Coasting acts through downregulation of VEGF gene expression and protein secretion

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BACKGROUND: This study was conducted to investigate the mechanisms by which coasting may be effective in decreasing the incidence of ovarian hyperstimulation syndrome (OHSS). METHODS: A total of 160 women (patients and oocyte donors) undergoing coasting and 116 controls were included in the study. Serum, follicular fluid and granulosa cells were collected on the day of oocyte retrieval. Vascular endothelial growth factor (VEGF) concentrations were determined using an enzyme-linked immunosorbent assay (ELISA). Real-time PCR was performed to evaluate VEGF gene expression in granulosa cells. Cell death was studied by flow cytometry using annexin V–fluorescein isothiocyanate (FITC) and counterstaining by propidium iodide, and double staining with CD45 monoclonal antibody was performed to distinguish the contamination of apoptotic leukocytes. RESULTS: Follicular cells aspirated from coasted patients showed a ratio in favour of apoptosis, especially in smaller follicles (48 versus 26%, \( P < 0.05 \)). Follicular fluid determinations confirmed that coasting reduces VEGF protein secretion (1413 versus 3538 pg/ml, \( P < 0.001 \)) and gene expression (2-fold decrease) in granulosa cells. Follicular fluid VEGF protein levels positively correlated with follicular size \( (r = 0.594, P = 0.001) \) and estradiol production \( (r = 0.558, P = 0.038) \). Women who underwent coasting showed a comparable IVF cycle outcome; however, a higher cancellation rate was found in cycles that were coasted. CONCLUSIONS: Coasting affects all follicles through apoptosis, especially immature follicles, without affecting oocyte/endometrial quality. The significant decrease found in VEGF expression and secretion explains why coasting is clinically effective in reducing the incidence and severity of OHSS.

Key words: coasting/ovarian hyperstimulation syndrome/VEGF

Introduction

Ovarian hyperstimulation syndrome (OHSS) is a severe complication of gonadotrophin treatment in infertile women that occurs in a severe life-threatening form in 0.1–2% of assisted reproduction treatment (ART) cycles (Wheelan and Vlahos, 2000). The main clinical characteristic is excessive fluid accumulation due to increased vascular permeability. Both clinical forms are HCG related: the early-onset form occurs 3–5 days after administration of exogenous HCG and is strongly related to high ovarian response, while the late-onset form presents 12–15 days after ovulatory HCG and is related to pregnancy-induced HCG production, highly associated with multiple pregnancies. Among the several preventive measures to avoid this life-endangering complication, diminishing the ovarian response (in early OHSS) and avoiding multiple pregnancies (in late OHSS) are key factors (Fluker et al., 1999).

A novel approach to avoid early OHSS is to withhold gonadotrophin stimulation during ovarian stimulation whilst continuing pituitary desensitization with GnRH analogues (coasting) until the serum estradiol (E2) levels drop (Delvigne and Rozenberg, 2002). The clinical benefit of this approach of reducing the incidence of OHSS without hampering oocyte and/or embryo quality has been shown by our group and others (Sher et al., 1993; Benadiva et al., 1997; Lee et al., 1998; Tortoriello et al., 1998; Fluker et al., 1999; Waldestrom et al., 1999; Al-Shawaf et al., 2001; Isaza et al., 2002). Of all the different options to reduce the incidence of OHSS, coasting is the first choice among physicians, as shown in a recent survey (Delvigne and Rozenberg, 2001). However, there are no experimental data offering a plausible biological explanation about why coasting is effective. Probably, mature follicles will survive for a few days without exogenous FSH/HMG while smaller follicles will enter apoptosis/necrosis, reducing the potential granulosa cell population that will release vascular mediators after HCG administration.

Vascular endothelial growth factor (VEGF) is clearly implicated in the pathogenesis of OHSS, and it seems to be the principal mediator of the action of HCG (Rockwell et al., 2002; Wang et al., 2002). VEGF, and more specifically its
isoform VEGF\textsubscript{165}, is expressed and produced in granulosa-lutein cells in response to HCG, and it is released into the follicular fluid, inducing increased capillary permeability (Yan \textit{et al.}, 1993; McClure \textit{et al.}, 1994). We have shown recently that the ovary is the main source of VEGF, which acts through the VEGF receptor-2 to increase vascular permeability, an event that may be blocked by a specific VEGF receptor-2 inhibitor (Gómez \textit{et al.}, 2002).

