytes retrieved), whereas the remaining 20 were documented poor responders, determined based on the collection of ≤3 oocytes in the current IVF cycle and an abnormal ovarian reserve test result (AFC<5 and AMH<1.1 ng/mL). All the normal and poor responders fulfilled the previously defined criteria of normal ovarian response and POR to stimulation (2, 3, 15). Oocyte retrieval was performed 36 hours after ovulation trigger. The recovered luteal GCs were processed and analyzed separately for each individual patient. All the patients underwent fresh embryo transfers (ETs), which took place on days 3 and 5 for the poor and normal responders, respectively. All eligible patients undergoing IVF were invited to participate in this study over a 12-month period between May 2020 and May 2021 until the required sample numbers, determined based on power analysis calculations, were reached. Normal and poor responders with ovarian pathology and/or infertility etiologies (ovarian surgery, cysts, endometriosis, and so forth) and high responders, including those with polycystic ovary morphology and/or syndrome, were excluded from the study to reduce the risk of the confounding effect of infertility etiology-related factors on the parameters investigated. Patients with metabolic or endocrine disorders, chronic and/or inflammatory systemic diseases, autoimmune disorders, malignancies, previous exposure to radiation and chemotherapy drugs, a history of previous ovarian surgery, and a family history of premature menopause were also excluded from the study for the same reason. None of the patients underwent screening for genetic causes of premature ovarian failure.

Isolation and culture of human luteal GCs

The luteal GCs were obtained from follicular aspirates during the oocyte retrieval procedure and cultured individually for each patient without pooling as described previously (16, 17). In brief, the recovered cells were cultured in 6-well format culture plates at a density of 100,000 cells per well using Dulbecco's modified Eagle medium-F12 culture medium supplemented with 10% fetal bovine serum at 37°C and 5% carbon dioxide.

Chemicals and reagents

All cell culture materials were obtained from Gibco, Inc. (Gaithersburg, Maryland). Hoechst 33342 (#4082) and all Western blotting buffers and reagents were purchased from Bio-Rad (Hercules, California). The antivinculin antibody (V9264) was purchased from Sigma-Aldrich (St. Louis, Missouri). Mouse antihuman monoclonal antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas) for the detection of human 3β-hydroxysteroid dehydrogenase (3β-HSD) type II (sc-100466) and steroidogenic acute regulatory protein (StAR) (sc-166821). The aromatase (CYP19A, ab34193) monoclonal mouse antibody was from Abcam, Inc. (Cambridge, United Kingdom). The SuperBlock reagent (#AAA125) was purchased from ScyTek Laboratories (Logan, Utah).

Gene expression analysis

Ribonucleic acid isolation was performed using the Quick-RNA MicroPrep Kit (Zymo Research, Irvine, California) according to the manufacturer's instructions. The RNA was quantified at a spectrophotometric read of 260 nm using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts), and 500 ng of complementary DNA was prepared using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Waltham, Massachusetts). The quantitative real-time expression of messenger RNAs (mRNAs) of interest was detected and compared using Light Cycler 480 SYBR Green I Master (Roche, Mannheim, Germany). The primers of the genes used in the study are shown in Supplemental Figure 1. The means and standard deviations (SDs) were calculated from 3 different readouts taken for each target gene in the quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assay. We used the ΔΔCt method for the relative quantitation of target genes (16–19).

Immunoblotting

The 3β-HSD type II (sc-100466) and StAR(sc-166821) monoclonal antibodies were purchased from Santa Cruz Biotechnology. The aromatase (CYP19A, ab34193) monoclonal mouse antibody was from Abcam. The low-density lipoprotein receptor (LDL-R) polyclonal antibody was purchased from Cayman Chemicals (Item no. 10007665; Ann Arbor, Michigan). The antivinculin antibody (Sigma-Aldrich) was used as a loading control at a dilution of 1:10,000. Cell lysates for Western blotting were prepared using a radioimmunoprecipitation assay buffer (Sigma-Aldrich) as described previously (18). Immun-Blot polyvinylidene fluoride membranes (BioRad) were incubated overnight at 4°C with the antibodies at the concentrations recommended by the manufacturers.

Conventional and confocal laser immunofluorescence imaging

The cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature (RT), washed twice with phosphate-buffered saline, and permeabilized (except for LDL-R) with 0.1% Triton X-100 in Dulbecco's phosphate-buffered saline (DPBS) for 8 minutes. Blocking was performed with Super-Block for 10 minutes. Steroidogenic enzymes (StAR and 3β-HSD), LDL-R, oil red O, and mitochondria (MitoTracker) were detected using the immunofluorescence staining method as described previously (20). Images were taken under appropriate channels using a confocal microscope (DMi8; Leica, Wetzlar, Germany). Oil red O (Sigma-Aldrich) is a fat-soluble dye and is, thus, used for staining lipid droplets in cells. An oil red O working solution (0.5%) was prepared by boiling 0.5 g of oil red O in 100 mL of 100% isopropanol. The cells were washed with DPBS and fixed with 4% paraformaldehyde for 20 minutes at RT. Following washing with DPBS, the cells were rinsed with 60% isopropanol and stained with oil red O (Sigma-Aldrich) for 20 minutes at RT. Subsequently, the cells were rinsed with 60% isopropanol and running tap water, respectively, and prepared for immunofluorescence staining steps. Permeabilization was performed (if necessary) in 0.2% Triton X-100 containing DPBS for 20 minutes at RT. The blocking of nonspecific epitopes was performed by incubation in the SuperBlock medium (ScyTek) for 20 minutes at RT. Thereafter, the cells were incubated with the primary antibodies overnight at 4°C. The cells were washed 3 times with DPBS-Tween (0.01%) and then incubated with the secondary antibodies for 1 hour at 37°C. The cells were washed 3 times and then covered with the Fluoroshield mounting medium with 4′,6-diamidino-2-phenylindole (Abcam). Alexa Fluor 405 (blue), Alexa Fluor 488 (green), and Alexa Fluor 594 (red) (Thermo Fisher Scientific) were used for multicolor imaging. Images were taken using fluorescent (LDMi8; Leica) and confocal (DMi8/SP8; Leica) microscopy. Image intensity and colocalization analyses were quantitatively performed using the ImageJ software (v2.1.0/1.53c, National Institutes of Health). Four different low-power field areas (×20) that contain at least 500 nuclei were randomly chosen for each sample of each patient to obtain the average numbers of signal intensity and colocalization. The intensity and colocalization measurements using the ImageJ software were analyzed based on the following principles: Intensity measurement: intensity of selected area / vastness of selected area = mean (mean gray value) or average intensity; and colocalization measurement: colocalized particle number / nucleus number = colocalization.

