In vitro viability and secretory capacity of human luteinized granulosa cells after gonadotropin-releasing hormone agonist trigger of oocyte maturation

Lawrence Engmann, M.D.,[a] *Jonathan Romak, M.D.,*[b] *John Nulsen, M.D.,*[a] *Claudio Benadiva, M.D.,*[a] *and John Peluso, Ph.D.*[c]

[a] Center for Advanced Reproductive Services, Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, and [c] Department of Cell Biology, University of Connecticut Health Center, Farmington, Connecticut; and [b] Department of Otorhinolaryngology, Mayo Clinic, Rochester, Minnesota

Objective: To evaluate viability of luteinized granulosa cells obtained from patients triggered with either GnRH agonist or hCG and to assess the secretion of steroids and vascular endothelial growth factor (VEGF) by cultured luteinized granulosa cells in the presence or absence of hCG.
Design: Prospective, randomized controlled trial.
Setting: University-based fertility center.
Patient(s): A subset of patients who underwent a randomized trial involving GnRH agonist trigger after GnRH antagonist protocol vs. hCG trigger after pituitary suppression with GnRH agonist protocol.
Intervention(s): In vitro fertilization cycles.
Main Outcome Measure(s): Proportion of apoptosis; basal and hCG-induced secretion of E_2, P, and VEGF by luteinized granulosa cells; follicular-fluid VEGF and luteal-phase serum E_2, P, and plasma VEGF concentrations.
Result(s): There were no differences in the proportion of granulosa/luteal cell apoptosis, follicular-fluid or luteal-phase plasma VEGF concentration, or basal culture media E_2, P, and VEGF concentrations between the two groups. Addition of hCG to the culture media significantly increased the P concentration in both groups, but there were no changes in E_2 or VEGF concentrations. Serum E_2 levels were lower at 5 and 9 days after GnRH agonist compared with hCG trigger.
Conclusion(s): The granulosa/luteal cells obtained on the day of oocyte retrieval after GnRH agonist trigger are still viable and have the capacity to respond to hCG by increasing the secretion of steroids. (Fertil Steril® 2011;96:198–202. ©2011 by American Society for Reproductive Medicine.)

Key Words: VEGF, apoptosis, granulosa cells, granulosa/luteal cells, follicular fluid, GnRH agonist trigger

Several studies have shown that the use of GnRH agonist to induce endogenous LH surge and subsequent oocyte maturation in high-risk patients prevents the development of ovarian hyperstimulation syndrome (OHSS) (1–3). The exact mechanism for the prevention of OHSS has not been fully elucidated. Nevertheless, it has been proposed that the shorter half-life of the endogenous LH surge induced by a single administration of GnRH agonist and the subsequent pituitary suppression and withdrawal of LH support for the corpora lutea (CL) leads to early CL demise (3).

The CL dysfunction may result in a consequent failure to secrete steroids and express certain angiogenic factors, such as vascular endothelial growth factor (VEGF), which have been implicated in the pathogenesis of OHSS. Previous studies showing low luteal-phase serum steroidal concentration (4, 5), as well as other markers of luteal activity, such as inhibin A and pro-alphaC (6), confirm that CL

dysfunction occurs after GnRH agonist trigger. However, there are relatively few previous studies evaluating luteal-phase circulating VEGF levels after GnRH agonist trigger (1, 7) and no studies evaluating the functional capacity of granulosa/luteal cells after GnRH agonist trigger.

One of the associated consequences of CL dysfunction is a potential detrimental effect on implantation rates (8, 9). In an effort to restore partial CL function and improve implantation rate, certain groups have proposed that administration of low-dose hCG approximately 35 hours after GnRH agonist administration will restore CL function and endometrial receptivity (10). However, there is no evidence to support the notion that the granulosa/luteal cells are still viable and responsive to exogenous hCG administration 35 hours after GnRH agonist trigger.

