Luteal granulosa cells from natural cycles are more capable of maintaining their viability, steroidogenic activity and LH receptor expression than those of stimulated IVF cycles

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STUDY QUESTION: Are there any differences in the molecular characteristics of the luteal granulosa cells (GC) obtained from stimulated versus non-stimulated (natural) IVF cycles that may help explain the defective luteal phase in the former?

SUMMARY ANSWER: Luteal GC of stimulated IVF cycles, particularly those of agonist-triggered antagonist cycles, are less viable ex vivo, express LH receptor and anti-apoptotic genes at lower levels, undergo apoptosis earlier and fail to maintain their estradiol (E2) and progesterone (P4) production in comparison to natural cycle GC.

WHAT IS KNOWN ALREADY: Luteal function is defective in stimulated IVF cycles, which necessitates P4 and/or hCG administration (known as luteal phase support) in order to improve clinical pregnancy rates and prevent miscarriage. The luteal phase becomes shorter and menstruation begins earlier than a natural cycle if a pregnancy cannot be achieved, indicative of early demise of corpus luteum (premature luteolysis). Supra-physiological levels of steroids produced by multiple corpora luteae in the stimulated IVF cycles are believed to inhibit LH release directly via negative feedback actions on the hypothalamic–pituitary–ovarian axis resulting in low circulating levels of LH and a defective luteal phase. We hypothesized that some defects in the viability and steroidogenic activity of the luteal GC of the stimulated IVF cycles might contribute to this defective luteal phase in comparison to natural cycle GC. This issue has not been studied in human before.

STUDY DESIGN, SIZE, DURATION: A comparative translational research study of ex vivo and in vitro models of luteal GC recovered from IVF patients undergoing natural versus stimulated IVF cycles was carried out. Luteinized GC were obtained from 154 IVF patients undergoing either natural (n = 22) or stimulated IVF cycles with recombinant FSH and GnRH agonist (long) (n = 44), or antagonist protocol triggered conventionally either with recombinant hCG (n = 46) or with a GnRH agonist (n = 42). GC were maintained in vitro for up to 6 days.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Cellular viability (YO-PRO-1 staining), the expression of the steroidogenic enzymes, pro-apoptotic genes [Bcl-2-associated death promoter (BAD), Bcl-2-associated X protein (BAX) and Caspase-3 (CASP3)], anti-apoptotic genes [RAC-alpha serine/threonine-protein kinase (AKT-1) and Bcl-2-like protein 2 (BCL2-L2)], LH receptor, vascular endothelial growth factor (VEGF) (using real-time quantitative PCR at mRNA level and western blot immunoprecipitation assay at protein level) and in vitro E2 and P4 production (electrochemiluminescence immunoassay) were compared in GC among the groups.

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**Main Results and the Role of Change:** Natural cycle GC were significantly more viable ex vivo (88%) compared to their counterparts of the stimulated IVF cycles (66, 64 and 37% for agonist and antagonist cycles triggered with hCG and GnRH agonist respectively, $P < 0.01$). They were also more capable of maintaining their vitality in culture compared to their counterparts from the stimulated IVF cycles: at the end of the 6-day culture period, 74% of the cells were still viable whereas only 48, 43 and 22% of the cells from the agonist and antagonist cycles triggered with hCG and agonist respectively, were viable ($P < 0.01$). The mRNA expression of anti-apoptotic genes (AKT-1 and BCL2-L2) was significantly lower, while that of pro-apoptotic genes (BAD, BAX and CASP3) was significantly higher in the stimulated cycles, particularly in the agonist-triggered antagonist cycles, compared to natural cycle GC ($P < 0.01$ for long protocol and antagonist hCG trigger, $P < 0.001$ for agonist trigger). The expression of steroidogenic enzymes (stAR, SCC, 3β-HSD and aromatase) and VEGF was significantly higher in the agonist and hCG-triggered antagonist cycles compared to natural cycle GC. Therefore, in vitro $E_2$ and $P_4$ production in cells from the stimulated IVF cycles was significantly higher than their counterparts obtained from the natural cycles in the first 2 days of culture. However, after Day 2, their viability and hormone production began to decline very rapidly with the most drastic decrease being observed in the agonist-triggered cycles. By contrast, natural cycle GC maintained their viability and produced $E_2$ and $P_4$ in increasing amounts in culture up to 6 days. In vitro $P$ production and the mRNA and protein expression of LH receptor, VEGF and 3β-HSD were most defective in the agonist-triggered antagonist cycles compared to natural and agonist and hCG-triggered antagonist cycles. In vitro hCG treatment of a subset of the cells from the agonist-triggered cycles improved their viability, increased $E_2$ and $P_4$ production in vitro and up-regulated the mRNA expression of anti-apoptotic gene BCL2-L2 together with steroidogenic enzymes stAR, SCC, 3β-HSD, LH receptor and VEGF.