Confocal time-lapse live-cell imaging for cholesterol uptake or trafficking

For the timeline visualization of the cholesterol uptake process and its transportation to mitochondria, the cells were

live stained using NBD-cholesterol (22-(*N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino)-23,24-Bisnor-5-Cholen-3β-Ol) (Invitrogen) and MitoTracker (Invitrogen), respectively. Briefly, the cells were cultured in glass-bottom dishes (Thermo Fisher Scientific) a day before staining. The next day, the medium was replaced with 1 mL of fresh medium, which contained NBD-cholesterol (1 μg/mL), MitoTracker (100 nM), and Hoechst (1 μg/mL). Subsequently, the cells were transferred to the confocal microscope (DMi8; Leica), which was equipped with an incubation chamber (37°C and 5% carbon dioxide) for live-cell imaging. Previous in vitro and in vivo findings have demonstrated NBD-cholesterol to be useful as a fluorescent probe for the real-time imaging of lipoprotein-mediated cholesterol uptake and trafficking within cells (21).

Hormone assays

The E_2 and progesterone (P_4) levels in the culture media were determined using the electrochemiluminescence immunoassay "ECLIA" (Elecsys and Cobas immunoassay analyzers; Roche Diagnostics, Indianapolis, Indiana). The lower detection limit of the E_2 and P_4 levels was 5.00 pg/mL and 0.030 ng/mL, respectively.

Statistical analysis

The samples size required for statistical significance and proper interpretation of the results was calculated based on the qRT-PCR and immunoblot assays. We used the $\Delta\Delta$Ct method for the relative quantitation of target gene mRNAs (16–19). The mean and SD values were calculated from 3 different readouts taken for each target gene in the qRT-PCR assay. The mean and SD of the target gene (*stAR*) were calculated after 3 different readouts taken for each individual sample of the 20 poor-responding patients undergoing IVF, yielding a total of 60 (20 $\times$ 3) readouts, with the SD ranging from 0.05 to 0.1. Hence, an analysis of samples from at least 20 women would provide an 80% power to detect a difference between the means of 0.09 with a significance level of 0.05. The mRNA levels of the target genes used in the qRT-PCR assay (steroidogenic enzymes, FSH receptor, and luteinizing hormone [LH] receptor) and hormone levels are continuous variables and, therefore, expressed as mean $\pm$ SD. Analysis of variance/Bonferroni or Kruskal Wallis/Dunn post hoc tests were used to compare the groups if the data were parametric or nonparametric, respectively. The paired *t*-test was used to compare the signal intensity of the steroidogenic enzymes and hormone levels before and after treatment with FSH and hCG. The significance level was set at 5% ($P<.05$), and Graphpad Prism, version 9, was used to analyze the data and create figures.

RESULTS

The clinical characteristics and outcomes of IVF cycles

The demographic and IVF cycle characteristics of the patients are summarized in Supplemental Table 2 (available online). The IVF cycles were comparable in terms of age, gonadotropin dose, and the duration of stimulation. The serum E_2 and P_4 levels on the day of ovulation trigger, number of oocytes retrieved, and clinical pregnancy rates were significantly lower in the poor responders than in the normal responders despite similar daily and total doses of the gonadotropins used being comparable between the groups. A comparison of the molecular characteristics of the steroidogenic function of the GCs of the normal and poor responders was performed using several different experimental methodologies, as illustrated in Supplemental Figure 1.

Basal and gonadotropin-stimulated steroidogenesis and gonadotropin response were defective in the poor responders

First, we simply compared the mRNA expression of the steroidogenic enzymes and gonadotropin receptors using qRT-PCR and found that the levels of the transcripts of StAR, 3β-HSD, aromatase, and FSH and LH receptors were significantly lower in the GCs of the poor responders than in those of the normal-responding patients undergoing IVF (Fig. 1A). Consistent with the qRT-PCR results, the immunoblot analysis showed that the expression of StAR, 3β-HSD, and aromatase was significantly reduced in the poor responders compared with that in the normal responders (Fig. 1B and C).