The specific aims of this study were to evaluate viability of granulosa/luteal cells obtained from patients triggered with either GnRH agonist or hCG and to assess the secretion of steroids and VEGF by cultured granulosa/luteal cells in the presence or absence of hCG. We also evaluated the follicular-fluid VEGF and luteal-phase circulating VEGF and steroid profile after triggering of oocyte maturation with either GnRH agonist or hCG.

MATERIALS AND METHODS
Study Population
The study population consisted of a subgroup of a prospective, randomized, controlled trial involving patients undergoing IVF treatment at the Center for

Advanced Reproductive Services at the University of Connecticut Health Center. The clinical outcome of the whole study population has been previously reported elsewhere (2). A subgroup of these patients agreed to take part in a substudy that involved obtaining follicular fluid and plasma for further analysis. The subjects were recruited for a period of 20 months between August 2004 and March 2006. Approval for this study was obtained from the institutional review board at the University of Connecticut Health Center.

Treatment Protocol

The treatment protocols used for the two groups, as well as the recruitment and randomization of the patients, have been previously described (2). In brief, the patients underwent a controlled ovarian stimulation protocol consisting of either GnRH agonist trigger (leuprolide acetate, 1.0 mg) after cotreatment with GnRH antagonist (study group) or hCG trigger after pituitary suppression with GnRH agonist (control group). Transvaginal ultrasound-guided oocyte retrieval was performed 35 hours after leuprolide or hCG administration, and ET was performed 72–76 hours after oocyte retrieval. Both groups received luteal-phase and early pregnancy supplementation with IM P, and patients in the study group also received additional E_2 patches, and their doses were adjusted according to serum levels as previously described (2).

Follicular Fluid and Granulosa/Luteal Cell Isolation

The procedure for cell isolation and culture has previously been described elsewhere (11). In brief, once oocytes have been isolated after oocyte retrieval, follicular aspirates were pooled and centrifuged at $250 \times g$ for 10 minutes. The follicular fluid was then decanted and stored in a freezer at $-20°C$ for future analysis. The cell pellet was then suspended in serum-free culture medium, layered on histopaque-1077, and centrifuged for 30 minutes at $400 \times g$. After centrifugation, the opaque interface containing the granulosa/luteal cells was carefully aspirated and transferred into a 15-mL sterile conical centrifuge tube. The cells were then washed by resuspending in 12 mL of phosphate-buffered saline and centrifuging them at $250 \times g$ for 10 minutes. This step was then repeated two additional times. The cell pellet was then resuspended in 1 mL of 0.25% trypsin–ethylenediaminetetraacetic acid solution and incubated for 5 minutes to dissociate the cells. After trypsinization, 5 mL of serum-supplemented medium was added and the cells centrifuged at $250 \times g$ for 10 minutes. The cells were then resuspended in serum-free culture medium, counted in a hemocytometer, and resuspended to yield a final concentration of 1×10^6 cells/mL.

Detection of Apoptotic Nuclei

Identification of apoptotic nuclei was achieved by in situ DNA staining done by adding hydroethidine directly to the culture medium at a final concentration of 3.5 μg/mL (12), and the cultures were incubated for 15 minutes at room temperature in the dark. The cells were then observed under an epifluorescent optics after staining. Under these conditions, only cells with condensed or fragmented nuclei were intensely stained with hydroethidine. These cells were considered to be apoptotic (12). One hundred cells per culture well were then counted and the percentage of apoptotic nuclei in each well documented. These values were used to determine the percentage of apoptotic granulosa/luteal cells.

