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"LOCAL INTERACTION BETWEEN THE DOPAMINERGIC SYSTEM AND THE VASCULAR ENDOTHELIAL GROWTH FACTOR AND ITS TYPE 2 RECEPTOR (VEGF/VEGFR2) IN THE REGULATION OF CORPUS LUTEUM ANGIOGENESIS IN PHYSIOLOGIC AND PATHOLOGIC CONDITIONS"

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CERTIFICAN:

Que Dña. **Mª Hortensia Ferrero Cháfer**, licenciada en Biologia por la Universidad de Valencia y postgraduada en Biotecnología y Biomedicina, especialidad en técnicas de reproducción asistida, por la Universidad de Alicante ha realizado bajo nuestra dirección el trabajo que, con el título: ***"Local interaction between the dopaminergic system and vascular endothelial growth factor (VEGF) and its type 2 receptor (VEGF/VEGFR2) in the regulation of corpus luteum angiogenesis in physiologic and pathologic conditions"*** presenta para optar al grado de doctor internacional en Biotecnología por la Universidad de Valencia.

Y para que conste así a los efectos oportunos, firmamos la presente certificación en Valencia a 12 de Marzo de 2014.

Fdo. Prof. D. Antonio Pellicer Martínez Fdo. Dr. D. Raúl Gómez Gallego

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A Pablo

ACE: Angiotensin Converting Enzyme

ACTH: Adrenocorticotropic Hormone

AES: Androgen Excess Society

AI: Angiotensin I

AII: Angiotensin II

AMH: Anti-Müllerian Hormone

ARDS: Acute Respiratory Distress Syndrome

ART: Assisted Reproductive Techniques

ASRM: American Society for Reproductive Medicine

BFA: Brefeldin-A

BMI: Body Mass Index

Cb2: Cabergoline

CI: Confidence Interval

CL: Corpus Luteum

CL-6: Insulin-induced growth response protein

COH: Controlled Ovarian Hyperstimulation

DAG: Diacylglycerol

D2: Dopamine receptor D2

D2-ag: D2-Agonist

DHEA-S: Dehydroepiandrosterone Sulfate

DOPAC: 3,4-Dihydroxyphenylacetic acid

Dp: Dopamine (3,4-dihydroxyphenethylamine)

E2: Estradiol

EC: Endothelial Cell

LIST OF ABBREVIATIONS

ESHRE: European Society for Human Reproduction and Embryology

FACS: Fluorescence-Activated Cell Sorting

FAK: Focal Adhesion Kinase

FF: Follicular Fluid

FSH: Follicle Stimulating Hormone

GC: Granulosa Cell

GnRH: Gonadotropin-Releasing Hormone

GnRHant: GnRH antagonist

HBD: Heparin-Binding Domain

hCG: Human chorionic gonadotropin

Hct: Hematocrit

HES: Hydroxyethyl Starch

hMG: Human Menopausal Gonadotropin

HUVEC: Human Umbilical Vein Endothelial Cell

Ig: Immunoglobulin

IL-1β: Interlukin-1-beta

IL-2: Interleucina-2

IL-6: Interleucina-6

IL-8: Interleucina-8

IP_3: Inositol Triphosphate

IVF: *In vitro* Fertilization

IVM: *In vitro* Maturation

LGC: Luteinized Granulosa Cell

LH: Luteinizing Hormone

MACS: Magnetic-Activated Cell Sorting

MADPH: Reduced form of Nicotinamide Adenine Dinucleotide Phosphate

MAPK: Mitogen-Activated Protein Kinase

MRI: Magnetic Resonance Imaging

mRNA: Messenger Ribonucleic Acid

NIH: National Institutes of Health

NO: Nitric oxide

OHSS: Ovarian Hyperstimulation Syndrome

P: Probability

P4: Progesterone

PCOS: Polycystic Ovarian Syndrome

PDGF: Platelet-Derived Growth Factor

PGE2: Prostaglandin E2

PGs: Prostaglandins

PIGF: Placental Growth Factor

PLC: Phospholipase C

Prl: Prolactin

R: Range

RAS: Renin-Angiotensin System

rLH: Recombinant LH

RR: Relative Risk

RTKS: Receptor Tyrosine Kinase

SQE: Squalene Epoxidase

sVEGFR-1: Soluble VEGF Receptor 1

T2DM: Type 2 Diabetes Mellitus

TG/HDL: Triglyceride to High-density Lipoprotein

LIST OF ABBREVIATIONS

TGF-beta: Transforming Growth Factor-Beta

TH: Tyrosine Hydroxylase

TRAP: Tartrate-Resistant Acid Phosphatase

US: Ultrasound

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

VP: Vascular Permeability

WBC: White Blood Cells

ZO-1: Zone Occludens-1

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SUMMARY

El Síndrome de hiperestimulación ovárica (SHO) es una complicación iatrogénica provocada por el uso de gonadotropina coriónica humana (hCG) para la inducción de la ovulación. La sobreexpresión ovárica del Vascular Endothelial Grown Factor (VEGF) produce un aumento de la angiogénesis y de la permeabilidad vascular (PV), síntomas que desencadenan el SHO. Pese a su gravedad, los tratamientos del SHO en mujeres sometidas a técnicas de reproducción asistida (TRA) se han limitado a cuidados paliativos, al desconocer su fisiopatología. Estudios previos realizados por nuestro grupo describieron la presencia del receptor D2 de dopamina (D2) en ovario y observaron que la activación de este receptor inhibe la PV, sin afectar a la angiogénesis, en ratas hiperstimuladas (Gómez R et al., 2006). Estas observaciones se confirmaron en humanos con mujeres con riesgo de sufrir SHO (Álvarez C, et al., 2007), en las que se describió también la expresión de D2 en las células de la granulosa (CG). En base a las evidencias científicas publicadas, el presente trabajo de tesis doctoral hipotetiza que los D2 agonistas (D2-ag) puede ejercer un efecto inhibitorio en la producción de VEGF por las CG y con ello disminuir la PV ovárica. Esta hipótesis sugiere la existencia de un sistema dopaminérgico en el ovario, el cual determina la susceptibilidad de cada mujer a los cambios de PV a través del sistema de regulación VEGF/VEGFR2, y con ello a desarrollar patologías de ovario dependientes, tales como SHO. Por todo ello, se postula que mujeres con riesgo de desarrollar el SHO, como son mujeres con Síndrome de Ovario Poliquístico (SOP), pueden presentar un tono dopaminérgico desregulado y/o disfuncional.