**Limitations, Reasons for Caution:** The limitations include analysis of luteinized GC only might not reflect the in vivo mechanisms involved in survival and function of the whole corpus luteum; GC recovered during oocyte retrieval belong to a very early stage of the luteal phase and might not be representative; effects of ovulation triggered with hCG may not equate to the endogenous LH trigger; the clinical characteristics of the patients may vary among the different groups and it was not possible to correlate stimulation-related molecular alterations in luteal GC with the clinical outcome, as no oocytes have been utilized yet. Therefore, our findings do not conclusively rule out the possibility that some other mechanisms in vivo may also account for defective luteal function observed in stimulated IVF cycles.

**Wider Implications of the Findings:** Ovarian stimulation is associated with significant alterations in the viability and steroidogenic activity of luteal GC depending on the stimulation protocol and mode of ovulation trigger. Reduced survival and down-regulated expression of 3β-HSD, LH receptor and VEGF leading to compromised steroid production in stimulated cycles, and particularly in the agonist-triggered cycles, may at least in part help explain why the luteal phase is defective and requires exogenous support in these cycles.

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**Key words:** natural cycle / ovarian stimulation / IVF cycles / luteal granulosa cells / steroidogenesis / apoptosis / ovulation trigger / GnRH agonist / GnRH antagonist / protocols

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**Introduction**

The natural ovarian cycle in human is characterized by the recruitment, development and ovulation of a single follicle (mono-ovulatory) and hence, the presence of a single corpus luteum. By contrast, in IVF cycles, controlled ovarian stimulation (COS) initiated at early follicular phase with exogenously administered gonadotrophin hormones overrides the natural selection process of a single dominant follicle and produces multiple ovulatory follicles. As a result, these cycles are also characterized by the presence of multiple corpora lutea after ovulation is triggered either conventionally with hCG or with a GnRH agonist. The GnRH agonist trigger is exclusively used when there is a risk of ovarian hyperstimulation syndrome (OHSS) in which the ovaries hyper-respond to gonadotrophin stimulation by producing too many growing follicles along with development of ascites, pleural effusion and hemo-concentrations as a result of increased vascular permeability and leakage of intravascular volume into third spaces (Humaidan et al., 2010, 2013; Mascarenhas and Balen, 2017). The GnRH agonist ovulation trigger strategy is also used when early luteolysis and rapid reduction in serum estrogen level are indicated such as random-start ovarian stimulation protocols employed for oocyte and embryo freezing in patients with estrogen sensitive tumors, for example breast cancer (Okjay et al., 2010). It is well documented that the function of corpus luteum is defective in stimulated IVF cycles (Fatemi, 2009). This necessitates progesterone ($P_4$) and/or hCG administration, known as luteal phase support, in order to improve clinical pregnancy rates and prevent miscarriage (Pritts and Atwood, 2002; van der Linden et al., 2011). Luteal phase becomes shorter and menstruation begins earlier than a natural cycle if pregnancy could not be achieved, indicative of the early demise of corpus luteum (premature luteolysis). If conception occurs, the pregnancy rate declines without luteal phase support, suggesting that existing corpora lutea do not sufficiently support the maintenance of pregnancy (Pritts and Atwood, 2002). Those IVF cycles in which ovulation is triggered with a GnRH agonist without co-administration of hCG appear to have the most defective luteal phase such that clinical pregnancy rate is drastically reduced even with exogenous progesterone supplementation if a fresh embryo transfer in that cycle is performed (Humaidan et al., 2013). It is unclear why luteal...
function is defective and requires exogenous support in stimulated IVF cycles. According to our current understanding, supra-physiological levels of steroids produced by multiple corpora lutea during COS cause a luteal phase defect by directly inhibiting LH release via negative feedback actions on the hypothalamic-pituitary-ovarian axis (HPO) (Fauser and Devroey, 2003; Fatemi, 2009). We hypothesized that differences may exist between natural and stimulated IVF cycles in terms of the ability of GC to survive and maintain their viability and steroidogenic activity that potentially may help to explain, at least in part, defective luteal function in stimulated cycles. To the best of our knowledge, this issue has not been studied at a molecular level in human female.

Materials and Methods
This study was approved by the institutional review board of Koc University (IRB# 2017.141.IRB2.049). Informed written consents were obtained from all participants.

Patients
All expected normo-responders and predicted high-responders without polycystic ovary syndrome (PCOS) were included in this study. We did not include predicted or documented poor responders (defined according to the Bologna criteria; Ferraretti et al., 2011) and high-responders with PCOS because patients in these categories may have distinct underlying molecular aberrations that may confound the results. All eligible IVF patients were invited to participate in this study over a 6-month period between September 2017 and April 2018. Since the minimum number of patient samples required for the experiments were determined after power analysis calculations, active enrollment of the patients was continued until the required sample numbers were reached. A total of 154 IVF patients who consented to allow their GC to be used for research were finally included in the study. The high-responders had high-ovarian reserve but were not diagnosed with PCOS based on ovarian morphology, clinical and laboratory parameters.