Isolation of Spent Media from Cultured Granulosa/Luteal Cells

Sixty-millimeter dishes (BD Bioscience) that had been previously coated with Growth Factor Reduced Matrigel Matrix (BD Bioscience) were plated with the isolated granulosa/luteal cells at 5×10^5 cells per dish in 5 mL of serum-free medium with and without the addition of 2 IU/mL of hCG. The cells were then incubated for 3 hours to allow the granulosa/luteal cells to plate down. The cells were then washed twice with serum-free media to remove any remaining blood cells or nonattached granulosa/luteal cells. The cultures in the presence and absence of hCG were then continued for an additional hour to allow secretion of steroids and VEGF into the media. After 1 hour, the spent media was then aspirated and centrifuged in the

cold room and 3 mL aspirated and stored in the freezer at $-20°C$ for future analysis of E_2, P, and VEGF.

Measurement of VEGF, E_2, and P Concentrations

Two blood samples were obtained from each patient on the day of trigger of oocyte maturation, day after trigger, day of ET (5 days after trigger), and 7 days after oocyte retrieval (9 days after trigger or midluteal). One sample was placed in a serum tube and immediately analyzed for steroid levels. The other sample was placed in tubes containing ethylenediaminetetraacetic acid as anticoagulant and immediately centrifuged and the plasma obtained and immediately frozen at $-80°C$ for subsequent analysis of VEGF concentrations.

Culture media, plasma, and follicular-fluid samples were assayed for VEGF using an immunoassay kit (BioSource) according to the manufacturer's instructions. The sensitivity of the VEGF assay was <5 pg/mL, and the intra- and interassay coefficients of variation were $<5.5\%$ and $<9.3\%$, respectively, as quoted by the producer. Each sample was run in duplicate.

Estradiol and P levels in culture media were determined using an ELISA kit (BioQuant) according to the manufacturer's instructions. The assay sensitivity for E_2 was 10 pg/mL, and the intra- and interassay coefficients of variation were $<24.1\%$ and $<26.7\%$, respectively. The sensitivity of the assay for P was 0.3 ng/mL, and the intra- and interassay coefficients of variation were $<7.1\%$ and $<12.6\%$, respectively. All analyses were performed by one investigator who was blinded to the treatment allocation.

Outcome Variables

The main outcome variables were the proportion of apoptotic granulosa/luteal cells, as well as baseline and hCG-stimulated secretion of E_2, P, and VEGF by the granulosa/luteal cells. Other outcome variables included follicular-fluid VEGF and luteal-phase serum E_2, P, and plasma VEGF profile. The percentage of viable and apoptotic cells was evaluated in the two groups by determining the total number of granulosa/luteal cells and apoptotic cells.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences. (release 17.0; SPSS). The independent-sample t test and paired t test were used where appropriate for continuous variables. A two-way analysis of variance was also used for continuous variables where appropriate. Data are presented as mean $\pm$ SD unless otherwise stated. All P values quoted are two-sided, and values $<.05$ were taken to indicate statistical significance.

RESULTS

Blood samples for circulating VEGF and steroids were obtained from 33 patients, 16 in the study group and 17 in the control group. A subset of these patients consisting of 16 subjects also underwent further analysis of granulosa/luteal cells and culture media secretion of steroids and VEGF and follicular-fluid VEGF, 10 in the study group and 6 in the control group.

There were no differences between the whole subset of patients in the study and control groups in terms of age (32.5 ± 3.1 years vs. 33.0 ± 3.6 years) and baseline serum FSH levels (5.4 ± 1.5 IU/L vs. 5.2 ± 1.3 IU/L). There were also no differences in the duration of ovarian stimulation (9.8 ± 2.0 days vs. 9.7 ± 2.0 days), total dose of gonadotropins required for ovarian stimulation ($1,618 \pm 481$ IU vs. $1,477 \pm 564$ IU), number of oocytes retrieved (22.5 ± 10.3 vs. 19.4 ± 11.3), proportion of mature oocytes ($86.7\% \pm 12.6\%$ vs. $73.0\% \pm 18.4\%$), or fertilization rate ($68.8\% \pm 12.6\%$ vs. $73.0\% \pm 18.4\%$).