The aims of the present study were (i) to evaluate the outcome of IVF in coated cycles as well as the clinical usefulness of the coating procedure in preventing OHSS in women undergoing controlled ovarian stimulation (COS); (ii) to investigate the mechanism by which coating may be acting, probably by VEGF regulation; and (iii) to determine which follicle population is more likely to be affected by coating.

\section*{Materials and methods}

\subsection*{Subjects and protocols}

From May 1999 to March 2003, 85 patients and 74 oocyte donors underwent COS for IVF, showing a high response to ovarian stimulation and therefore coated as previously described (Isaza \textit{et al.}, 2002). The age of the patients ranged from 24 to 40 years (mean age 33.6 years); donors' age ranged from 18 to 34 years (mean age 25.4 years). Body mass index [weight (kg)/height squared (m\textsuperscript{2})] was 22.5 and 22.1 for patients and donors, respectively. They were thoroughly informed about the coating procedure and the fact that it does not completely abolish the risk of developing OHSS. All patients and donors gave informed consent to participate in the study, which was approved by our scientific board.

All subjects underwent a long protocol of downregulation with a GnRH agonist (tiptoremil; Decapeptyl\textsuperscript{R}, 0.1 mg; Ipsen Pharma, Barcelona, Spain). A basal vaginal ultrasound was performed to ascertain ovarian quiescence on the first 3 days of menses, and then ovarian stimulation was started as previously described (Garcia-Velasco \textit{et al.}, 2001). Briefly, they received recombinant FSH (Gonal F\textsuperscript{R}, Serono, Madrid, Spain) 300 IU/day for the first 3 days and then individual dose adjustment as required according to serum E\textsubscript{2} levels and ovarian response until the leading follicles were >17 mm in mean diameter. HCG (Profasi\textsuperscript{R}, 5000 IU; Serono) was administered and ovarian puncture was performed 36 h later.

\subsection*{Coasting procedure}

According to our institutional criteria as well as to the described series, if more than 20 follicles >18 mm and/or a serum E\textsubscript{2} level >4000 pg/ml were observed, patients/donors were considered to have a high response to ovarian stimulation, which made them an extremely high risk population for the development of OHSS. Gonadotrophin administration was withheld and the GnRH agonist was maintained (coasting), until daily measurements of serum E\textsubscript{2} levels showed that the level had decreased to <3500 pg/ml. HCG (5000 IU) was then administered and ovarian puncture was performed 36 h afterwards. If an abrupt fall was observed in serum E\textsubscript{2} levels (<1000 pg/ml) or if coasting required >5 days, egg retrieval was cancelled as oocyte quality might be affected (Isaza \textit{et al.}, 2002).

Follow-up was performed by ultrasonographic and clinical evaluation of the patient 7 days after oocyte retrieval, and they were encouraged to contact us at any time if minimum symptoms of OHSS developed. OHSS was classified (Golan \textit{et al.}, 1989) as: mild, when abdominal discomfort/distension appeared; moderate, when there also was sonographic evidence of ascites; and severe, when there was ascites plus changes in renal function, coagulation abnormalities, haemoconcentration and trouble breathing with or without hydrothorax. When OHSS occurred, treatment for each individual case was recorded.

\subsection*{Control group}

Current knowledge of the risks of severe OHSS in women with extremely high serum E\textsubscript{2} levels made it unethical to establish a control group of patients in similar conditions in terms of ovarian stimulation (number of follicles/serum E\textsubscript{2} levels). Thus, we compared the clinical outcome as well as the experimental data from patients who were coated with a group of patients with a good response to COS who did not require any strategy to prevent OHSS (E\textsubscript{2} on the day of HCG <5500 pg/ml, but >1000 pg/ml and >6 mature follicles). Cycles from the control group (n = 116) were selected as the next patient who met the requirements whose oocyte retrieval was performed immediately after the coated patient.

\subsection*{Sample collection}

\textit{Follicular fluid and granulosa-lutein cells.} Follicular fluids were aspirated from 200 follicles into 10 ml tubes by transvaginal ultrasonographic-guided oocyte retrieval. Selected follicles of >14 mm and <14 mm were individually aspirated after being measured in two dimensions. The needle was withdrawn and completely emptied prior to each puncture, and no culture medium was used in the collection tubes. After removal of oocytes, follicular aspirates were centrifuged at 200 g for 5 min and supernatant was stored at ~80°C until assayed. The cell pellets were resuspended in 2 ml of Dulbecco’s modified Eagle’s medium (DMEM), layered over 45% Percoll gradients (Pharmacia, St Albans, UK) and centrifuged for 20 min at 4°C and 400 g to isolate granulosa-lutein cells from erythrocytes. Then, luteinized granulosa cells were recovered from the interface, washed in DMEM and centrifuged (600 g, 5 min). The supernatant was discarded, and cells were frozen at ~80°C for isolation of RNA.