The natural cycle IVF group consisted of 22 patients who presented for oocyte freezing at pre-ovulatory phase and therefore underwent oocyte retrieval after ovulation was induced with recombinant hCG (rhCG). The indications for oocyte freezing were elective (retreival after ovulation was induced with recombinant hCG (rhCG). The oocyte freezing at pre-ovulatory phase and therefore underwent oocyte power analysis calculations, active enrollment of the patients was continued between September 2017 and April 2018. Since the minimum number of patient samples required for the experiments were determined after power analysis calculations, active enrollment of the patients was continued until the required sample numbers were reached. A total of 154 IVF patients who consented to allow their GC to be used for research were finally included in the study. The high-responders had high-ovarian reserve but were not diagnosed with PCOS based on ovarian morphology, clinical and laboratory parameters.

The natural cycle IVF group consisted of 22 patients who presented for oocyte freezing at pre-ovulatory phase and therefore underwent oocyte retrieval after ovulation was induced with recombinant hCG (rhCG). The indications for oocyte freezing were elective (n = 18) and fertility preservation prior to cancer therapy (n = 4). The remaining 132 patients underwent COS with recombinant FSH using the GnRH agonist long protocol (n = 44) and antagonist protocol triggered with rhCG (n = 46) or GnRH agonist leuprolide acetate (Abbott Pharmaceutical Products, USA) (n = 42). While hCG-triggered GnRH agonist and antagonist protocols were mainly used for the patients who were predicted to have normal response to ovarian stimulation (4–15 oocytes), the agonist-triggered antagonist protocol was mainly used for the predicted high responders (≥16 oocytes) (Drakopoulos et al., 2016).

Isolation and culture of human luteinized GC from follicular fluid
Follicular fluids were obtained from IVF patients during the oocyte retrieval procedure and luteinized GC were isolated from this fluid as we described previously (Bildik et al., 2015). Oocyte retrieval was performed 36 h after ovulation trigger. Recovered luteal GC were processed and analyzed separately for each individual patient. The follicular aspirates from each patient were pooled in conical bottomed 50-ml centrifuge tubes and centrifuged at 500 x g for 5 min at room temperature. At this speed, the resulting pellet shows no layering. The supernatant was aspirated using a Pasteur pipette connected to a vacuum line. To remove erythrocyte contamination, the hypo-osmotic lysis technique was performed as described previously (Lobb and Youngblut, 2006). Sterile distilled water (9 ml) was added to the cell slurry remaining in the bottom of the tube after supernatant aspiration, and the tube was capped and mixed. After 30 s, 1 ml of 10x concentrated PBS, pH 7.4, was added and the tube was capped and mixed. The tubes were then centrifuged at 500 x g for 5 min at room temperature. The cell pellet was re-suspended in 0.5 ml of culture media and counted for cell number and viability in 0.2% trypsin blue on a hemocytometer. The recovered cells were either cultured or processed for other experiments. For culture, the cells were seeded in six-well format culture plates at a density of 25 000–50 000 cells per well using DMEM-F12 culture medium supplemented with 10% fetal bovine serum at 37°C and 5% CO2.

Viability assay
A live/dead cell assay was performed with YO-PRO-1 (1 μM), a green fluorescent carboxyfluor and acid stain absorbed by only apoptotic cells, whereas live cells are impermeable to it. Hoechst 33342 (1 μg/ml) was used for counterstaining. Live/dead cell imaging of the cells was undertaken under appropriate channels using a fluorescence microscope (IX71; Olympus, Tokyo, Japan).

Gene expression analysis
RNA isolation from GC was performed with Quick-RNA MicroPrep Kit (Zymo Research, Irvine, CA, USA) according to the manufacture’s instructions. RNA was quantified with a spectrophotometric read at 260 nm using Nanodrop 2000 (Thermo Fisher Scientific, MA, USA) and 500 ng cDNA was prepared by using the M-MLV Reverse Transcriptase (Invitrogen, CA, USA). The mean coefficient of variation in RNA yields of the samples was 7.2%, which appeared to have a small effect on the reliability of the results. The expression of mRNAs of interest was compared using quantitative real-time RT-PCR (qRT-PCR) and Light Cycler 480 SYBR Green I Master (Roche, Germany). The PCR primers for the genes investigated in the study (steroidogenic enzymes, LH receptor, progesterone/anti-steroidogenic genes, vascular endothelial growth factor (VEGF)) are shown in Table I. RT-PCR experiments were repeated four times. For each experiment, 3 different readouts were obtained for each gene of interest.

Hormone assays
Estradiol (E2) and P4 levels in culture media were determined using an electrochemiluminescence immunoassay (ECLIA) (Elecys and Cobas immunoassay analyzers; Roche Diagnostics, Germany). Lower detection limits for E2 and P4 were 5.00 pg/ml (18.4 pmol/ml) and 0.030 ng/ml (0.095 nmol/ml), respectively.