Despite the use of luteal-phase E_2 supplementation in the study but not in the control group, the serum E_2 levels 5 days after trigger and during the midluteal phase were significantly lower in the study compared with the control group (Fig. 1A). Further, the serum P was significantly lower 5 days after trigger in the study compared with the control group, although the midluteal levels did not differ

FIGURE 1

Serum E₂ (**A**), P (**B**), and VEGF (**C**) concentrations on the day of trigger, day after trigger, and 5 days and 9 days after trigger between the GnRH agonist and hCG trigger. *$P < .01$.
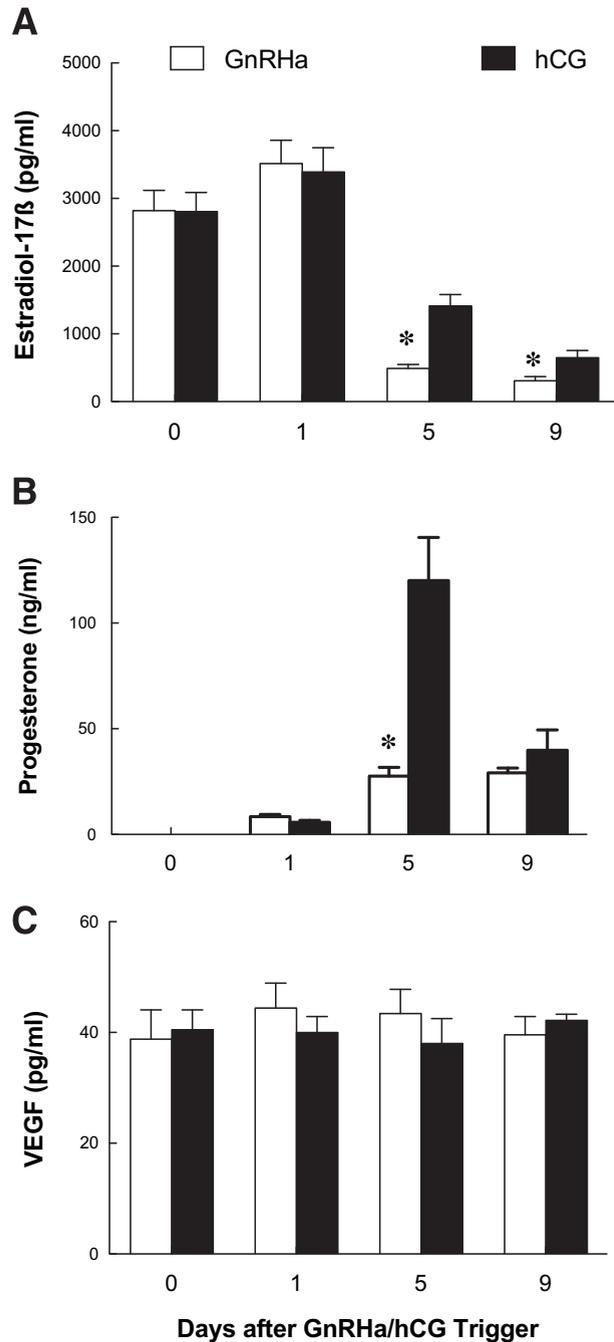
Engmann. Granulosa cell viability after GnRHa trigger. Fertil Steril 2011.