\textit{Serum samples.} Blood was withdrawn by venipuncture and collected in 10 ml sterile tubes, and were kept at room temperature. No more than 2 h elapsed between blood collection and serum processing in order to avoid changes in serum VEGF concentrations according to clotting duration. Serum aliquots were stored at ~80°C until further analysis.

\subsection*{Immunoreactive VEGF protein measurement}

Concentrations of soluble VEGF\textsubscript{165} in serum and follicular fluid were determined with a commercially available enzyme-linked immuno-sorbent assay (ELISA) kit (Quantikine; R&D Systems, Abingdon, UK), according to the manufacturer’s instructions. The assay sensitivity was 5 pg/ml. The inter- and intra-assay coefficients of variation were <7 and < 4.5\%, respectively.

\subsection*{Hormonal measurements}

Serum and follicular fluid E\textsubscript{2} was analysed using microparticle enzyme immunoassay kits (Abbot AxSYM, Abbot Park, Chicago, IL). The inter- and intra-assay variability for E\textsubscript{2} at a concentration of <40 pg/ml was 2.8 and 4.3\%, respectively.

\subsection*{mRNA expression of VEGF in granulosa-lutein cells}

\textit{RNA isolation.} RNA extraction was performed according to the method described by Chomczynski and Sacchi (1987) with minor modifications using the Trizol reagent. Briefly, cell weight was quantified and 500 \textmu l of Trizol reagent/100 mg cell weight were added. Total RNA was separated from DNA and proteins by adding
250 μl of chloroform/1000 μl of Trizol and was precipitated with 1000 μl of isopropanol (overnight, −20°C). The precipitate was washed twice in 500 μl of ethanol, air-dried, and resuspended in diethylpyrocarbonate (DEPC)-treated water. The amount of RNA was quantified by spectrophotometry on a SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Inc., Barcelona, Spain).

Reverse transcription was carried out using the Advantage RT-for-PCR Kit (Clontech Laboratories, Inc., Palo Alto, CA). Mastermix per sample was prepared as follows: 4 μl of 5X reaction buffer, 1 μl of deoxy-NTP mix (10 mmol/l each), 0.5 μl of recombinant RNase inhibitor and 1 μl of Moloney murine leukemia virus reverse transcriptase. A 1 μg aliquot of each sample was diluted to a final volume of 12.5 μl in DEPC-treated water plus 1 μl of oligo(dT)18; the mixture was heated at 70°C for 2 min and kept on ice until Mastermix (6.5 μl) was added. For each reverse transcription, a blank was prepared using all of the reagents except the RNA sample, for which an equivalent volume of DEPC-treated water (12.5 μl) was substituted. The reverse transcription blank was used to prepare the PCR blank (below). Once all components were mixed, the samples were incubated at 42°C for 1 h, then heated at 94°C for 5 min to stop cDNA synthesis and destroy DNase activity. The product was diluted to a final volume of 100 μl with DEPC-treated water and stored at −20°C until PCR analysis.

**Real-time PCR**

Primers for quantitative PCR were designed using the Primers Express Software (PE Applied Biosystems, Warrington, UK) and synthesized (PE Applied Biosystems, Barcelona, Spain). The sense GAPDH primer was 5’-216CCCATCACCATCTTCCAGGA3’-3’, and the antisense GAPDH primer was 5’-268CATCGCCCCACTTGGATTTTG248-3’. Primers for quantitative PCR were designed using the Primers Express Software (PE Applied Biosystems, Warrington, UK) and synthesized (PE Applied Biosystems, Barcelona, Spain). The sense GAPDH primer was 5’-216CCCATCACCATCTTCCAGGA3’-3’, and the antisense GAPDH primer was 5’-268CATCGCCCCACTTGGATTTTG248-3’. (NCBI accession number J04038), giving rise to an expected PCR product of 52 bp, that encompassed exons 3 and 4.