Immunoblotting
The 3β-hydroxysteroid dehydrogenase (3β-HSD) Type II (sc-100466), steroidogenic acute regulatory protein (StAR) (sc-166821) and cholesterol side-chain cleavage enzyme (p450Scc) (CYP11A1, sc-292456) monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (TX, USA). Mouse monoclonal antibody against human progesterone (LS-C194163) was purchased from LifeSpan BioSciences (Seattle, WA, USA). Aromatase (CYP19A, ab34193) monoclonal mouse antibody was from Abcam (Abcam Inc., MA, USA). Anti-vinculin antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as loading control at a dilution of 1:10 000. Cell lyses for western blot were prepared using radioimmunoprecipitation assay buffer (Sigma-Aldrich) as we described previously (Oktew et al., 2017). Immun-Blot® PVDF Membranes (BioRad, CA, USA) were
incubated overnight at 4°C with the antibodies at the concentrations recommended by the manufacturers.

### Statistical analysis
Samples size required for statistical significance and proper interpretation of the results were calculated based on the qRT-PCR assays. We have used the ΔΔCt method for relative quantitation of target gene mRNAs (Livak and Schmittgen, 2001; Oktem et al., 2017). The mean and SD were calculated from 3 different readouts taken for each target gene in the RT-PCR assay. As an example, the mean and SD of the target gene stAR were calculated after 3 different readouts taken for each individual sample of 22 natural cycle IVF patients, giving a total 66 (22 × 3) readouts with SD ranging from 0.05 to 0.1. Similar SDs were obtained in the readouts of other target genes. Therefore, the experiment will have a 80% power to detect a difference between the means of 0.09 with a significance level of 0.05 if we had at least 20 samples. mRNA levels of the target genes used in the qRT-PCR assay (steroidogenic enzymes, LH receptor, pro-apoptotic/anti-apoptotic genes, VEGF) and hormone levels are continuous variables therefore, expressed as the mean ± SD. ANOVA/ Bonferroni or Kruskal–Wallis/Dunn’s post hoc tests were applied to compare the groups if data are parametric or non-parametric respectively. The percentages of viable and apoptotic cells were compared between the groups using Fisher’s exact test. Significance level was set at 5% (P < 0.05), and the SPSS statistical program (version 22) (IBM Software, NY, USA) was used to analyze the data.

### Results
Demographic and IVF cycle characteristics of the patients are summarized in Table II. Natural and stimulated IVF cycles were comparable in terms of age, gonadotrophin dose and duration of stimulation. However, the peak E2 levels on the hCG day and the number of oocytes retrieved were significantly higher in the agonist-triggered antagonist cycles compared to the others. Serum P4 level on the hCG day was significantly lower in the natural cycles in comparison to the other types of stimulated cycles.

### Viability assay
First, we carried out a simple viability assay with green fluorescent carbocyanine uptake in the luteal GC immediately after they were recovered from follicular aspirates during oocyte retrieval procedure. We found that natural cycle GC were significantly more viable (88% viable cells) compared to their counterparts of the stimulated IVF cycles (66, 64% and 37% for agonist and antagonist cycles triggered with hCG and GnRH agonist, respectively, respectively, Fig. 1A and B).

### Comparison of the expression of steroidogenic enzymes and in vitro E2 and P4 production of the luteal GC
The expression of ovarian steroidogenic enzymes stAR, SCC, 3β-HSD and aromatase was significantly higher in GC from the agonist and hCG-triggered antagonist cycles compared to natural cycle in both RT-PCR and quantitative immunoblotting analyses (Fig 2A–C). 3β-HSD, VEGF and LH mRNA expressions in the cells obtained from agonist-triggered antagonist cycles were significantly lower than natural, agonist and hCG-triggered antagonist cycles (Fig 2A–C). In line with this finding, when plated at equal density, in vitro E2 and P4 production in the cells from the stimulated cycles was significantly higher than in those of natural cycles in the first days of culture. However, on the following days the hormone production in cells from the stimulated cycles began to decline gradually. By contrast, such a decline was not observed in the natural cycle GC, which continued to produce E2 and P4 hormones in increasing amounts during the same culture period.