Con el propósito de corroborar esta hipótesis se pretendió identificar las poblaciones celulares del ovario que expresan D2 y aislarlas mediante un método que nos permitiera obtener el mayor número de células puras, con el fin de cultivarlas *in vitro*. En segundo lugar, se pretendió determinar la relación entre sistema dopaminérgico (dopamina (Dp)/D2) y sistema VEGF/VEGFR2 en condiciones fisiológicas y patológicas (SOP), con el fin de determinar si existen diferencias entre ellas.

De los métodos de aislamiento de GC analizados, el método de aislamiento mediante filtros fue el que ofreció mayor efectividad en cuanto a la cantidad de células recuperadas, sin comprometer significativamente a la pureza.

Una vez aisladas, estas células fueron cultivadas *in vitro* con el fin de estudiar la relación entre sistema dopaminérgico (Dp/D2) y sistema VEGF/VEGFR2. Los resultados de dicho estudio demostraron que la secreción del VEGF fue inhibida por el D2-ag de una forma dosis-dependiente. Así mismo, se evidenció que el D2-ag estaba mediado por su receptor D2. Dicha expresión del receptor D2 se correlaciona con la inhibición de VEGF, existiendo una relación dosis-dependiente entre la cantidad de D2 expresada y el grado de inhibición del VEGF observado.

Finalmente, al comparar el tono dopaminérgico y sistema VEGF/VEGFR2 en condiciones fisiológicas y patológicas (SOP), se observó una disminución en la expresión de D2 y un aumento en la vascularización en las pacientes SOP. Además, observamos en estas pacientes, un metabolismo acelerado de la Dp y un porcentaje de inhibición del VEGF disminuido en comparación con pacientes sanas.

Con todo ello, podemos concluir en este trabajo de tesis doctoral que el D2-ag inhibe la secreción de VEGF mediante la activación de D2 de manera dosis dependiente, tanto en condiciones fisiológicas como patológicas. Sin embargo, en condiciones patológicas (SOP) la administración de D2-ag es menos efectiva en prevenir el SHO debido a que dichas pacientes presentan un metabolismo acelerado de la Dp junto con un tono dopaminérgico desregulado, el cual provoca que el D2 presente una baja eficiencia en inhibir la secreción del VEGF cuando es activado por D2-ag. Puesto que dicha capacidad del D2 en inhibir el VEGF es dosis-dependiente, un aumento de la concentración intraovárica de D2-ag podría ser suficiente para prevenir el SHO.

1. INTRODUCTION

1. INTRODUCTION

1.1. OVARIAN HYPERSTIMULATION SYNDROME BACKGROUND

In the second half of the twentieth century, the management of human infertility, and the development of advanced assisted reproduction techniques (ART) by modern medicine has progressed considerably. Ovarian stimulation, used in order to obtain the highest possible number of oocytes, has been key to such treatments. The use of ovulation-inducing drugs, such as gonadotropin, is highly effective in ovarian stimulation and was a breakthrough for these techniques. However, although these techniques bring benefits, we must also acknowledge the possibility of complications, which in some cases are associated with the appearance of ovarian hyperstimulation syndrome (OHSS).

OHSS has been known of since 1943, when gonadotropins were first used to induce ovulation (Rydberg E, et al., 1943, Davis E, et al., 1944), and at that time the disease was referred to as *"syndrome d'hyperluteinisation massive des ovaires"* in French. The first fatal cases were described in 1951 (Altirriba E, et al., 1961). In 1957, Le Dall described this 'syndrome' in his thesis and subsequently published acute cases in which laparotomy and unilateral ovariectomy were required (Le Dall R, 1957). Although, the term 'ovarian hyperstimulation syndrome' was not found in the literature until 1968 (Mingeot R, et al, 1968), based on the clinical and laboratory parameters described by many authors (Rabau E, et al., 1967, Schenker J. and Weinstein D, 1978; Golan A, et al., 1989; Navot D, et al., 1992; Whelan I. and Vlahos N, 2000; Aboulghar M and Mansour, 2003) and despite the different terminology used (Southam A. and Janowskin A, 1962; Sassi D, et al., 1964), the disease was already defined before this official classification. OHSS appeared to be a possible complication of the induction of ovulation by using gonadotropins in controlled ovarian hyperstimulation (COH) protocols. The presentation and severity of this syndrome has evolved over time in relation to such protocols, for example, the development of *in vitro* fertilization (IVF) techniques in the late 80s and early 90s led to more aggressive treatments in order

to obtain sufficient numbers of oocytes and embryos, consequently leading to a greater risk of OHSS (Golan A, et al., 1988; Aisaka K, et al, 1989; McClure N, et al, 1992; Urman B, et al, 1992, MacDougall M, et al., 1993).

Due to the importance of this syndrome many groups have studied its symptoms and pathophysiology, and have developed techniques that can prevent or reduce its incidence. Although there have been many different lines of investigation which have brought great advances in the understanding and prevention of OHSS, its pathophysiology, the intrinsic molecular mechanisms at the origin of this syndrome, and knowledge of why these mechanisms function differentially in different patients remains unclear.