The VEGF primers were designed to amplify a region common to all VEGF isoforms, that encompassed exons 2 and 3, so the sense VEGF primer was 5’-109GGGCAGAATCTCAGCGATGT230-3’, and the antisense VEGF primer was 5’-164ATTGGATGGCAGTACGTGCCG143-3’, where a 65 bp PCR product was expected (NCBI accession number AL136131).

To amplify cDNA, single oligonucleotides still remaining were removed from the reverse transcription samples using the ultraclean clean-up kit (MoBio, Carlsbad, CA) and the purified cDNA diluted to a final concentration of 12.5 pg/μl. In each reaction, a total of 4 μl (50 pg of cDNA) from each reverse transcription tube was mixed with 12.5 μl of SYBR Green PCR master mix (PE Applied Biosystems) containing nucleotides, Taq DNA polymerase, MgCl2, and reaction buffer with SYBR green; 1–3 μl of 0.5 μmol/l VEGF or GAPDH primers, and double-distilled water were added to a final volume of 25 μl.

Real-time PCR was performed using an ABI PRISM 7700 Sequence Detection System (Perkin Elmer Corp., Norwalk, CT) according to the manufacturer’s instructions, with a heated lid (105°C), using an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was amplified in duplicate for VEGF or GAPDH, giving rise to four reactions per sample. In parallel, 6-fold serial dilutions of known concentrations of VEGF and GAPDH cDNA were run with each analysis as a calibration curve. Quantification data were analysed at the beginning of the exponential phase (cycles 18–25) with ABI PRISM 1.7 analysis software. Background fluorescence was removed by setting a noise band. Duplicates showing a >5% variation were discarded. To validate a real-time PCR, standard curves with r > 0.95 and slope values between 3.1 and 3.4 were required.

For each sample, the amounts of VEGF cDNA and GAPDH cDNA were determined in relation to the standard curves. The VEGF/GAPDH ratio was used to estimate and compare the relative VEGF expression. The results of each PCR experiment were confirmed in a minimum of three consecutive experiments.

At the end of the PCR, all products reached a plateau. To determine whether other non-expected products were also amplified, the PCR products from each VEGF or GAPDH after 40 cycles were subjected to melting analysis with a starting and final temperatures of 60 and 90°C, and 20 min ramp time, with the fluorescence signal collected every 10 s. Curves related to VEGF or GAPDH products were discarded when more than one peak appeared after melting analysis. A final subsequent 4% agarose gel electrophoresis with ethidium bromide was performed to confirm that those products we had expected to be amplified were, in fact, the only ones to be affected in such a way.

**Flow cytometry study of granulosa-lutein cell death and phenotypical analysis**

Cell death was studied using the annexin V–fluorescein isothiocyanate (FITC) apoptosis detection Kit (Bender Medsystem, Vienna) according to the manufacturer’s instructions. Briefly, luteinized granulosa cells obtained by Percoll isolation (see above) were washed twice with phosphate-buffered saline (PBS), resuspended in binding buffer and adjusted at 5 × 106 cells/ml concentration. Then, cells were incubated with annexin V–FITC for 10 min at room temperature. Finally, cells were washed twice with PBS, resuspended in PBS containing 10 μg/ml propidium iodide (PI) and analysed by flow cytometry. When cells undergo apoptosis, a phosphatidylserine residue normally on the inside of the plasma membrane flips to the outside and is specifically recognized by annexin V. Counterstaining by PI allows the discrimination of apoptotic from necrotic cells. Necrotic cells stain only with PI, whereas early apoptotic cells stain only with annexin V, and late apoptotic cells stain with both annexin V and PI.

To distinguish the contribution of apoptotic leucocytes in the obtained samples, immunofluorescence studies were performed in parallel. To do that, an aliquot of annexin V-stained cells was washed twice with PBS and incubated with phycoerythrin (PE)-conjugated CD45 monoclonal antibody (mAb; BD Biosciences, Mountain View, CA) for 30 min at 4°C. After two washes with PBS, cells were then analysed by flow cytometry.

All samples were measured on an Epics Elite Analyzer flow cytometer (Coulter Electronics Inc., Hialeah, FL) with an argon laser at 488 nm for excitation. In all cases, data were collected on 104 viable cells by electronic gating on forward and side scatter light parameters. For comparative stainings, we used the mean fluorescence intensity (MFI), defined as the average fluorescence value of the corresponding staining referred to the logarithmic scale of fluorescence intensity along the x-axis of the histograms. When necessary, isotype-matched irrelevant mAb was used to define background fluorescence.