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**Table I: Primers used in RT-PCR analysis.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>5′-Sequence-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ATGGAAATCCCATACCATCTTT</td>
</tr>
<tr>
<td>Reverse</td>
<td>CGCCCCACCTTGATTTTGG</td>
</tr>
<tr>
<td>STAR</td>
<td>AAACCTTAGGCTCTACGACATC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GACCTGGTGTAGTGTCTCTTG</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>GCCTTCAGACCAAGATGTAGAGA</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCCCTCAAGTGACTGACTTGG</td>
</tr>
<tr>
<td>CYP11A1 (SCC)</td>
<td>CAGGAGGGGGTGACAGCACAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGTGTGCGTCCCATCTCATAC</td>
</tr>
<tr>
<td>CYP19A1 (Aromatase)</td>
<td>GGTCACACGTTCTCTGCT</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCAAGCTCTCCTCATCAACCA</td>
</tr>
<tr>
<td>LH-R</td>
<td>TTGAACCTAGGTGTTGCTCTCACCA</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCCCTCAGGGTGTATGAGAGC</td>
</tr>
<tr>
<td>VEGFA</td>
<td>TTGGTCGGCGCTGTTGCTTAA</td>
</tr>
<tr>
<td>Reverse</td>
<td>TAACCTCAAGCTGCCTCGCT</td>
</tr>
<tr>
<td>AKT</td>
<td>GCAGCATCGCTCTTGGCG</td>
</tr>
<tr>
<td>Reverse</td>
<td>GCTGTCATCTGTCAGTGG</td>
</tr>
<tr>
<td>BCL2-L2</td>
<td>CGGGAGCTACAGCTCATCAC</td>
</tr>
<tr>
<td>Reverse</td>
<td>AAAAGCCCTCTACAGTACCA</td>
</tr>
<tr>
<td>BAD</td>
<td>CTCCGGAGGATGAGGTGAGAGT</td>
</tr>
<tr>
<td>Reverse</td>
<td>TTTCCCCTGCCAAAGGTCGA</td>
</tr>
<tr>
<td>BAX</td>
<td>CGGTGTTGTCGCCCCCTTCTAC</td>
</tr>
<tr>
<td>Reverse</td>
<td>GACAGGGGACATGACTGCTTCCAG</td>
</tr>
<tr>
<td>CASP3</td>
<td>CATGGAACGGAATCAATGGACT</td>
</tr>
<tr>
<td>Reverse</td>
<td>CTGTAACAGACGAGATGTC</td>
</tr>
</tbody>
</table>

Granulosa cell functions vary with mode of stimulation

The most defective steroid output was observed in the agonist-triggered antagonist cycles, with E2 and P4 production drastically reducing during the 6-day culture period (Fig 3A and B).

We also investigated if patient age and serum P4 level on the hCG day have any impact on the expression of steroidogenic enzymes and viability of the cells. There was an inverse correlation between patient age and the number of oocytes retrieved. However, neither patient age nor serum P4 level on the day of hCG trigger were associated with the level of expression of steroidogenic enzymes and viability of the cells (Supplementary Figs S1 and S2).

**Table II** Demographic characteristics of the patients in the study and details of the IVF cycles.

<table>
<thead>
<tr>
<th></th>
<th>Natural cycle</th>
<th>GnRH antagonist protocol (hCG trigger)</th>
<th>GnRH antagonist protocol (agonist trigger)</th>
<th>GnRH agonist (long) protocol</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>22</td>
<td>46</td>
<td>42</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>32.8 ± 3.5</td>
<td>31.2 ± 6.1</td>
<td>32.4 ± 4.4</td>
<td>31.8 ± 5.0</td>
<td>0.17</td>
</tr>
<tr>
<td>Duration of infertility (years)</td>
<td>-</td>
<td>3.9 ± 3.5</td>
<td>4.0 ± 2.9</td>
<td>4.0 ± 3.8</td>
<td>0.22</td>
</tr>
<tr>
<td>Starting gonadotrophin dose (IU)</td>
<td>-</td>
<td>295.1 ± 51.0</td>
<td>241.1 ± 60.9</td>
<td>313.6 ± 58.9</td>
<td>0.34</td>
</tr>
<tr>
<td>Duration of stimulation (days)</td>
<td>-</td>
<td>10.9 ± 1.9</td>
<td>11.1 ± 2.0</td>
<td>12.2 ± 2.0</td>
<td>0.19</td>
</tr>
<tr>
<td>Peak estradiol (pg/d)</td>
<td>224.6 ± 37.6</td>
<td>2799.5 ± 959.3</td>
<td>3987.7 ± 407.1</td>
<td>2810.2 ± 823.7</td>
<td>0.004</td>
</tr>
<tr>
<td>hCG day progesterone (ng/ml)</td>
<td>0.27 ± 0.1c</td>
<td>0.71 ± 0.26</td>
<td>0.94 ± 0.31</td>
<td>0.64 ± 0.29</td>
<td>0.006</td>
</tr>
<tr>
<td>Total number of oocytes</td>
<td>1.0 ± 0.0d</td>
<td>11.8 ± 4.9</td>
<td>17.7 ± 4.0d</td>
<td>12.5 ± 4.7</td>
<td>0.010</td>
</tr>
<tr>
<td>Metaphase II oocytes</td>
<td>1.0 ± 0.0</td>
<td>8.1 ± 2.1</td>
<td>14.2 ± 3.4</td>
<td>8.7 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Number of embryos transferred</td>
<td>N/A</td>
<td>1.5 ± 0.5</td>
<td>N/A</td>
<td>1.5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Clinical pregnancy rates</td>
<td>N/A</td>
<td>47.5%</td>
<td>N/A</td>
<td>48.3%</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SD. P values in the column were calculated by ANOVA or Kruskal–Wallis tests. The letters in superscripts show the p values obtained after multiple comparison post hoc test.

a, c, d: P < 0.01 natural cycle versus all other stimulated cycles.
b, c, d: P < 0.05 agonist-triggered antagonist cycle versus agonist and antagonist cycles triggered with hCG.
e: P < 0.01 agonist-triggered antagonist cycle versus agonist and antagonist cycles triggered with hCG. N/A: not applicable because freeze all strategy was adopted in these cycles.