FIGURE 2

In vitro secretion of E₂ (**A**), P (**B**), and VEGF (**C**) concentrations at basal conditions and after addition of hCG in the GnRH agonist and hCG trigger groups. *$P < .05$.
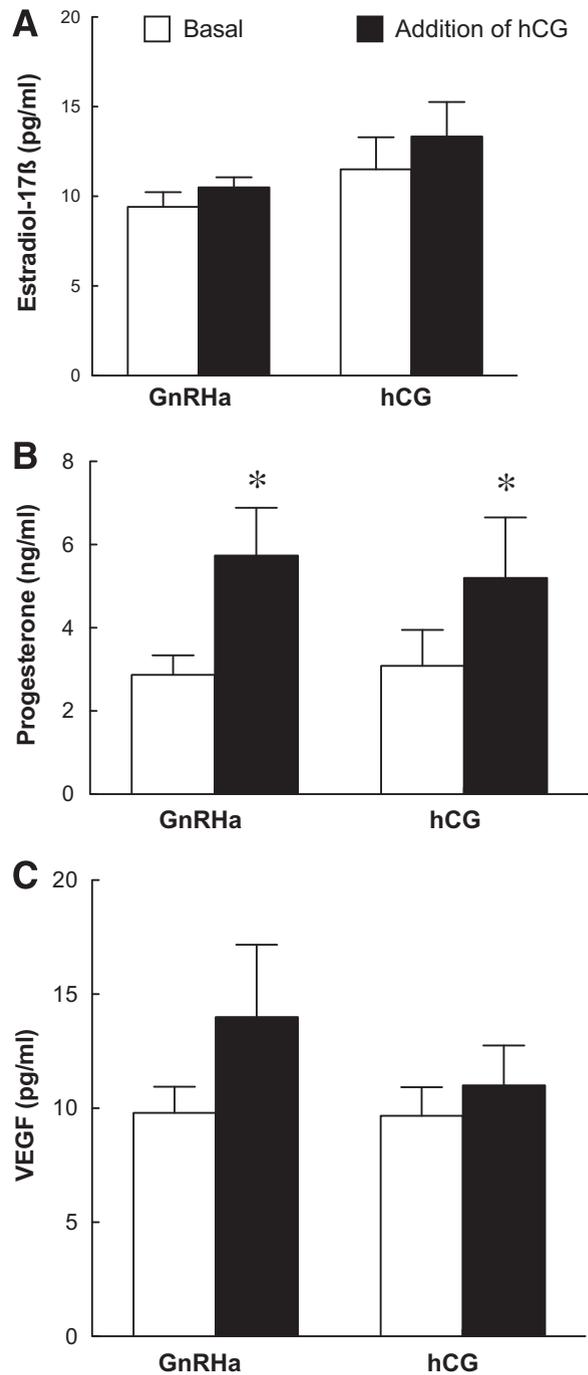
Engmann. Granulosa cell viability after GnRHa trigger. Fertil Steril 2011.

(Fig. 1B). There were no significant differences between the two groups in terms of luteal-phase plasma VEGF levels (Fig. 1C). The follicular-fluid VEGF levels did not differ between the study (468 ± 272 pg/mL) and control groups (556 ± 213 pg/mL) ($P > .05$).

There were no significant differences in the proportion of granulosa/luteal cell apoptosis between the study and control groups ($22.2\% \pm 8.2\%$ vs. $22.2\% \pm 7.2\%$, respectively; $P > .05$). There were also no significant differences between the study and control groups in the basal culture media E₂, P, and VEGF concentrations (Fig. 2). Addition of hCG to the culture media significantly increased the P concentration in both groups but did not affect E₂ or VEGF secretion in either group (Fig. 2).

DISCUSSION

To the best of our knowledge, this is the first study to evaluate the viability and steroidogenic capacity of luteinized granulosa cells after GnRH agonist trigger. The results suggest that the granulosa/luteal cells obtained 2 days after GnRH agonist trigger are viable in view of the similar proportion of apoptotic granulosa/luteal cells as that obtained after hCG trigger. Moreover, the granulosa/luteal cells are functionally responsive to exogenous hCG administration 35 hours after GnRH agonist trigger: they had the same capacity to secrete steroids in response to hCG.