**Statistical analysis**

Data were expressed as the mean ± SEM. Statistical calculations were performed using Sigma Stat for Windows, version 2.03 (Jandel Scientific Corporation, San Rafael, CA). For comparisons, t-test or χ2 test were used when appropriate. Correlations were performed using linear regression analysis. P < 0.05 was considered statistically significant.

**Results**

**Coasting decreases the risk of severe OHSS without affecting cycle outcome**

In both patients and egg donors, coasting effectively reduced their risk of severe OHSS. All of them had serum E2 levels...
>5000 pg/ml, putting them at a very high risk of OHSS. Withholding gonadotrophin administration until the serum E₂ dropped to levels <3500 pg/ml diminished their risk and avoided cycle cancellation; however, it did not abolish severe OHSS completely, as 3.5% of the patients and 2.7% of the donors showed severe OHSS that required paracentesis but not hospital admission (Table I). We observed an excellent IVF cycle outcome in terms of implantation and clinical pregnancy rates, comparable with the control group that did not require coasting (26.1 and 43.9% versus 24.8 and 42.4%, respectively). The oocyte donation outcome, which was an indicator of oocyte quality, was also not affected (26.4% implantation rate and 53.5% pregnancy rate). There was a trend towards a lower implantation rate with the longer duration of the coasting procedure (2 days, 25.3%; 3 days, 22.7%; 4 days, 21.9%; 5 days, 14.3%), but differences were not significant. In 2–3% of the patients/donors, cycles had to be cancelled, due either to an abrupt fall in E₂ levels or to a prolonged coasting (>5 days).

Coasting reduces VEGF protein secretion and gene expression in granulosa cells

In order to investigate the mechanisms by which coasting may be acting, we studied serum and follicular fluid VEGF protein levels, a well known mediator of HCG action in the development of OHSS.

**Serum VEGF concentrations.** Soluble VEGF protein concentrations were measured in 159 women during the coasting procedure (Figure 1). Levels initially rose on the first day that gonadotrophins were withheld, and gradually declined until the day of HCG administration. When compared with control patients who were not coasted (n = 116), serum VEGF concentrations were significantly higher on the day of HCG administration (273 ± 34 versus 152 ± 60 pg/ml, P < 0.05). However, values were comparable on the day of oocyte retrieval.

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Table I. Clinical data and IVF outcome from patients and egg donors/egg recipients undergoing coasting

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 85)</th>
<th>Donors (n = 74)/recipients (n = 134)</th>
<th>Controls (n = 116)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>33.6 ± 1.3</td>
<td>25.4 ± 1.1</td>
<td>34.1 ± 1.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.5 ± 0.5</td>
<td>22.1 ± 0.7</td>
<td>21.9 ± 0.8</td>
</tr>
<tr>
<td>Total FSH (IU)</td>
<td>2848 ± 156</td>
<td>2630 ± 185</td>
<td>2185 ± 135</td>
</tr>
<tr>
<td>Serum E₂ on the first day of coasting (pg/ml)</td>
<td>5904 ± 395</td>
<td>5143 ± 328</td>
<td>–</td>
</tr>
<tr>
<td>Serum E₂ on the day of HCG (pg/ml)</td>
<td>3312 ± 270</td>
<td>2430 ± 310</td>
<td>2914 ± 298</td>
</tr>
<tr>
<td>Duration of coasting (days)</td>
<td>3.8 ± 0.4</td>
<td>3.9 ± 0.3</td>
<td>–</td>
</tr>
<tr>
<td>No. of oocytes retrieved</td>
<td>19.5 ± 3.5</td>
<td>18.2 ± 2.5</td>
<td>15.8 ± 2.8</td>
</tr>
<tr>
<td>Egg retrieval cancellation rate (%)</td>
<td>2.3</td>
<td>2.7</td>
<td>–</td>
</tr>
<tr>
<td>Embryo transfer cancellation rate (%)</td>
<td>22.3</td>
<td>24.6</td>
<td>12.1*</td>
</tr>
<tr>
<td>Moderate OHSS (%)</td>
<td>10.6</td>
<td>10.8</td>
<td>8.2</td>
</tr>
<tr>
<td>Severe OHSS (%)</td>
<td>3.5</td>
<td>2.7</td>
<td>0</td>
</tr>
<tr>
<td>Mean no. of embryos transferred</td>
<td>2 ± 0.1</td>
<td>2.6 ± 0.2</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>24.8</td>
<td>26.4</td>
<td>26.1</td>
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<td>Clinical pregnancy rate (%)</td>
<td>42.4</td>
<td>53.5</td>
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<td>Multiple pregnancy rate (%)</td>
<td>35.5</td>
<td>27.6</td>
<td>31.3</td>
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<tr>
<td>Miscarriage rate (%)</td>
<td>18.7</td>
<td>15.0</td>
<td>13.7</td>
</tr>
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*P = 0.026 when compared with patients and donors/recipients.