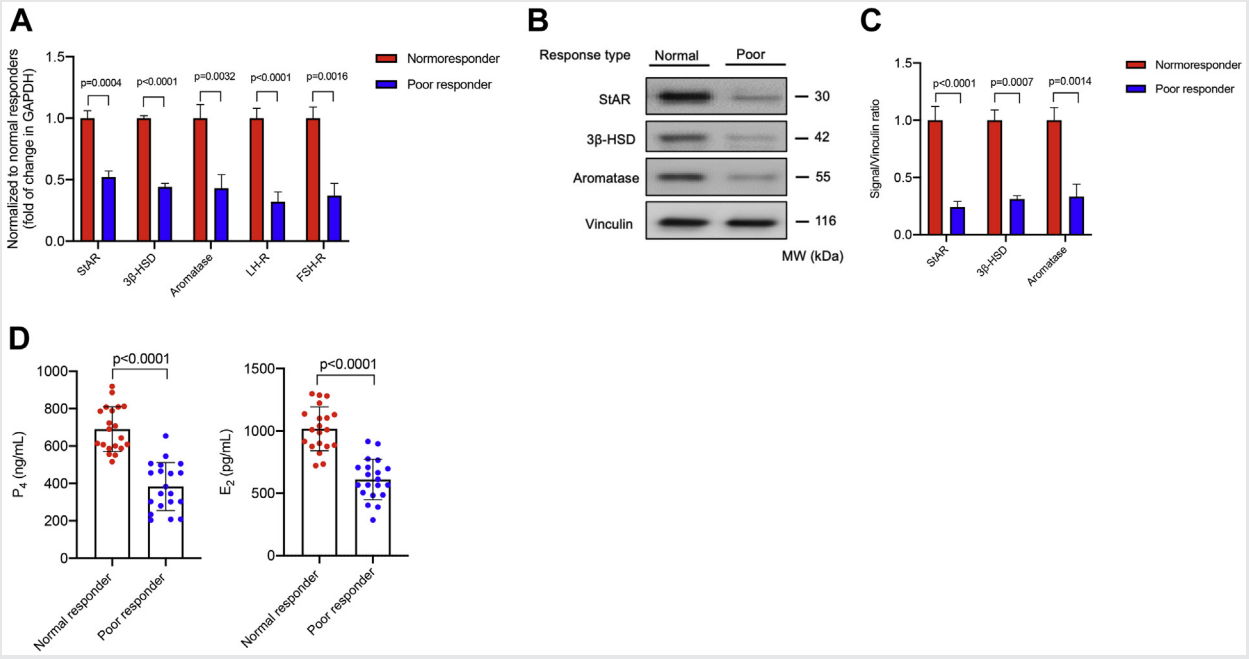
A comparison of the in vitro steroidogenic activity of the cells revealed that when plated at an equal density, the GCs of the poor responders produced significantly lower amounts of E_2 (628.8 $\pm$ 160 vs. 1,017 $\pm$ 176 pg/mL, respectively; $P<.0001$) and P_4 (382 $\pm$ 128 vs. 690 $\pm$ 119 ng/mL, respectively; $P<.0001$) after the 24-hour culture period compared with the GCs of the normal responders (Fig. 1D).

The defective expression of the FSH and LH receptors in the GCs of the poor responders, determined using the qRT-PCR analysis, led us to analyze the gonadotropin responsiveness of these receptors. For this purpose, in another set of experiments, the GCs were stimulated with recombinant hCG and recombinant FSH, and then, the increases in StAR expression and E_2 and P_4 output were compared between the normal and poor responders. We noticed that the increases in StAR expression and P_4 output were much smaller after treatment with hCG in the poor responders than in the normal responders (Fig. 2A to C). A similar blunted response was observed after FSH stimulation. The up-regulation of StAR expression and E_2 output was significantly lesser in the poor responders (Fig. 2D to F). Taken together, these findings so far indicate that the expression of steroidogenic enzymes and gonadotropin receptors is defective and that basal and gonadotropin-stimulated steroidogenesis is impaired in the GCs of poor responders.

Conventional and laser confocal immunofluorescence imaging

We noticed in the conventional immunofluorescence microscopy examination that the cytoplasmic accumulation of intracellular lipids, as assessed using oil red O staining, significantly diminished in the luteal GCs of the poor responders compared with that in the GCs of the normal responders

FIGURE 1



Comparison of the steroidogenic function of luteal granulosa cells between the normal and poor responders using gene expression studies with quantitative real-time polymerase chain reaction (**A**) and immunoblotting (**B** and **C**). The expression of the steroidogenic enzymes and gonadotropin receptors was significantly down-regulated in the poor responders, as determined using quantitative real-time polymerase chain reaction (**A**), the immunoblotting images (**B**), and after a quantitative comparison of the signals of immunoblotting (**C**). The steroidogenic capacities of the cells were compared by measuring their 24-hour progesterone and estradiol output (**D**) in vitro. Progesterone and E_2 production was substantially compromised in the poor responders. 3β-HSD = 3β hydroxysteroid dehydrogenase; E_2 = estradiol; FSH-R = follicle stimulating hormone receptor; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; LH-R = luteinizing hormone receptor; MW = molecular weight; P_4 = progesterone; StAR = steroidogenic acute regulatory protein.

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(Fig. 3A and B). A more detailed analysis using confocal microscopy confirmed this finding (Fig. 3C) and revealed that the mitochondrial content, as evidenced by a weaker signal intensity of MitoTracker (Fig. 3D), and MitoTracker-oil red colocalization (Fig. 3E), the indicator of lipid content within mitochondria, was significantly reduced in the GCs of the poor responders compared with that in the GCs of the normal responders.

Consistent with the reduced expression of the steroidogenic enzymes in the poor responders, as evidenced by immunoblotting, we also observed in the confocal images that the signal intensities of StAR (Fig. 3F and G) and 3β-HSD (Fig. 3I and J) were significantly diminished and their colocalizations with mitochondria (Fig. 3H and K) were markedly reduced in the poor responders compared with those in the normal responders.