1.2. DEFINITION

OHSS is an iatrogenic complication of the luteal phase and/or early pregnancy (Kasum et al, 2010). It is an exaggerated response to COH, characterized by increased ovarian size, large internal cysts, and extravasation of intravascular fluid into the third space due to increased vascular permeability (VP) in response to administration of the exogenous gonadotropins (mainly human chorionic gonadotropin; hCG) used to induce ovulation (Gómez R, et al, 2010). While mild OHSS has almost no clinical relevance, severe OHSS is characterized by massive ovarian enlargement, ascites, pleural effusion, oliguria, hemoconcentration, edema in the third space, disturbed electrolyte balance, hydrothorax, and thrombotic events (Shenker and Weinstein, 1978; Navot D, et al., 1992; Schenker J. and Ezra Y, 1994; Serour G et al., 1998; Hock D, and Seifer D, 2000), which can endanger the life of the patient

1.3. SYMPTOMS

OHSS predictive signs and symptoms, such as increased ovary size, can be observed even at the early stages of the ovarian stimulation cycle, although the total manifestation of the syndrome, expressed as an increase in VP and the output of protein-rich fluid from the intravascular space, occurs in the post-ovulatory phase (or after oocyte recovery). The different manifestations which characterize OHSS are described below:

1.3.1. VASCULAR COMPLICATIONS

The mechanism responsible for the clinical manifestations of OHSS seems to be an increase in the VP, which causes fluid to shift into the intravascular space (Polishuk W, et al., 1969). Patients who suffer this clinical manifestation can suffer from several cardiovascular effects, which include low blood pressure, low blood volume, low central venous pressure, tachycardia, increased cardiac output, low peripheral resistance, hemoconcentration, and hypercoagulation (Cremisi H. and Mitch W, 1994). High hematocrit directly reflects a decrease in intravascular blood volume and an increase in viscosity which can both influence renal perfusion and thromboembolic events. Hypovolemia, associated with loss of fluid and proteins into the abdominal cavity, can induce oliguria and electrolyte imbalances. In some cases it may trigger clinical complications, such as decreased renal perfusion which occurs in approximately 1.4% of the severe forms of OHSS (Abramov Y, et al., 1999; Khalaf Y, et al, 2000).

Patients with severe OHSS exhibit compensatory endogenous vasoconstrictor activity: an increase in cardiac output and blood volume as a result of the retention of sodium and water, although this is not sufficient to maintain homeostasis. This is the most severe form of OHSS since the reduced blood volume can cause peripheral arteriolar vasodilation (Balasch J, et al, 1998). In patients with moderate OHSS, the degree of arteriolar vasodilation is not as intense, and so homeostasis and blood pressure are normal and no edema or hemoconcentration is observed (Balasch J, et al., 1998).

1.3.2. CLINICAL SYMPTOMS

The onset of clinical symptoms can be observed from the end of ovarian stimulation by a high ovarian response which implicates an increased ovary volume. The first symptoms, including nausea, vomiting, and abdominal pain, can appear 48 hours after hCG administration and these usually progress rapidly within the first few days after follicular aspiration, and become severe around 7-10 days after hCG administration. Some patients develop ascites on day 7 after hCG administration. Symptoms improve slowly with rapid diuresis within 48 hours of the onset of menstruation if pregnancy does not occur, but if there is a pregnancy, the symptoms become aggravated due to the secreted hCG. It is unusual to find serious cases of OHSS syndrome without pregnancy, however, when it does occur the duration is shorter.

There are several clinical complications that can occur, as a consequence of OHSS which are described in the following.

1.3.2.1. Obstetric complications

- Ectopic pregnancy: This is relatively common in cycles with conception, perhaps due to ovarian enlargement (Ballesteros A, et al., 1999).

- Obstetric morbidity: In complicated pregnancies which present severe OHSS, a higher rate of multiple pregnancies, spontaneous abortion, prematurity, low birth weight, preeclampsia, gestational diabetes, and placental abruption are observed in pregnancies generated by assisted reproduction but no associated with this syndrome (Mathur R. and Jenkins J, 2000a).

Most of these complications can be explained by the higher incidence of multiple pregnancies. The abortion rate in pregnancies complicated with OHSS ranges from 27-40%, which is higher than the abortion rate described in assisted-reproduction pregnancies not complicated by OHSS (18-22%; Chen C, et al., 1997; Schenker J. and Weinstein D, 1978).

1.3.2.2. Gynecological complications

- Intraperitoneal hemorrhage, caused by the rupture of a corpus luteum.
- Adnexal torsion: This may cause any combination of ovarian enlargement, abdominal pain, nausea, progressive leukocytosis, or anemia (Mashiach S, et al., 1990).
- The persistence of large ovarian cysts.

1.3.2.3. Ascites

Accumulation of fluid in the abdominal cavity can lead to tense ascites with increased intra-abdominal pressure. This generates a decrease in venous blood return, resulting in hypovolemia and oliguria, and secondary respiratory distress with dyspnea.

1.3.2.4. Thromboembolic events

Thromboembolic events are most frequent when hemoconcentration is present, but they may also appear in its absence and/or in the presence of low levels of antithrombin III (Kaaja R, et al., 1989). These findings support the hypothesis that high levels of estradiol and progesterone play a role in the etiology of deep venous thrombosis associated with OHSS.

1.3.2.5. Acute renal failure

This complication is usually due to hypovolemia and a renal perfusion deficit (Balasch J, et al., 1990). Acute renal failure presents clinical manifestations such as oliguria, disturbed electrolyte balance, azotemia, and dilutional hyponatremia associated with antidiuretic hormone hypersecretion.