Figure 1. Daily serum E₂ (black line) and soluble VEGF₁₆₅ (dotted line) were evaluated from the first day of coasting until the day of HCG administration. E₂ and VEGF behaved in a very similar fashion: after an initial rise on the first day, both gradually declined while gonadotrophins were withheld.

**Follicular fluid VEGF concentrations.** A total of 200 follicles were aspirated and evaluated in 50 women undergoing IVF/ICSI. A significantly lower concentration of soluble VEGF₁₆₅ was observed in mature follicles from women undergoing coasting when compared with non-coated patients (1413 ± 206 versus 3538 ± 409 pg/ml, P < 0.001). This difference in protein production and secretion was also observed when immature follicles (<14 mm) were assayed (757 ± 201 versus 3201 ± 705 pg/ml, P < 0.001).

**Granulosa-lutein cell VEGF mRNA expression.** Luteinized granulosa cells were obtained from follicular aspirates from both coated and non-coated patients, and mRNA was extracted. The real-time PCR signal was followed during all the PCR cycles and this allowed us to quantify VEGF and β-actin during the exponential phase and not after the over-expression of the PCR products (Figure 2A). Melting analysis revealed one-peak curves for both VEGF and GAPDH, with melting temperatures of 77.6 and 77.2°C, respectively.
Figure 2. (A) Granulosa-lutein cell mRNA was extracted and VEGF gene expression analysed by real-time PCR. The whole fluorescence signal was followed during the 40 cycle PCR, and a threshold was established in the log-linear phase for proper quantification. As seen if quantification had been performed during the non-linear phase, the products would be overexpressed (minimal or no clear separation between the PCR fluorescent signal curves), making quantification erroneous. (B) After 40 PCR cycles, melting was performed on the resulting PCR products to detect contamination. The melting curves for the VEGF PCR products showed a single peak, with a melting temperature (Tm) of 77.6°C, while GAPDH showed a Tm of 77.2°C. In both cases, the existence of a single peak indicated the presence of no PCR products other than those expected. (C) The VEGF/GAPDH ratio was used to compare VEGF expression between coasted and non-coasted patients. For a better visualization, VEGF gene expression in luteinized granulosa cells from coasted patients was normalized to that of non-coasted patients that served as control. As observed, VEGF gene expression in granulosa-lutein cells from coasted patients showed a 2-fold decrease when compared with non-coasted patients (P < 0.05).

(Figure 2B), while those not showing such characteristics were discarded. At the end of the whole process, the quantification of the real-time PCR products showed a 2-fold decrease in VEGF mRNA expression in granulosa cells from women after coasting when compared with control women (Figure 2C).

Coasting affects mainly the viability of small/medium follicles
To explore if there was a follicular population more sensitive to coasting, we evaluated cell cycle status in granulosa-lutein cells from mature (≥14 mm mean diameter) and immature (<14 mm) follicles as well as their VEGF protein secretion and E2 production.

Follicular fluid E2 concentrations. E2 concentrations were evaluated in follicular fluid from coasted and non-coasted patients. E2 production in matures follicles (≥14 mm) did not seem to be affected by coasting, as intrafollicular concentrations were comparable (333.5 ± 21.2 versus 301.9 ± 23.2 ng/ml, P = NS). Interestingly, smaller follicles (<14 mm) significantly reduced their capacity to secrete E2, as intrafollicular
concentrations were diminished after the coasting procedure (258.3 ± 33.6 versus 180.1 ± 21.2 ng/ml, P = 0.013).

Intrafollicular E2 concentrations positively correlated with VEGF protein concentrations in coasted patients, but not in controls (r = 0.558, P = 0.038).

**Follicular fluid VEGF protein concentration is related to follicular size.** Smaller follicles from coasted patients secreted significantly lower amounts of VEGF than mature follicles (757 ± 201 versus 1413 ± 206 pg/ml, P < 0.05). In contrast, this difference was not observed when a similar comparison was performed in non-coasted patients (3210 ± 705 versus 3538 ± 409 pg/ml, P = NS). Additionally, soluble VEGF concentrations found in follicular fluid positively correlated with the follicular size in coasted patients (r = 0.594, P < 0.001) (Figure 3).