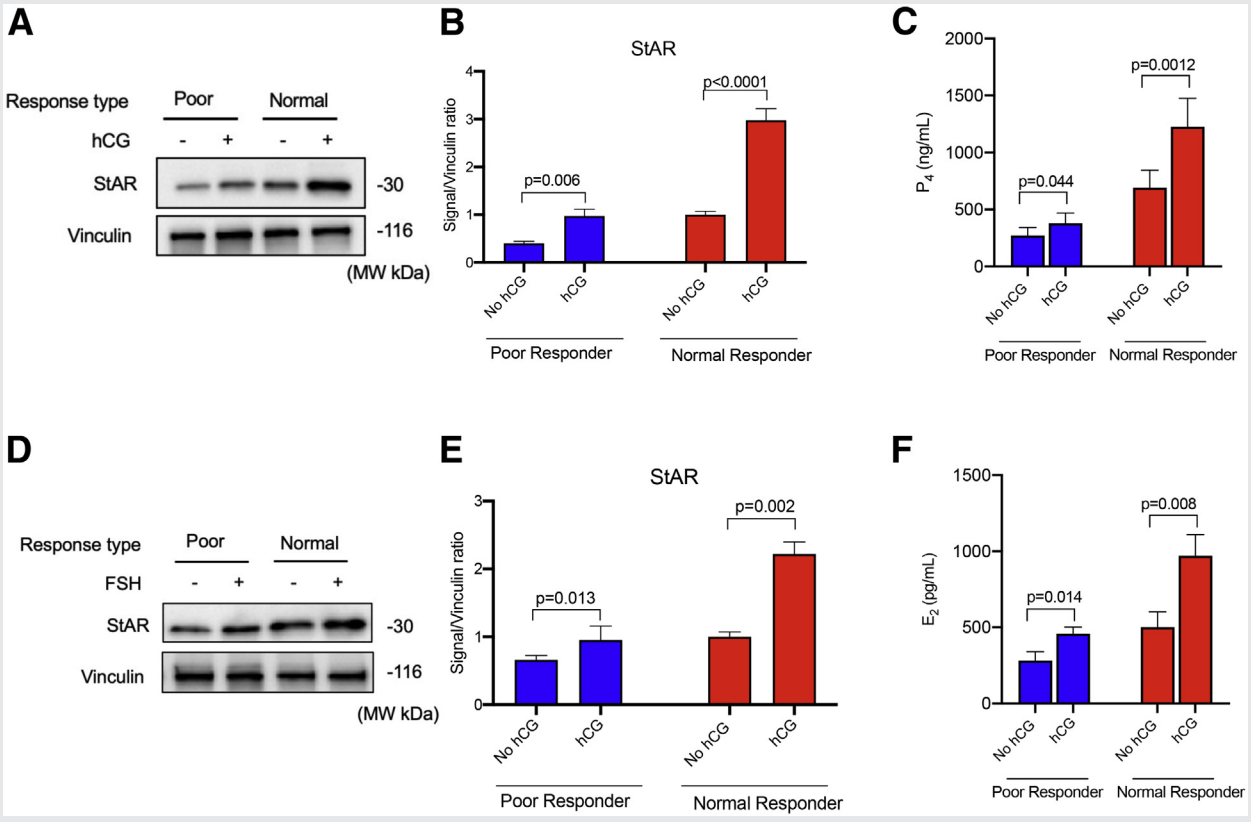
Monitoring and assessment of intracellular lipids and cholesterol trafficking in luteal GCs using confocal real-time live-cell microscopy

Defective steroidogenic function together with reduced lipid accumulation in the luteal GCs of the poor responders led us to investigate whether the uptake and intracellular trafficking of cholesterol are defective in these cells. For this purpose, we monitored the uptake of NBD-cholesterol using confocal real-time live-cell imaging. We observed that there was a substantial delay and reduction in its uptake, cytoplasmic accumulation, and transportation to mitochondria in the poor responders. The MitoTracker-NBD cholesterol colocalization was markedly diminished in the poor responders (Fig. 4A to D and Supplemental Movies 1 to 4, available online). Low-density lipoprotein receptor, which is expressed in the plasma membrane of steroidogenic cells, is primarily responsible for cholesterol uptake. Therefore, as the last set of experiments, we analyzed LDL-R expression using confocal microscopy (Fig. 4E to G) and immunoblotting (Fig. 4H and I) and observed that it was significantly reduced in the luteal GCs of the poor responders.

DISCUSSION

In this study, we tracked several steps of steroid biosynthesis in luteal GCs and uncovered some intrinsic defects in this pathway in the young poor-responding patients undergoing IVF, which are as follows: the reduced expression of the steroidogenic enzymes StAR, 3β-HSD, and aromatase as well as FSH and LH receptors; impaired response to FSH and hCG; defective basal and gonadotropin-stimulated E_2 and

FIGURE 2



Comparative analysis of the response of the gonadotropin receptors to exogenous follicle-stimulating hormone (FSH) and human chorionic gonadotropin in luteal granulosa cells in the normal and poor responders. Luteinizing hormone receptor responsiveness was assessed by comparing the increase in steroidogenic acute regulatory protein (StAR) expression (**A** and **B**) and progesterone output (**C**) after treatment with human chorionic gonadotropin. Follicle-stimulating hormone receptor responsiveness was analyzed in a similar fashion by analyzing the StAR expression pattern (**D** and **E**) and estradiol output (**F**) following treatment with FSH. Note the more robust increase in the expression of StAR and the production of estradiol and progesterone after gonadotropin stimulation in the normal responders in comparison with those in the poor responders. E_2 = estradiol; FSH = follicle-stimulating hormone; hCG = human chorionic gonadotropin; MW = molecular weight; P_4 = progesterone; StAR = steroidogenic acute regulatory protein.