1.3.2.6. Liver disease

Cholestatic or hepatocellular lesions have been associated with high estradiol (E2) levels and hepatic edema due to increased VP (Younis J, et al., 1988; Ryley N, et al., 1990).

1.3.2.7. Pulmonary alterations

- Pleural effusion by increasing VP.
- Acute hydrothorax with severe pulmonary involvement (Padilla S, et al., 1990).
- Acute respiratory distress syndrome (ARDS): severe hypoxemia associated with this syndrome can lead to cardiac arrest (Zosmer A, et al., 1987).

1.4. CLASSIFICATION

OHSS was first classified in 1967 (Rabau E, et al., 1967) by combining both laboratory and clinical findings. Several years later, in 1978, Schenker J. and Weinstein D. reorganized and modified the classification into three main clinical categories (mild, moderate, and severe) and six grades according to the severity of the symptoms. In 1989, a new classification system with three categories and five grades was introduced (Golan A, et al., 1989), which included echographic evaluation of the ovary size and the presence of ascites as a criteria associated with OHSS. This classification method is the most widely accepted, because it incorporates both clinical signs and symptoms, and echographic and analytical findings. This was later modified by further dividing the severe form into two subgroups (severe and critical) which allow severe cases and those that endanger the life of the patient to be distinguished (Navot D, et al., 1992). The most recent classification with further modifications was introduced in 1999 (Rizk B. and Aboulghar M, 1999) and is described in the table below (Tabla.I)

Table I.

Study	Mild	Moderate	Severe		
Rabau et al, 1967	Grade 1: estrogen >150 µg and pregnanediol >10 mg 24 h Grade 2: + enlarged ovaries and possibly palpable cysts Grade 1 and 2 were not included under the title of mild OHSS	Grade 3: grade 2+ confirmed palpable cysts and distended abdomen Grade 4: grade 3+ vomiting and possibly diarrhoea	Grade 5: grade 4+ ascites and possibly hydrothorax	Grade 6: grade 5+ changes in blood volumen, viscosity and coagulation time	
Schenker and Weinstein, 1978	Grade 1: estrogen > 150 µg/24h and pregnanediol> 10mg/ 24h Grade 2: grade 1+ enlarged ovaries, sometimes small cysts	Grade 3: grade 2+ abdominal distension Grade 4: grade 3+ nausea, vomiting and/or diarrhea	Grade 5: grade 4+ large ovarian cysts, ascities and/or hydrothorax	Grade 6: marked haemoconcentration + increased blood viscosity and possibly coagulation abnormalities	
Golan et al. 1989	Grade 1: abdominal distension and discomfort Grade 2: grade 1+ nausea, vomiting and/or diarrhoea, enlarged ovaries 5±12 cm	Grade 3. Grade 2+ ultrasound evidence of ascites	Grade 4: grade 3+ clinical evidence of ascites and/or hydrothorax and breating difficulties.	Grade 5: grade 4+ haemocroncentration , increase blood viscosity, coagulation abnormality and diminished renal perfusion	
Navot et al, 1992			Severe OHSS: variable enlarged ovary; massive ascites ± hydrothoras; Hct> 45%; WBC > 15000; oliguria; creatinine 1.0–1.5; creatinine clearance ≥ 50ml/min; liver dysfunction; anasarca	Critical OHSS: variable enlarged ovary; tense ascites ± hydrothorax; Hct> 55%; WBC ≥ 25000; oliguria; creatinine ≥ 1.6; creatinine clearance <50 ml/mim; renal failure; thromboembolic phenomena; ARDS	
Rizk et el. Aboulghar (1999)		Discomfort, pain, nausea, distension, ultrasonic evidence of ascites and enlarged ovaries, normal haematolgical and biological profiles	Grade A: Dyspnoea, oliguria, nausea, vomiting, diarrhoea, abdominal pain, clinical evidence of ascites, marked distension of abdomen or hydrothorax, US showing large ovaries and marked ascites, normal biochemical profile	Grade B: Grade A plus massive tensión ascites, markedly enlarged ovaries, severe dyspnoea and marked oliguria, increased haematocrit, elevated serum creatinine and liver dysfunction	Grade C: Complications as respiratory distress syndrome, renal shut-down or venous thrombosis

ARDS= acurate respiratory distress sindrome; Hct= haematocrit; US= ultrasound; WBC= white blood cells.
Classification of ovarian hyperstimulation sindrome (OHSS) (Aboulghar M.A. and Marsour R.T, 2003)

1.5. EPIDEMIOLOGY

It is not easy to estimate the incidence of OHSS as the mild grade cannot always be diagnosed, mainly because it is practically asymptomatic. Before the application of ART, Schenker J. and Weinstein D. estimated an incidence of 8-23% for the mild grades; 0.005-7% in moderate grades, and 0.008-10% in severe grades.

This incidence increased when new ARTs were applied; 0.1-2% of severe OHSS (Busso C, et al, 2009), constituting the most serious complications described above, now present in patients undergoing ovulation induction treatments and ART. On average, two cases of severe OHSS are observed per 200 cycles of ovulation induction with the follicle stimulating hormone hCG, and one or two cases are hospitalized in the intensive care unit. The frequency and risk factor for OHSS is increased by 50% in cycles in which the pregnancy is already present, and is four times higher than that of non-pregnant cycles (Golan A, et al., 1989). The late manifestation of the syndrome is concomitant with pregnancy in 96.7% of cases, and a severe manifestation is also more likely to develop in these cases than in early-diagnosed OHSS (Papanikolaou E, et al., 2005).