hCG treatment up-regulates the expression of steroidogenic enzymes, LH receptor and anti-apoptotic genes and improves survival and P production of the luteal GC

We hypothesized that declining in vitro E2 and P4 productions in the luteal GC from the stimulated IVF cycles, and particularly in the agonist-triggered cycles, might be due to increased cell death. To test our hypothesis, we monitored viability of the cells for 6 days in culture and observed that natural cycle GC maintained their vitality in culture compared to their counterparts from the stimulated IVF cycles: at the end of 6-day culture period, 74% of the natural cycle GC were still viable compared to their counterparts from the stimulated IVF cycles especially in the agonist-triggered cycles, leading to more severely compromised luteal function. hCG administration appears to reverse these defects and improves viability and P4 output from these cells (Supplementary Figs S1 and S2).

We also investigated if patient age and serum P4 level on the hCG day have any impact on the expression of steroidogenic enzymes and viability of the cells. There was an inverse correlation between patient age and the number of oocytes retrieved. However, neither patient age nor serum P4 level on the day of hCG trigger were associated with the level of expression of steroidogenic enzymes and viability of the cells (Supplementary Figs S1 and S2).

BCL-L2 in the cells from the agonist and hCG-triggered antagonist cycles as well as additional defective mRNA expression of 3β-HSD and VEGF in the agonist-triggered antagonist cycles may account for compromised luteal function in the stimulated IVF cycles. Therefore, in another set of experiments we treated GC from the agonist-triggered cycles with rhCG for 24 h and observed that hCG treatment significantly improved the viability, up-regulated the mRNA expression of 3β-HSD, LH receptor, VEGF and BCL-L2, and increased P4 output from the cells compared to their counterparts not incubated with hCG (Fig 5).

**Discussion**

We have shown in this study that there are several differences in the molecular characteristics of luteal GC obtained from natural versus stimulated IVF cycles that might be related to defective luteal function in the latter. First, luteal GC obtained from natural cycles are more viable ex vivo and more capable of maintaining their vitality and steroidogenic activity in vitro in comparison to the stimulated IVF cycles. Second, natural cycle GC express LH receptor and anti-apoptotic genes at higher levels compared to their counterparts obtained from stimulated IVF cycles. Third, the mRNA expression of LH receptor, VEGF and 3β-HSD, the enzyme responsible for conversion of pregnenolone to progesterone, are most defective in the luteal GC from the agonist-triggered antagonist cycles, leading to more severely compromised luteal function. hCG administration appears to reverse these defects and improves cell viability and P4 output from these cells.

It is not clearly understood why luteal function is defective and requires exogenous support in the stimulated IVF cycles despite the presence of multiple corpora lutea. Several theories have been proposed to explain this phenomenon. It was initially thought that traumatic injury of corpora lutea and removal of some portions of luteinized mural GC during oocyte retrieval procedure cause defects...
in luteal steroid production. But this theory was later refuted because it was demonstrated that aspiration of a single follicle did not cause any luteal defect in women with normal ovulatory cycles (Kerin et al., 1981). Another theory holds that GnRH agonist and antagonists used to prevent a premature LH surge may cause a prolonged pituitary recovery resulting in low LH levels and defects in corpus luteum in the stimulated cycles (Smitz et al., 1992; Pritts and Atwood, 2002). Lack of LH was thought to be caused by hCG administration, however it was shown later that hCG administration does not reduce LH secretion in the natural cycle of normal ovulatory women (Tavaniotou and Devroey, 2003). Our current understanding suggest that supra-physiological levels of steroids produced by multiple corpora lutea directly inhibit LH release via negative feedback actions on the HPO axis (Fauser and Devroey, 2003; Fatemi, 2009). Human and primate studies documented that a constant LH stimulus is required for maintenance and steroidogenic activity of the corpus luteum (Jones, 1991). Therefore, the length of luteal phase is shortened due to premature luteolysis occurring secondary to low-levels of endogenous LH in stimulated IVF cycles. As another possibility, this phenomenon might also be related to ovarian physiology in human itself, which is characterized by mono-follicular development and the formation of a single corpus luteum. COS overrides the process of dominant follicle selection and produces multiple ovulatory follicles. If dominant follicle selection from a cohort of antral follicles is not a random event and there is a quality check, COS bypasses this process and allows the growth of other antral follicles, which would otherwise undergo atresia in a natural cycle. Resultant multi-follicular development may exceed the ability of ovary to produce multiple corpora lutea with adequate luteal function.

The question of why the most defective luteal function is observed in the antagonist cycles triggered with a GnRH agonist might be explained by the fact that the short half-life of the endogenous LH surge induced by the flare effect of exogenously administered GnRH agonist might not be sufficient to induce adequate expression of 3β-HSD, LH receptor and VEGF, which appeared to be defective in these cycles. Furthermore, LH and hCG action on the same receptor does not necessarily result in activation of the same signaling pathways as there appear to be some quantitative and qualitative differences in intracellular signaling after hCG and LH stimulation of LH receptors. Also the highest serum E2 levels are attained in the high-responders in whom ovulation is triggered with a GnRH agonist. It is therefore not surprising to anticipate the strongest negative feedback inhibition of the HPO axis and hence the lowest LH levels and most defective luteal function.