**Flow cytometric analysis of necrotic/apoptotic luteinized granulosa cells.** Granulosa-lutein cells were obtained from follicular aspirates and stained with PI to evaluate cell death in this cell population, clearly differentiating necrotic/apoptotic (low PI fluorescence intensity) from G1 (high PI fluorescence intensity) cells. As shown in Figure 4, there was a significantly higher percentage of necrotic/apoptotic granulosa-lutein cells obtained from women undergoing coasting when compared with non-coasted controls (34 versus 18%, P < 0.05). Interestingly, this percentage was significantly higher when luteinized granulosa cells from immature follicles were compared with those obtained from mature follicles (48 versus 26%, P < 0.05).

Double staining with annexin V/PI enabled us to discriminate whether early apoptosis or necrosis was responsible for cell death. While follicles from non-coasted patients showed an annexin/PI ratio favouring necrosis (21%/37%, P < 0.05), follicular cells aspirated from coasted patients showed a ratio in favour of apoptosis, especially in smaller follicles (12 versus 7%, P < 0.05).

In order to rule out any confounding variable coming from the leukocyte population included in the follicular cells aspirated, we studied by double staining with CD45/annexin V the distribution of apoptotic leukocytes in the different subsets of the cell population studied. In follicles ≥14 mm, the leukocyte contribution (CD45-positive) was similar in coated and non-coasted patients (6 versus 7%, P = NS), with only 0.6–1% of these leukocytes being annexin V positive. In smaller follicles (<14 mm), the leukocyte contribution was similar (14 versus 16%), with only 2% of these cells being double positive for CD45 and annexin V, thus confirming an even contribution in both coasted and non-coasted patients and in mature and immature follicles (data not shown).

**Discussion**

The prevention of OHSS still remains a crucial step in the management of IVF cycles. Several studies have demonstrated the value of coasting in the prevention of severe OHSS without compromising oocyte/embryo quality and implantation rates (Sher et al., 1993; Benadiva et al., 1997; Lee et al., 1998; Tortoriello et al., 1998; Fluker et al., 1999; Waldestrom et al., 1999; Al-Shawaf et al., 2001; Isaza et al., 2002). We have observed from the oocyte donation model that oocyte quality is not affected and the comparable IVF cycle outcomes confirm that endometrial receptivity remains unaltered. The reasons why coasting is effective in preventing OHSS have remained speculative. Most authors hypothesized that there was a diminished functional granulosa cell cohort, resulting in a gradual decline in serum E2 levels and HCG mediators that increase vascular permeability. We have shown that coasting reduces VEGF gene expression and protein secretion by increasing granulosa-lutein cell apoptosis/necrosis, especially in small and medium (<14 mm) follicles.

Previous *in vitro* experiments have reported that VEGF production by cultured macaque non-luteinized granulosa cells diminishes over time in the absence of gonadotrophic support (Christenson and Stouffer, 1997). In coasted patients, we have seen that serum VEGF levels increase on the first day that gonadotrophin is withheld, probably due to the high doses administered and the long half-life of FSH; after 24 h, serum levels start to decline gradually, in a similar fashion to E2 production by cultured macaque non-luteinized granulosa cells. Granulosa-lutein cell apoptosis/necrosis, especially in smaller follicles, may influence the secretion of both steroids (E2) and vasoactive substances (VEGF). Although we are aware of the methodological difficulties in using serum samples for VEGF measurements, as activated platelets and other cellular components may release VEGF during clotting, as opposed to using plasma samples (Verheul et al., 1997), we consider that the use of serum is still representative if serum processing is performed under standardized conditions (Gagné...
et al., 2004). Obviously, plasma VEGF sampling would have been more reliable, but we did not perform those studies.