Severe OHSS currently occurs in approximately 1.4% of all cycles (Fiedler K and Ezcurra D, et al., 2012) and the risk of mortality is estimated to be 1 in 450,000 to 500,000 cases. This low incidence appears to be due to improved monitoring of ovulation induction, better identification of risk factors, and probably also because of greater understanding and prevention of OHSS.

1.6. RISK FACTORS

Patients undergoing assisted reproduction treatments using IVF techniques should follow a personalized stimulation protocol which is adapted to each patient, after assessing the presence or absence of risk factors which can lead to OHSS. In 1992, Navot D. et al. described some risk factors which have since been analyzed separately and confirmed by others (Delvigne A, et al., 1993). The risk factors

described by these studies allowed doctors to better predict OHSS and thus reduce its incidence.

- Age: the risk of patients developing OHSS depends on their age. There is a higher incidence in younger women (under 35 years) who might be explained by the fact that a greater number of follicles are recruited in these patients (Gaona R, et al., 2010) and that they have a higher number of gonadotropin receptors.
- Body mass index (BMI): There are conflicting results regarding the relationship about BMI in the development of OHSS (Lee T, et al., 2008; Delvigne A, et al, 2002). Therefore, BMI does not appear to be a useful marker for increased risk of OHSS.
- Number of follicles recruited: Several studies have demonstrated that recruitment of more than 35 follicles increases the risk factor (Navot D, et al., 1988; Enskog A, et al, 1999), even if these include small or intermediate follicles, due to their ability to produce E2 and vasoactive molecules.
- Estradiol levels: E2 concentrations higher than 4,000 pg/ml on the day of hCG administration are related with OHSS (Navot D, et al., 1992). High levels of E2 alone are not good predictors of the syndrome (Alper M, et al., 2009; Humaindan P et al., 2010; Papanikolau E. G., 2010). Nevertheless, a combination of the number of follicles recruited and the E2 serum levels is a good predictor of OHSS, providing high sensitivity and specificity (Papanikolaou E, et al., 2006).

- hCG administration: Administration of exogenous hCG to induce ovulation is associated with OHSS due to its role in stimulation by neovascularization and increasing VP (Brinsden P, et al., 1995). Use of hCG in the luteal phase after the IVF cycle instead of progesterone is also associated with OHSS (Navot D, et al., 1992).
- Anti-Müllerian Hormone (AMH): AMH is expressed in the granulosa cells (GCs) of preantral and small antral follicles (Humaidan P, et al., 2010; Durlinger A, et

al, 2002) and measuring it gives a good estimation of ovarian reserve. Initial studies found that all the cycles that were cancelled due to a risk of OHSS were in patients whose AMH levels were greater than 7 ng/ml. This suggests that AMH is a predictor of ovarian response (La Marca A, et al., 2007; Gnoth C, et al., 2008), which can differentiate normal responders (AMH levels of 1.26 ng/ml) with a success rate of 98% (Hehenkamo W, et al., 2006).

- Polycystic ovary syndrome (PCOS): women with PCOS have a higher incidence of developing OHSS after administration of exogenous gonadotropins than women who present other causes of anovulatory infertility. This is due to the high number of follicles recruited by these patients. Studies show that 14.7% of patients with OHSS are PCOS patients, thereby indicating that PCOS is an OHSS risk factor.

  The implication of this risk factor in the OHSS and the relationship between them has been studied in this thesis. For this reason, a special section to describe the PCO syndrome has been engaged.

1.7. POLYCYSTIC OVARIAN SYNDROME (PCOS)

PCOS was first described in 1935 and was characterized by the presence of Polycystic ovaries (PCOs), menstrual irregularities, and clinical/biochemical hyperandrogenism (Stein I. and Leventhal M. et al., 1935). Development of PCOS has been linked to hereditary and environmental factors including genetics, insulin resistance, obesity, and birth weight, and is associated with chronic oligoanovulation and a typical PCO morphology (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004).

1.7.1. The clinical features of polycystic ovary syndrome

The commons symptoms which characterize PCOS include:

- **Menstrual disorder:** PCOS patients are mostly oligomenorrheic, which is defined as a menstrual interval of more than 35 days or amenorrhea (which is defined as the absence of menstrual bleeding for more than 90 days). Some

women with PCOS may have normal menstruation patterns but they may not ovulate (chronic anovulation).

- **Infertility:** this symptom results from anovulation (lack of ovulation). Menstrual disorders are one of the main reasons why PCOS women have difficulty becoming pregnant.
- **Clinical hyperandrogenism:** Hyperandrogenism is one of the most noticeable features of PCOS. This symptom is caused by increased ovarian and/or adrenal androgen production. The primary clinical indicator of hyperandrogenism is the presence of hirsutism (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004
- **Biochemical hyperandrogenism:** Androstenedione and testosterone serum levels should also be used in PCOS diagnosis. However specific cut-off points for hyperandrogenism have not been identified.
- **Metabolic signs:** PCOS is also associated with obesity, insulin resistance, diabetes, and hyperinsulinemia.
- **Polycystic ovaries:** The first definition of polycystic ovary (PCO) morphology in 1935 considered PCOs as "usually being bilateral, enlarged, tense ovaries which were often distinctly globular in shape, in addition to the presence of multiple cysts, which are rarely larger than 15 mm" (Stein I. and Leventhal M. et al., 1935). With the introduction of gynecological transvaginal ultrasound in the 1970s, the morphological diagnosis of PCO started to be made using ultrasound instead of ocular inspection or histology. Thus, PCOs are identified by the presence of at least one ovary with a size greater than 10 cm^3 or the presence of 12 or more follicles between 2-9 mm in diameter.