It has now been shown by several studies that the administration of GnRH agonist to induce final oocyte maturation is an effective protocol in the prevention of OHSS (1–3). Although the exact pathophysiology of OHSS prevention has not been fully elucidated, the administration of a single dose of GnRH agonist results in a defective CL function, as shown by the lower luteal-phase serum P and E_2 levels in this study as well as previous studies (4, 5). Studies in primates have demonstrated the presence of GnRH receptors in the ovary, as well as a local effect of GnRH agonist on the induction of oocyte maturation (13–15), which will suggest a direct local effect on the granulosa/luteal cells, leading to apoptosis. However, the present in vitro studies demonstrate that the granulosa/luteal cells isolated from patients triggered with GnRH agonist have the same viability and capacity to secrete P as those of patients triggered with hCG. Moreover, granulosa/luteal cells from both groups secreted the same amount of P in response to hCG. Thus, the lower luteal-phase serum P levels observed after GnRH agonist trigger is not due to a fundamental alteration in the granulosa/luteal cells but rather a lower exposure to endogenous factors that stimulate steroidogenesis, such as the shorter half-life of the GnRH agonist–induced LH surge.

Similarly, it has been hypothesized that defective CL function induced by GnRH agonist trigger results in a decreased release of vasoactive peptides such as VEGF, which has been implicated in the pathogenesis of OHSS (16). In fact, previous studies have shown that GnRH agonist trigger results in significantly lower VEGF levels in follicular fluid (7, 17) and in mRNA expression in granulosa cells (17). However, the present study did not show any differences in the follicular-fluid VEGF concentrations after GnRH agonist or hCG trigger. This is consistent with the other findings of this study that the granulosa/luteal cells are viable 2 days after trigger and have normal functional secretory capacity and therefore normal follicular-fluid VEGF secretion.

The observation that granulosa/luteal cells obtained after GnRH agonist trigger are viable is important because we have also shown that these cells respond to hCG in a manner identical to those cells

isolated after hCG treatment. Thus, our results provide a scientific basis for previous studies that have shown improvement in pregnancy rates after GnRH agonist trigger when low-dose hCG is administered at the time of oocyte retrieval (18). This implies that administration of low-dose hCG approximately 35 hours after GnRH agonist trigger may be effective in stimulating partial CL function to improve implantation rates because the granulosa/luteal cells will still be viable. The findings may also suggest that although CL demise occurs after GnRH agonist trigger, this may not occur until several days later. This is corroborated by the fact that serum LH levels after GnRH agonist trigger peak at 4 hours and return to baseline levels by the day of oocyte retrieval (19). The high proportion of viable cells may also represent normal oocyte competence on the day of retrieval, which has been confirmed by the excellent number of oocytes and proportion of mature oocytes and fertilization rates after GnRH agonist trigger reported by several studies (2, 8).

Circulating VEGF level increases significantly after hCG administration and then declines during the midluteal phase in patients who do not develop OHSS (20). In view of the early luteolysis that occurs after GnRH agonist trigger, it may be postulated that circulating VEGF levels will be significantly lower in the luteal phase, which may be the pathophysiological basis for the significantly lower rate of OHSS reported in several studies (1, 2, 21, 22). However, we did not show any significant differences in luteal-phase VEGF levels between patients triggered with either GnRH agonist or hCG. These findings are consistent with those of other studies that did not find any significant differences in luteal-phase serum or plasma VEGF levels (1, 7, 17) and may be related to methodologic issues with the VEGF analysis (23).

One may argue that the use of different ovarian stimulation and luteal-phase steroid supplementation protocols in the two groups may have introduced bias in the study and influenced interpretation of the hormonal profile results of the study. However, it is unlikely the differences in protocols affected the main findings of the study, which showed that luteinized granulosa cells obtained after GnRH agonist trigger were still viable and responsive to hCG.

In conclusion, we have shown that the granulosa/luteinized cells obtained on day of oocyte retrieval after GnRH agonist trigger are still viable and have the capacity to respond to hCG by increasing the secretion of steroids. This serves as a basis for adjuvant low-dose hCG administration after GnRH agonist trigger to improve CL function.

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