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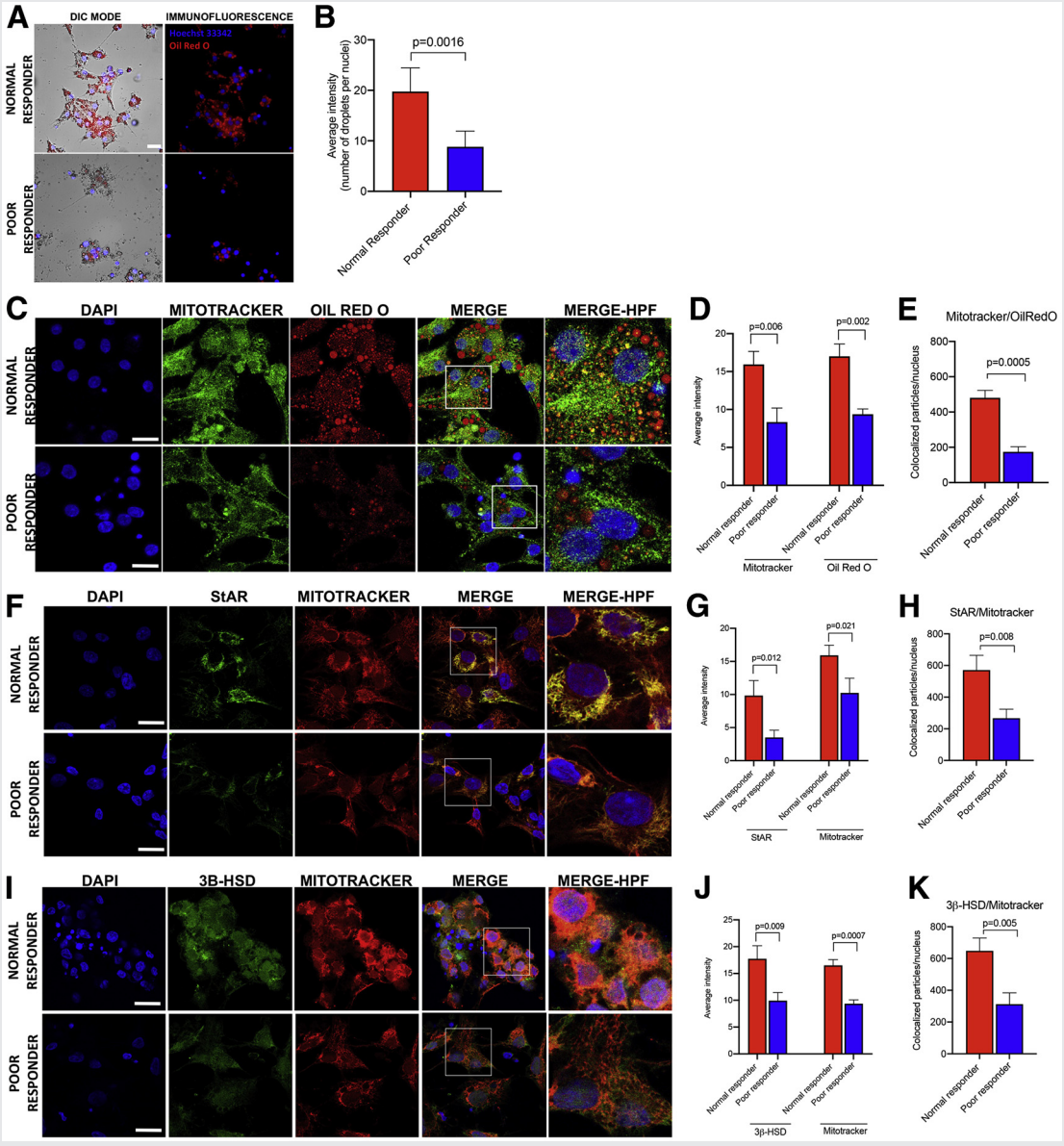
P_4 production; reduced mitochondrial content and defects in its colocalizations with the steroidogenic enzymes and lipids; and reduced LDL-R expression, impaired cholesterol uptake and its intracellular trafficking, and intracytoplasmic accumulation. Taken together, these findings become much more meaningful when translated into clinical practice because they indicate that POR to stimulation in young individuals with DOR should not simply be considered as a state of lesser follicle growth or oocyte yield due to low ovarian reserve. Rather, the underlying molecular perturbations of POR are much more complex and involve multiple steps of steroidogenesis and gonadotropin responsiveness. This is perhaps the most important message of our study.

Steroidogenesis entails a multistep physiological process in which cholesterol is converted into steroid hormones. In brief, steroidogenic cells take up circulating cholesterol mainly in the form of low-density lipoproteins via LDL-R–mediated endocytosis and then direct the cholesterol for steroid hormone synthesis to mitochondria or store it in lipid droplets after esterification. Steroidogenic acute regulatory protein is the rate-limiting enzyme of steroid biosynthesis and facilitates the rapid flux of free (unesterified) cholesterol into mitochondria in steroidogenic cells, whereas 3β-HSD catalyzes the conversion of Δ^5 steroids to the corresponding Δ^4 steroids, including the conversion of pregnenolone to progesterone within the mitochondria aromatase (CYPA1) as a CYP450 enzyme catalyzes the conversion of androgens to estrogens (22).

To date, a limited number of studies have analyzed the steroidogenic characteristics of luteal GCs obtained from a heterogenous population of patients with DOR, advanced age, and POR. Thus, the existing data are still limited and somewhat inconsistent mainly because of methodologic or technical limitations. To our knowledge, no study so far has conducted a detailed comparative analysis of the different steps of steroidogenesis in patients with POR. Seifer et al. (13) compared the E_2 and P_4 production of luteal GCs between 7 women with low day-3 FSH levels ($\leq$6 IU/mL) and 8 women