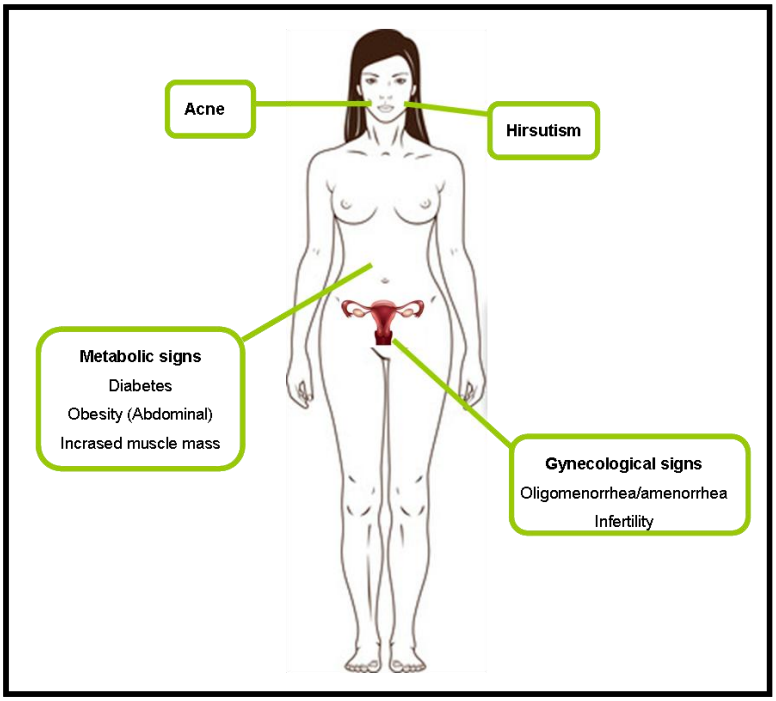

Figure 5: Illustration of the characteristic clinical features of PCOS in women of fertile age and the possible consequences.

1.7. 2. Polycystic ovary syndrome definitions and phenotypes

The most recent PCOS definitions used are the following:

- **National institutes of health criteria**

Since it was first identified in 1935, there was no formal tool for diagnosing PCOS until it was defined and described in an expert conference sponsored by the national institutes of health (NIH) in April 1990. Patients who presented ovulatory dysfunction (oligo- or anovulation) and hyperandrogenism, with the exclusion of known disorders, such as Cushing's syndrome, hyperprolactinemia, non-classical congenital adrenal hyperplasia, and androgen-secreting tumors were diagnosed with PCOS (Table 2).

- **Rotterdam criteria**

The Rotterdam criteria were established in 2003 and revised in 2004 (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2004) by the European society for human reproduction and embryology (ESHRE) in collaboration with the American society for reproductive medicine (ASRM). Experts in Europe believed that PCO morphology should be considered as a diagnostic criterion. This definition required at least two of the three following criteria: oligo- or anovulation, clinical and biochemical signs of hyperandrogenism, and/or PCOs (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004; Table 2). However, the Rotterdam 2003 criteria did not replace the NIH 1990 criteria, because all women diagnosable by the NIH criteria also meet the Rotterdam definition (Table 2).

- **Androgen excess society criteria**

In 2006, the androgen excess society (AES) proposed that the criteria for the diagnosis of PCOS should be tightened (Azziz R, et al., 2006). They included a requirement for hyperandrogenism in combination with ovarian dysfunction. The diagnosis criteria were changed again to include excess androgen as a necessary component along with the exclusion of other etiologies.

Study	Criteria
NIH, 1990	To include all of the following: 1) Oligoovoulation 2) Hyperandrogenism and/or hyperandrogenemia 3) Exclusion of related disorders
ESHRE/ASRM (Rotterdam, 2003)	To include two of the following, in addition to exclusion of related disorders: 1) Oligo- or anovulation 2) Clinical and/or biochemical signs of hyperandrogenism 3) Polycystic ovaries
AES, 2006	To include hyperandrogenism in combination with: 1) Oligo- or anovulation 2) Polycystic ovaries

Depending on the PCOS definition used, different phenotypes exist. The division into phenotypes is based on the characteristics: oligo-/amenorrhea, hyperandrogenism, and PCO. Currently, the NIH, Rotterdam and AES criteria remain the three main diagnostic tools for PCOS. In this thesis, PCOS is defined according to the Rotterdam criteria, which currently seem to be the most commonly used criteria (at least in Europe).

1.7.3. Etiology and pathophysiology of polycystic ovary syndrome

Although the cause of PCOS is currently unknown, multiple mechanisms have been discussed which involve interactions between heritable and environmental factors (Norman R, et al., 2007; Ehrmann D, 2005). These factors include genetics, insulin resistance, obesity, and birth weight (which is related to the adipose tissue hypothesis.

1.7.3.1. Genetics

In spite of numerous studies on genes associated with the synthesis and metabolism of androgens and insulin, it is not known how PCOS is inherited. Recent studies provide support for a genetic component of PCOS with evidence of the disorder occurring among women in the same family (Govind A. et al., 1999; Carey A, et al., 1993; Lenarcik A, et al., 2011). Although it appears to be an autosomal dominant disease, there is not currently enough evidence to prove this theory.

1.7.3.2. Insulin resistance

Insulin resistance is a pathogenic characteristic feature of PCOS (Dunaif A, et al, 1989), which occurs in 5-70% of the PCOS population. The molecular mechanism of insulin resistance involves defects in the insulin-receptor signaling pathway in both adipocytes and in skeletal muscle (Dunaif A, 1997). Insulin resistance causes compensatory hyperinsulinemia and may contribute to hyperandrogenism and gonadotropin aberrations. It is known that ovarian stimulation is exerted by a synergistic effect of insulin upon LH theca cell-stimulation (Diamanti-Kandarakis E, et al., 2008; Nestler J, et al., 1997).Hyperinsulinemia also stimulates androgen production, which starts and/or maintains a positive feedback loop in which hyperandrogenism leads to hypothalamus/pituitary abnormalities, ovarian dysfunction, insulin resistance, and obesity, which in turn stimulates further androgen production (Escobar-Morreale H. and San Millan J, 2007).