**Figure 1**  Live/dead cell assay with YO-PRO-1 green fluorescent carbocyanine uptake. Viability of the luteal granulosa cells (GC) immediately after they were recovered from follicular aspirates during oocyte retrieval procedure were analyzed by YO-PRO-1 uptake. Natural cycle GC were significantly more viable (88%) compared to their counterparts of the stimulated IVF cycles (66, 64 and 37% for agonist and antagonist cycles triggered with hCG and GnRH agonist respectively, scale bar: 50 μm).
Function in this group of IVF patients. Up-regulated expression of LH receptor, 3β-HSD and VEGF, plus enhanced viability and increased P₄ output after hCG “rescue” treatment of the cells obtained from these cycles not only provides a biological basis for the co-administration of hCG with a GnRH agonist to induce ovulation but also confirms one more time its critical role as a luteal phase support.

Figure 2 Comparison of the mRNA and protein expression of the steroidogenic enzymes in the luteal GC from natural versus stimulated IVF cycles. The expression of ovarian steroidogenic enzymes steroidogenic acute regulatory protein (stAR), cholesterol side-chain cleavage enzyme (SCC), 3β-Hydroxysteroid dehydrogenase (3β-HSD) and aromatase was significantly higher in the GC from the agonist and hCG-triggered antagonist cycles compared to natural cycle in both quantitative RT-PCR (qRT-PCR) (A) and quantitative immunoblotting analyses (B and C). However, 3β-HSD, vascular endothelial growth factor (VEGF) and LH expression was significantly lower in the cells from agonist-triggered antagonist cycles than natural, agonist and hCG-triggered antagonist cycles.

Figure 3 Comparison of in vitro estradiol and progesterone production of the luteal GC from natural versus stimulated IVF cycles. In vitro estradiol (E₂) and progesterone (P₄) production of the cells from the stimulated cycles were significantly higher than those of natural cycles in the first days of culture. However, on the following days their hormone production began to decline gradually. By contrast, such a decline was not observed in the natural cycle GC. They continued to produce E₂ and P₄ hormones in increasing amounts during the same culture period. The most defective steroid output was observed in the agonist-triggered antagonist cycles. Steroid production from the cells obtained from these cycles drastically reduced during 6-day culture period (A and B).
Previous studies showed that gene expression profiles of human GC might differ according the COS protocol as well as the mode of triggering for final oocyte maturation. Similar to our findings, Kaneko et al. (2000) observed that the incidence of GC apoptosis was lower in natural cycles compared to GnRH agonist stimulation cycles but somehow higher compared to hCG plus HMG stimulation cycles. Increased apoptosis of GC was initially linked to a reduction in oocyte quality, fertilization, pregnancy and live birth rate in several studies (Nakahara et al., 1997; Oosterhuis et al., 1998; Kaneko et al., 2000; Sifer et al., 2002). However, it was later recognized that apoptosis of luteal GC is a crude marker and has a poor predictive capability regarding oocyte quality and IVF success (Regan et al., 2018). With advancement of
molecular techniques later studies were more focused on the molecular characterization of GC. Borgbo et al. (2013) reported significant differences in human mural (MGC) and cumulus granulosa cell (CC) transcriptomes when hCG or GnRH agonist were used for triggering ovulation. One hundred and fifty-seven genes displayed a lower expression, and 234 genes displayed a higher expression in GnRH agonist-triggered CC as compared with CC from hCG-triggered follicles. Seventy-eight genes displayed a lower expression, and 174 genes displayed a higher expression in GnRH agonist-triggered MGC as compared with MGC from hCG-triggered follicles. The mRNA expression of LH receptor, CYP1A1 and HSD3B1 were found at significantly higher levels in the CC of agonist-triggered antagonist cycles in comparison to hCG-triggered ones. The authors did not observe such an increase in the expression of these genes in the MGCs, of which up-regulated expression was more significant for the genes involved in cell adhesion and extracellular matrix re-modeling. In both CC and MGC, LH was suggested as possible up-stream regulator. The up-stream regulator analysis predicted an activation of the LH and ERK regulatory cascade (extracellular signal regulated kinase of mitogen activated protein kinase family) in the CCs as differentially expressed genes, while no significant direction (activation or inhibition) prediction was present in the analysis of the MGC (Borgbo et al., 2013). Later on, the same group reported that follicular fluid steroid levels and gene expression patterns did not differ according to the dose of GnRH agonist (triptorelin) used to trigger ovulation (Vuong et al., 2017). Haas et al. (2016) compared hCG and dual trigger (hCG plus GnRH agonist) in terms of differential gene expression and showed that mRNA expression of amphiregulin and epiregulin (downstream regulators of epidermal growth factor signaling coupled to LH receptor activation during the ovulation process) were significantly higher in the dual trigger group compared with the hCG group.