FIGURE 3



Conventional and confocal microscopic images of the lipid stores, steroidogenic enzymes, and mitochondria in the granulosa cells of the normal and poor responders. The cytoplasmic lipids and mitochondria were assessed after staining with oil red O and the fluorescent mitochondrial marker MitoTracker, respectively, using conventional (**A** and **B**) and confocal microscopy (**C**–**E**). MitoTracker (*green signal*) and oil red staining (*red signal*) and their colocalization (*yellow signal* in merge images) in the confocal image (**C**). Their intensity (**D**) and colocalization (**E**) are shown as *graphic bars*. Note the weaker signal intensity of MitoTracker and oil red O, indicative of less mitochondrial content and lipid accumulation, respectively, and the paucity of *yellow signals* in the poor responders. The signal intensity of steroidogenic acute regulatory protein (*green signal*) and MitoTracker (*red signal*) and their colocalization (*yellow signals* in the merge images) are significantly diminished in the poor responders (**F** and **G**). Note the paucity of the *yellow signals*, indicative of the presence of less steroidogenic acute regulatory protein enzyme within the mitochondria in the merge and HPF merge images. The colocalization is also expressed as a *graphic bar* (**H**). A similar staining pattern was observed for 3β hydroxysteroid dehydrogenase and MitoTracker (**I**–**K**). The areas marked as *white squares* are also depicted as high magnification merge images. The areas marked as *white squares* in the merge images are also shown as high magnification merge images (merge HPF). *Scale bars*, 25 μ. 4′,6-diamidino-2-phenylindole (*blue signals*) fluorescently stained the nucleus. 3β-HSD = 3β hydroxysteroid dehydrogenase; DAPI = 4′,6-diamidino-2-phenylindole; DIC = differential interference contrast microscopy; HPF = high power field; StAR = steroidogenic acute regulatory protein.

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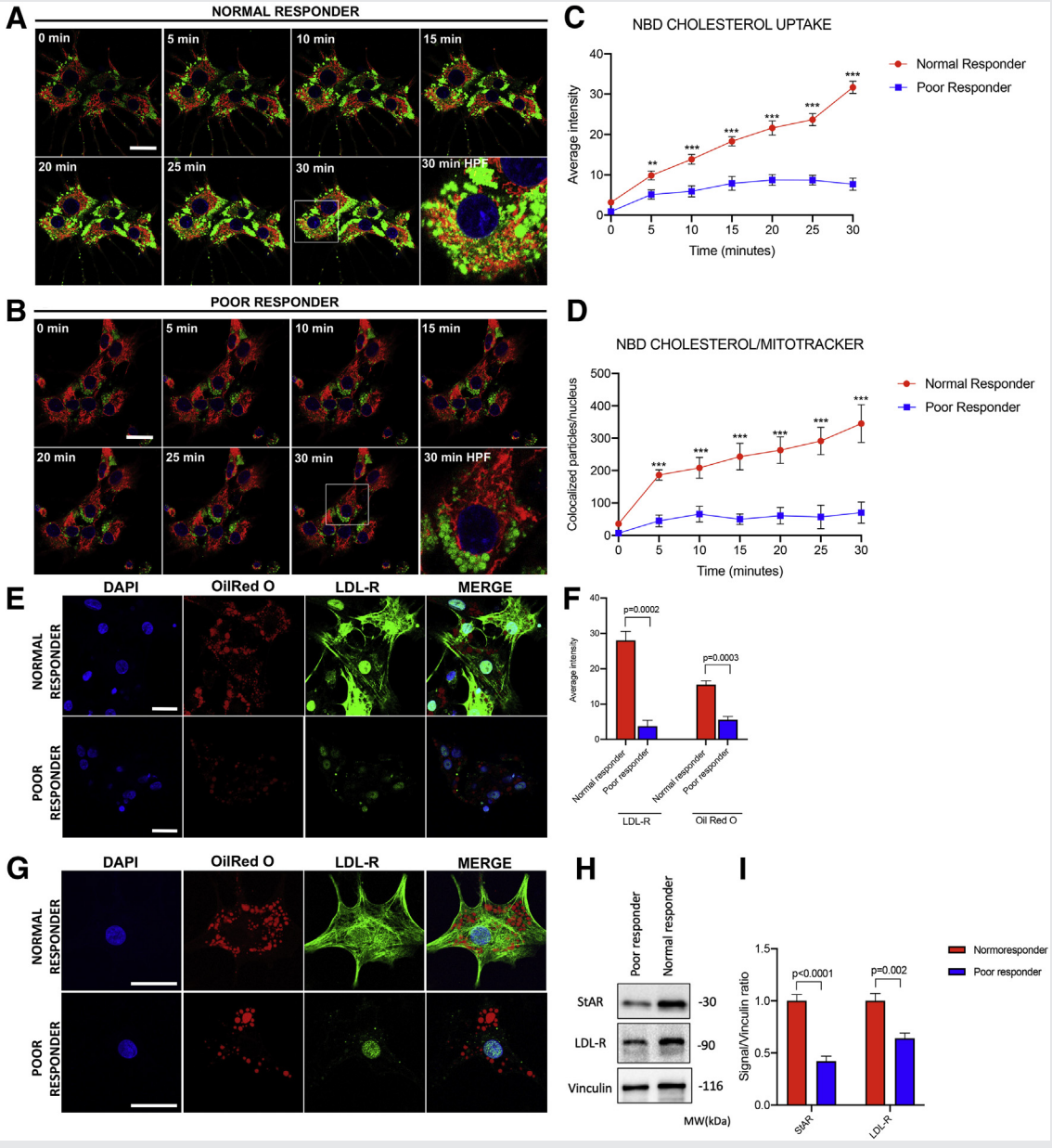
with high day-3 FSH levels ($\geq$10 IU/mL) and did not observe any difference in the steroidogenic activity between the groups. The sample size was low, and the number of oocytes retrieved was within the normal range even in the high-FSH group (8.6 $\pm$ 1.3 IU/mL), raising the question of whether these patients were real poor responders (13). Skiadas et al. (6)