1.7.3.3. Obesity

Obesity is another component of PCOS which may contribute to its pathogenesis. In PCOS women, excess weight is primarily in the abdominal region; when abdominal adipose tissue is broken down free fatty acid levels in the circulation rise leading to chronic hyperinsulinemia. As mentioned above, insulin resistance is a key feature in the development of PCOS, further helping to explain why obesity increases the symptoms of PCOS.

1.7.4. Reproductive implications of polycystic ovary syndrome

Anovulation (oligo- or amenorrhea) and concordant infertility are frequently the first clinical manifestations of PCOS (Goverde M, et al., 2008). Approximately 70% of PCOS patients remain undiagnosed (Teede H, et al., 2011) therefore making it difficult to know what proportion of PCOS patients are infertile. In general, PCOS is associated with a lower chance of conceiving and higher miscarriage rates (Pasquali R, et al. 2006); this may be due to the severe features of PCOS, such as amenorrhea and grave hyperandrogenism, which are associated with enduring anovulation and worse treatment outcomes (Balen A, et al. 1995). Moreover, once pregnancy is established in PCOS women, they are at an increased risk of pregnancy complications such as gestational diabetes, pregnancy-induced hypertension, and preeclampsia (Boomsma C, et. al., 2006; Kjerulff L, et al., 2011).

The first line of treatment for infertility associated with PCOS is weight reduction (Norman R, et al., 1998). The second option is conventional ovulation induction treatment, which is widely used in PCOS although it may lead to a higher risk of OHSS (Van Wely M, et al., 2003): 15% of PCOS women undergoing ovulation treatment suffer from severe OHSS, compared to 3% of control women (Swanton A, et al., 2009). Towards the end of the 1990s, *in vitro* maturation (IVM) was developed, mainly with the purpose of avoiding OHSS in PCOS women. Although thus far the IVM technique is not widely used because there is a higher cancellation rate of IVM cycles compared with IVF, the aspiration process is more difficult, and the success rate is lower than IVF (Suikkari A, 2008).

1.8. PATHOPHYSIOLOGY OF HYPERSTIMULATION SYNDROME

The pathophysiological mechanisms of OHSS remain unclear, nevertheless it is widely accepted that this pathology is caused by an exaggerated response to COH. This response is characterized by generalized capillary leakage and an acute shift of protein-rich fluid (mainly albumin) from the vascular space into the peritoneal pleural, and to a lesser extent into the pericardial cavities. This process

is triggered when ovaries increase their size due to follicular stimulation, and leads to the characteristic hemodynamic alterations of OHSS, such as ascites, hydrothorax, and generalized edema.

This shift of protein-rich fluid is due to an increase in the VP after hCG stimulation. The severity of this process depends on the degree of the ovarian follicular response to the ovulation induction agents (Schenker J, et al., 1999). Hence, the root cause of OHSS is the development of multiple follicles. In a spontaneous ovulatory cycle, there are very efficient negative feedback mechanisms which ensure the development of only one dominant follicle. This follicle is ovulated in response to luteinizing hormone (LH) in the middle of the cycle. In a gonadotropin-stimulated cycle, this endogenous self-regulation does not exist; to ensure successful therapy exogenous hCG is administered in order to activate and maintain several antral follicles at once. This allows multiple follicles (rather than only one) to start and finish development, thus increasing the risk of developing OHSS.

1.8.1. Human chorionic gonadotropin

hCG is used to stimulate the LH receptor in assisted reproductive cycles, in order to initiate the final phases of both follicular and oocyte development. The biological activity of hCG is approximately 6-7 times that of LH due to its long half-life and its affinity for the LH receptor (Soares S, et al., 2008). hCG is considered an essential factor in the development of OHSS, even though this hormone does not have vasoactive properties, making it hard to believe that it is directly responsible for the development of OHSS (Gómez R, et al., 2002). If hCG were a vasoactive factor which acted directly on the ovaries, all women with OHSS would present elevated levels of hCG. However, despite high hCG levels being a risk factor, not all women with high hCG levels develop the syndrome. This data suggests that mechanisms other than hCG also trigger OHSS, most likely some mechanism which is triggered by hCG administration.

1.8.2. VASOACTIVE FACTORS

Experiments carried out on animals have demonstrated a direct and positive correlation between increase in the ovary size, increased VP, the severity of ascites, and the gonadotropin dose (Schenker J. and Weinstein D, 1978; Gómez R, et al., 2003a), suggesting that the development of ascites is secondary and is related to the rise in VP, which is in turn related to the dose of hCG. Given that hCG does not have vasoactive properties *per se* we assumed that the increase in VP, which is the origin of and characterizes OHSS, must be mediated by vasoactive factors which are secreted at the systemic or ovarian level. This explains why most studies on OHSS aim to elucidate which substances or factors with vasoactive properties might mediate the action of hCG in increasing in VP.

1.8.2.1. Estradiol

Due to the high levels of sexual hormones that are associated with OHSS, the role of E2 in the pathophysiology of this disease has been particularly studied (Asch R, et al., 1991). At first it was considered that high E2 levels could be responsible for the increase in VP, although later this hypothesis was discarded when several studies proved that E2 is not necessary for the development of the OHSS. In fact, one patient had a deficiency in CYP17A1 enzyme activity but still developed OHSS during IVF treatment despite the low levels of E2 resulting from this deficiency (Pellicer A. et al., 1991). Moreover, high E2 levels only appear if hCG levels are high (Aboulghar M, et al., 2003). Additionally, recent work has shown that administration of gonadotropin-releasing hormone (GnRH) causes a reduction in E2 levels which is directly linked with a decrease in vascular endothelial growth factor (VEGF) levels, reducing the incidence and severity of OHSS (Gustofson R, et al., 2006; Tong X, et al., 2008; Cerrillo M, et al., 2009; Cerrillo M, et al., 2010). Hence E2 levels are now considered to be one of the best OHSS risk predictors: women at a high risk of developing OHSS present high levels of E2 (more than 2500 pg/ml) or a fast increase in E2 serum levels (The Practice Committee of the American Society for Reproductive Medicine, 2008).