When looking more closely at the inside of the follicle, we have confirmed that VEGF concentrations in follicular fluid are significantly reduced in coasted patients when compared with controls. This reduction is particularly marked in small/medium follicles. It has been postulated that smaller follicles have a higher threshold to gonadotrophins than larger follicles (Fluker et al., 1999). In fact, pre-ovulatory follicles apparently have the cellular machinery to maximize the diminishing circulating gonadotrophins, as they continue to enlarge despite decreasing gonadotrophin levels (Sher et al., 1993). FSH not only stimulates follicular steroidogenesis and upregulates gonadotrophin receptor, but also inhibits granulosa cell apoptosis (Chun et al., 1996). Thus, during the coasting period, the smaller, immature follicles, which are less receptive to FSH, will undergo developmental arrest and enter necrosis/apoptosis. As a consequence, steroidogenesis will be reduced/stopped in the granulosa cells of these follicles, as reflected by declining E2 concentrations, and, similarly, vasoactive mediators, such as VEGF. To confirm this fact further, we observed that VEGF gene expression was also reduced in coasted granulosa-lutein cells, a decline that was even lower in small/medium follicles when compared with mature follicles. If coasting is prolonged beyond a certain point, even the large follicles will undergo atresia, affecting the clinical outcome of the IVF cycle, as changes in the follicular milieu may occur (E2, androgens, E2:androgen ratio). According to our previous work (Isaza et al., 2002), we cancelled the cycle if coasting lasted >4 days, so we do not have data on VEGF expression and secretion beyond that point.

FSH is the most important regulator of folliculogenesis (Fauser and Van Heusden, 1997). Exogenous FSH together with endogenous secretion will determine the effect at the follicular level. Benadiva et al. (1997) were the first to report that serum FSH declined during the coasting period, but the level remained adequate to sustain follicular development. Later, Al-Shawaf et al. (2001) tried to find a more sensitive method of determining when to decrease or even stop gonadotrophin injection in those at high risk of severe OHSS by measuring serum E2 and FSH levels. They found that FSH levels declined 25% per day during the coasting period, and

![Figure 4](image_url)

**Figure 4.** Flow cytometric analysis with propidium iodide was performed to evaluate granulosa-lutein cell cycle status: (a) a significantly higher percentage of necrotic/apoptotic cells was found in the luteinized granulosa cell population obtained from coasted patients compared with non-coasted patients (34 versus 18%, P < 0.05); (b) when granulosa-lutein cells were obtained from small/medium sized follicles, the percentage of necrotic/apoptotic cells was higher than luteinized granulosa cells from larger follicles (48 versus 26%, P < 0.05).
when FSH reached 5 IU/l or less, E₂ was at a ‘safe’ level to trigger ovulation with HCG, considering the combined measure useful in predicting when to stop coasting. The recent finding that FSH stimulates VEGF production to a similar degree compared with HCG (Agrawal et al., 2002) emphasized the importance of circulating FSH levels in the pathogenesis of OHSS. In fact, the step-down protocol which acts by tapering the dose of FSH during the IVF cycle reduces the risk of OHSS (Simón et al., 1998). We could hypothesize, as recently suggested by Meldrum (2002), that reduction of circulating FSH may be the mechanism through which coasting reduces the incidence of OHSS.

The fact that small and medium (≤14 mm) follicles are more sensitive to undergo atretic changes is of crucial relevance in both steroid and vasoactive mediator secretion. We have observed that a significantly higher percentage of granulosalutein cells become necrotic/apoptotic after coasting when compared with non-coasted cells, and this difference is even greater for immature follicles. Previous work has demonstrated that it is the small and medium size follicles that are mostly responsible for the high serum E₂ concentration and vasoactive mediator secretion in granulosa cells and follicular fluid from the study group explains why coasting is clinically useful in predicting when to stop coasting. Considering that E₂ also upregulates VEGF receptor-2 expression in endothelial cells (Gargett et al., 2002), we could closely monitor serum levels of this simple marker before triggering ovulation with HCG. The high inter-patient variability due to different body mass index, granulosalutein cell sensitivity, pre-treatment with GnRH agonists or ultrasonographic polycystic ovary pattern explains the different behaviour with similar E₂ levels. Additional measures together with coasting, such as specifically blocking VEGF action with soluble VEGF receptor-1 (Hazzard et al., 2002) or with VEGF receptor-2 inhibitors (Gómez et al., 2002), may provide new insights into the development of future strategies to prevent and treat OHSS.

In summary, the significant decrease found in VEGF expression and secretion in granulosalutein cells and follicular fluid from the study group explains why coasting is clinically effective in reducing the incidence and severity of OHSS. A lower VEGF production after HCG administration in coasted patients will have a reduced effect on endothelial cells, diminishing its effect in increasing vascular permeability.

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References
Hazzard T, Xu F and Stouffer R (2002) Injection of soluble vascular endothelial growth factor receptor 1 into the preovulatory follicle disrupts