FIGURE 4



NBD-cholesterol uptake assay with time-lapse live-cell imaging in the confocal microscopy and low-density lipoprotein receptor LDL-R expression analyses using confocal microscopy and immunoblotting. Green fluorescent cholesterol analog NBD-cholesterol was rapidly taken up by luteal GCs in the normal responders and began to accumulate in their cytoplasm within 30 minutes after its administration, as evidenced by a rapid and steady increase in the intensity of the *green signal* in the cytoplasm of the cells (**A**) and after the quantification of the signals as a *graphic bar* (**C**). At the end of the monitoring period, its transportation to the mitochondria (*red signal*) was easily identified as strong *yellow signals* (HPF) (**A**). The NBD-cholesterol-MitoTracker colocalization is also shown as a curve as the number of colocalized particles in the *graphic bar* (**D**). In contrast, such drastic uptake and colocalization were not observed in the poor responders (**B**–**D**). The signal intensity of LDL-R (*green signal*) was much weaker, and intracytoplasmic lipid accumulation, as assessed using oil red O staining (*red signal*), was significantly reduced in the luteal GCs of the poor responders (**E**–**G**). Similarly, the expression of LDL-R was significantly diminished along with the expression of steroidogenic acute regulatory protein in the poor responders in the immunoblot images (**H**) and is depicted as a *graphic bar* after a quantitative comparison of the immunoblot signals (**I**). *Scale bars*, 25 μ. Hoechst (*blue signals*) fluorescently stained the nucleus. The comparison was statistically significant at $P<.001$ (**) and $P<.0001$ (***). DAPI = 4′,6-diamidino-2-phenylindole; LDL-R = low-density lipoprotein receptor; NBD = NBD cholesterol; StAR = steroidogenic acute regulatory protein.

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performed a detailed comparative analysis of the gene expression profiles of the luteal GCs of patients (age $\leq$ 35 years) without DOR vs. patients with DOR undergoing IVF using a microarray and confirmatory qRT-PCR. Their results demonstrated that the expression of LH receptors, StAR, and StARD4 (a member of the StAR-related lipid transfer [START] family)

was significantly higher and the AMH level was significantly lower in the patients with DOR. Although the lower expression of AMH in the patients with DOR is consistent with previous reports that showed that the serum AMH level is well correlated with AFC and that both AMH and AFC are reduced in patients with DOR, the higher expression of StAR as the other major finding of the investigators is in contrast with our findings and those of Phy et al. (23), who demonstrated lower StAR expression and P_4 production in the luteal GCs of patients with DOR (24). Nevertheless, it appears from the IVF data set of Skiadas et al. (6) that not all patients with DOR fulfill the current criteria of POR that are based on the Bologna and POSEIDON criteria, which together with stimulation protocol differences may potentially explain the contrasting results. In fact, the discrepancies among the published studies also underscore the need for more research on steroid biosynthesis pathways in more homogeneous cohorts of patients who are more clearly differentiated into 4 distinct groups of poor responders based on the POSEIDON criteria (3).

Apart from steroidogenic function, several other studies have compared the viability of luteal GCs between normal younger vs. poor older responders, with inconsistent results (5, 7, 14, 25). Reactive oxygen species and mitochondrial DNA content play critical roles in cellular aging (26). In this regard, Tatone et al. (27) demonstrated that the mean relative levels of mRNAs coding for superoxide dismutases, copper (Cu), zinc superoxide dismutase also known as superoxide dismutase-1 (ZnSOD [SOD1]), manganese superoxide dismutase, also known as superoxide dismutase-2 (MnSOD [SOD2]), and catalase were significantly decreased in the luteal GCs of women older than 38 years. Similar to our findings, the investigators also observed that the GCs of older women (range, 38–41 years) showed defective mitochondria and fewer lipid droplets than the GCs of a younger group (range, 27–32 years) and attributed these findings in older patients to age-dependent oxidative stress injury (27). In addition to this study, several other groups have demonstrated reduced mitochondrial DNA content in the oocytes and cumulus GCs of older patients with DOR and ovarian insufficiency undergoing IVF (10, 11, 28, 29).

We obtained these findings in a relatively specific patient population of ''predicted'' poor responders consisting of young patients (age<35 years) with DOR. Therefore, it is unclear whether the observed defects are universally present in all subtypes of poor response, regardless of the chronologic age and ovarian reserve status, and whether they vary depending on the infertility etiology. Although advanced age and DOR are generally the most common etiologic factors that explain the presence of POR to ovarian stimulation, at least some proportions of poor responders are still young (3), indicating that there must be some other factors that are implicated in the pathophysiology of poor response other than aging and ovarian reserve status. The development of poor response to stimulation in young patients with normal ovarian reserve indices further complicates the scene. It is likely that different pathogenetic mechanisms are operative for young vs. older poor responders. Advanced maternal age and aging-related decline in ovarian reserve are associated with a reduction in not only oocyte quantity but also oocyte quality, and these significantly increase the risk of embryo aneuploidy (30, 31). Thus, the prognosis is differentially affected by age and ovarian reserve (oocyte quantity) because the former is more closely associated with embryo aneuploidy. However, there is also evidence that links low ovarian reserve, regardless of the chronologic age, to an increase in oocyte aneuploidies and miscarriage risk as well as lower fertilization rates, indicative of some intrinsic defects in oocytes that cannot be attributed to aging (32–36).