Taken together, available data suggest that the mode of ovulation trigger is associated with an alteration in the gene expression profiles of CC and MGC. CC with their close proximity to, and extrusion with, the oocyte are more likely to be involved in the regulation of signaling pathways involved in oocyte maturation while the abundant MGC are a major source of progesterone production in corpus luteum (Russell et al., 2016). Given that the LH surge during ovulation has two specific purposes, namely final oocyte maturation and transition from a follicular phenotype to a functioning corpus luteum, it is not surprising to observe the differences in the up and down-regulation of different genes post activation of LH receptor in these two different types of luteal GC, even after an ovulation trigger with the same hormone or agent. While there is sufficient evidence that GnRH agonist as a final oocyte maturation in fresh autologous cycles is not only associated with a lower risk of OHSS but also with lower live birth rates, lower ongoing pregnancy rates and high risk of miscarriages in the first trimester (Youssef et al., 2014), it is unclear if triggering ovulation with a GnRH agonist alone or in combination with hCG improves mature oocyte yield, fertilization and pregnancy rates in comparison to hCG-triggered cycles (Humaidan et al., 2005; Oktay et al., 2010; Ding et al., 2017; Elias et al., 2017; Chen et al., 2018). In our study, we did not find any significant difference between the antagonist cycles triggered with hCG versus agonist in terms of the numbers of total and mature oocytes retrieved.

Our study has several importation limitations. First, we simply showed that luteal GC obtained from the stimulated IVF cycles are less capable of maintaining their vitality and steroidogenic activity in vitro and have reduced LH receptor expression when compared to their counterparts from natural cycles as a possible explanation of defective luteal function in the former. We did not identify the underlying novel molecular pathogenetic mechanism(s) that links ovarian stimulation itself to the observed defects in luteal function in a cause and effect relationship. Second, the corpus luteum is a transient endocrine organ that is composed of different compartments and cell types (Duncan, 2000). Its formation and regression (luteolysis) are characterized by a marked tissue re-modeling and vascular involution that involves many different cell types, signaling pathways, inflammatory mediators and the intriguing interactions between them. Therefore, luteinized GC are only one type of cell and might not reliably represent the actual in vivo mechanisms that regulate the survival and function of the whole corpus luteum. Third, the luteal phase of a typical menstrual cycle consists of different stages known as early, mid and late (Duncan, 2000). Murine luteinized GC immediately recovered during an oocyte retrieval procedure belong to a very early stage of the luteal phase and information gathered during this phase might not be true for the rest of the phase. Fourth, ovulation was triggered with hCG in the natural cycles in our study in order to time oocyte retrieval accurately. Therefore, murine luteinized GC obtained from these cycles may not truly represent native cycles in which ovulation is triggered naturally by the mid-cycle surge of endogenous LH. Fifth, since ovarian stimulation was achieved with recombinant FSH in our study, we do not know if LH supplementation would have any effect on the viability and the expression of steroidogenic enzymes, LH receptor and VEGF in the luteal GC. Sixth, there could be some differences inherent to the clinical characteristics of the patients undergoing natural versus stimulated IVF cycles that might have affected the results that we obtained. As a final limitation, it was not possible to correlate the stimulation-related molecular alterations in luteal GC with the clinical outcome since the oocytes retrieved in natural IVF cycles were all frozen and none have been utilized yet.

**Conclusion**

In this study we have provided the molecular evidence that COS itself and the mode of ovulation trigger are associated with significant alterations in the viability and steroidogenic activity of luteal GC. In addition to the negative feedback action of supra-physiological steroid levels on LH release in the HPO axis, the reduced cell viability, and suppressed expression of 3B-HSD, LH receptor and VEGF as well as an increased propensity to undergo apoptosis might all contribute to compromised steroid production in luteal GC and the luteal phase defects observed in the stimulated IVF cycles. It is unclear if a low-LH environment created during the course of COS is the only factor responsible for the observed defects in the luteal GC or some other mechanisms are also operative. As an additional finding, we have demonstrated that molecular characteristics of the luteal GC obtained from normal responding IVF patients undergoing ovarian stimulation with GnRH agonist long versus hCG-triggered antagonist protocols are similar in terms of viability and the expression of steroidogenic enzymes and LH receptor expression. A more thorough analysis of luteal function at the molecular level may help us to better understand the luteal phase and develop new strategies to improve it in assisted reproduction.
Supplementary data

Supplementary data are available at Human Reproduction online.

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Authors’ roles

G.B., N.A., A.S. and Y.E. conducted experiments. K.Y. conducted experiments and helped in writing the manuscript. B.B., B.A., I.K. and B.U. contributed to the provision of the cells. O.O. contributed to the conception of the work, designing experimental methodologies, provision of the cells, conducting statistical analyses, interpretation of the results and writing and approving the final form of the manuscript. All authors approved the final form of the manuscript.

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Conflict of interest

All authors declare no conflict of interest.

References


Granulosa cell functions vary with mode of stimulation