At this point, another intriguing question that arises is whether premature senescence has a role in the development of DOR and POR to stimulation in young patients. Although there is still no universal consensus on the exact definition of premature aging or senescence and its role in ovarian function or senescence, 2 recent studies from the same group of investigators were able to identify the signs of premature aging in GCs and oocytes in young patients with low ovarian reserve indices (8, 9). The investigators compared luteal GCs between patients without DOR and those with DOR using whole-genome methylation array data based on DNA methylation variability, age acceleration, DNA methylation telomere length estimator, and the accumulation of epimutations. Their findings demonstrated that the luteal GCs of women with DOR have a distinctive epigenetic profile and harbor a high frequency of epimutations, suggestive of premature aging, and that these appear epigenetically more like the luteal GCs of women with advanced age (>40 years) (8, 9). On the other hand, another interesting study assessed the accuracy of the "epigenetic clock" concept in women of reproductive age undergoing fertility treatment by applying the age prediction algorithm in peripheral (white blood cells [WBCs]) and follicular somatic cells (cumulus cells) to investigate whether women with premature reproductive aging (DOR) are at the risk of accelerated aging in their age prediction. The study used the methylation level of 353 CpG sites from the WBCs and cumulus GCs of younger and older patients undergoing IVF with normal and poor responses and demonstrated that although the WBCs helped accurately predict the chronologic age of the patients, the cumulus GCs did not. Furthermore, the cumulus GCs were found to have longer telomere lengths than the WBCs, and the investigators observed that the categorization of patients based on their ovarian response did not appreciably change age prediction in the WBCs or cumulus cells, and neither was it associated with the relative telomere DNA length (4). Taken collectively, it remains elusive whether premature aging develops in the ovary and, if any, to what extent it causes reproductive senescence by depleting ovarian reserve and/or impairing gonadotropin response in young poor responders who have low ovarian reserve discordant with their chronologic age.

Animal data have shown an aging-related decline in LH responsiveness, cyclic adenosine monophosphate production, and cholesterol transport to mitochondria in rats (37–39). However, to our knowledge, no conclusive evidence in human exists yet to link the perturbations in steroidogenesis and gonadotropin response to the detrimental effect of aging or oxidative stress or another pathologic process. This is also true for our findings. On the

other hand, there is solid evidence that ovarian steroidogenesis is well preserved in older women with regular cycles and even continues unhampered up until a few years before the final cessation of menses despite an age-related decline in ovarian reserve, higher FSH levels, and lower inhibin-B and AMH levels (12, 40–42).

One last question remains to be answered, which is whether the observed perturbations in the steroidogenic function of the luteal GCs of poor responders contribute to lower IVF success rates when a fresh ET is performed. To date, several observational or retrospective studies have compared fresh vs. freeze-all strategies in poor responders, with inconsistent results (43–46). In the meantime, the indications of the freeze-all strategy, such as P_4 elevation on hCG day, a history of 1 previously failed fresh cycle, and differences in the endometrium between fresh and frozen cycles, should be taken into consideration because the possible confounders that may affect the IVF success rate while remembering at the same time the very fact that many poor responders do not have the luxury of elective embryo freezing and undergo compulsory fresh ET cycles. This is also true for the poor responders in our study who underwent fresh ET on day 3.

In conclusion, this study unveils previously unknown intrinsic defects in the steroidogenic function of the GCs of young poor responders, underscoring the complexity of this phenomenon rather than simply being a state of lesser follicle growth or oocyte yield due to DOR. Caution should be exercised while interpreting these data because it is unclear whether the observed defects exhibit variations based on race, poor response type, ovarian response type, stimulation protocol, infertility etiology, and the mode of ovulation trigger.

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El consumo o tráfico de colesterol, la biosíntesis de esteroides y la respuesta a las gonadotropinas son defectuosas en jóvenes pobres respondedoras.

Objetivo: Investigar si la baja respuesta ovárica en pacientes jóvenes sometidas a fecundación in vitro se debe simplemente a un menor crecimiento folicular por disminución de la reserva ovárica o si existen perturbaciones intrínsecas en el ovario.

Diseño: Estudio de investigación traslacional.

Ubicación: Centro de Investigación Traslacional de Hospital Universitario.

Paciente(s): Fueron incluidas en el estudio un total de 40 pacientes sometidas a fertilización in vitro (20 con respuesta normal y 20 con respuesta deficiente) con estimulación ovárica utilizando un antagonista de la hormona liberadora de gonadotropina y hormona recombinante estimulante del folículo.

Intervención(es): Ninguna.

Principal(es) medida(s) de resultado: Para los experimentos se utilizaron células de la granulosa luteínicas obtenidas durante los procedimientos de recuperación de ovocitos. Se utilizó cultivo celular, reacción en cadena de la polimerasa cuantitativa en tiempo real, inmunotransferencia, obtención de imágenes de células vivas con lapso de tiempo confocal y ensayos hormonales.

Resultado(s): Rastreamos la vía esteroidogénica desde el paso inicial de absorción de colesterol hasta el paso final de producción de estradiol y progesterona en las células de la granulosa luteínicas e identificamos algunos defectos intrínsecos previamente desconocidos en las bajas respondedoras. En particular, la expresión de los receptores de lipoproteínas de baja densidad se hipo reguló significativamente y la captación de colesterol y su acumulación citoplasmática y transporte a las mitocondrias se retrasó y redujo sustancialmente en las respondedoras deficientes. Además, la expresión de las enzimas esteroidogénicas, la proteína reguladora aguda esteroidogénica, la 3b-hidroxiesteroide deshidrogenasa y la aromatasa, así como los receptores de gonadotropina, fue defectuosa, y la respuesta de las células a la hormona estimulante del folículo exógena y a la gonadotropina coriónica humana fue mitigada, lo que condujo a un compromiso de la producción de estradiol y progesterona basal y estimulada por gonadotropinas en las bajas respondedoras.

Conclusión(es): Este estudio demuestra que la respuesta ovárica deficiente en individuos jóvenes no debe considerarse simplemente como un menor crecimiento del folículo debido a la disminución de la reserva ovárica porque los mecanismos patogénicos subyacentes parecen ser mucho más complejos.