1.8.2.2. Renin-angiotensin system (RAS)

The existence of a direct correlation between the renin activity in plasma and the severity of OHSS has been described (Navot D, et al., 1987). The renin-angiotensin system has an important role in the pathophysiology of the ovary, especially in the transition from the preovulatory follicle to the corpus luteum, a highly vascularized structure. Theca cells from the ovarian follicles contain pro-renin as an inactive form of the renin enzyme (Paulson R, et al., 1989), which is activated in mid-cycle and catalyzes the conversion of angiotensinogen to angiotensin I (AI; Balen A, et al., 2008). AI is converted into angiotensin II (AII) by the action of the angiotensin-converting enzyme (ACE), after which AII promotes angiogenesis, increases VP, and stimulates the release of prostaglandins (Delbaere A, et al., 1997) and aldosterone.

Due to the important role of the renin-angiotensin system (RAS) in the induction of new vessel formation and in increasing VP (Navot D, et al., 1987), it might be responsible for the ovarian enlargement and accumulation of extracellular fluid which occurs during OHSS. High levels of renin, ACE, AI, and AII were observed in follicular fluid (FF) and plasma in OHSS patients (Sealey J, et al., 1986; Navot D, et al, 1987; Derkx F, et al., 1987). Furthermore, these patients also had high levels of AII and ascites, indicating activation of the RAS pathway in the OHSS ovaries (Delbaere A, et al., 1997). However, RAS cannot be responsible for ascites, but AII might act to mediate the formation of ascites via other vasoactive cytokines involved in the development of OHSS, such as VEGF (McClure N, et al., 1994, Revel A, et al., 1996). Administration of ACE inhibitors during ovarian stimulation with gonadotropins in a rabbit OHSS model reduced the incidence and severity of OHSS (Teruel M, et al., 2001). However, teratogenic effects have been associated with use of ACE inhibitors and so they cannot be used in women attempting to conceive.

1.8.2.3. Prostaglandins

It was initially thought that prostaglandins (PGs) had a key role in the development of OHSS (Schenker J. and Polishuk W, 1976) because they can induce OHSS directly or indirectly by increasing AII production. However, using the PG inhibitor indomethacin in rabbits, it was observed that blocking PG synthesis did not block the formation of ascites and enlarged ovaries (Pride S, et al., 1984), making it unlikely that they play a role in inducing OHSS.

1.8.2.4. Histamine

Histamine was originally seen as a possible OHSS mediator because the use of histamine receptor 1 inhibitors in rabbits inhibits the formation of ascites (Gergly R, et al., 1976) but several studies refute this possibility (Erlik Y, et al., 1979; Zaidise I, et al., 1983) because the levels of histamine in OHSS rabbits compared to controls is not different. It is not currently thought to be involved in mediating OHSS.

1.8.2.5. Endothelin

Vasoactive substances secreted by the endothelium, such as nitric oxide (NO), or endothelin (Vane J, et al, 1990, Battistini B, et al., 1993) maintain and regulate blood pressure. Although different roles for endothelin in the pathophysiology of reproduction have been suggested, its role as a possible OHSS mediator should also be highlighted (Goldsman M, et al., 1995; Elchalal U. and Schenker J, et al, 1997), because of its homeostatic effect on the endocrine system and circulation, and also its ability to interact with other endogenous vasoactive substances. There are studies which demonstrate that endothelin is found in FF and corpora lutea, and that its production is stimulated by hCG, (Masaki T, et al., 1993; Abae M. et al., 1994), which suggests that OHSS patient ovaries may overproduce this hormone.

1.8.2.6. Nitric oxide (NO)

NO has dilating effects on blood vessels and is produced by endothelial cells, macrophages, and certain groups of neurons. It is synthesized by NO synthase

(Griffith O. and Stuehr D, 1995; Snyder S, 1995) from L-Arginine, producing NO and L-Citrulline, and requires the presence of both the NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate; $NADP^+$) cofactor and oxygen. In neurons this molecule works as a neurotransmitter, conducting physiological signals through lipophilic cell membranes. When it is produced by endothelial cells in blood vessels it acts as a paracrine regulator, and in other cell types it is involved in cytotoxic signal transduction (Ben-Shlomo I, et al., 1994). During follicular development, an increase in NO is correlated with increased estrogen, and furthermore, NO can be increased with GnRH, human menopausal gonadotropin (hMG), and hCG treatment (Rosselli M, et al., 1994). In the process of ovulation, NO mediates the action of interlukin-1-beta (IL-1β), which induces the LH production necessary for ovulation (Ben-Shlomo I, et al., 1994; Bonello N, et al., 1996). The neovascular response, and many other angiogenic cellular mechanisms, require NO and are triggered by vasoactive agents through mechanisms autoparacrins (Ziche M, et al., 1994). It is also important to highlight its relationship with VEGF, which is considered to be the main trigger of this vasoactive agent in OHSS.

1.8.2.7. Inhibins

Inhibins are produced by the ovary and their main function is to regulate follicle stimulating hormone (FSH) production by the pituitary gland via endocrine mechanisms in order to regulate ovarian steroidogenesis by autocrine/paracrine mechanisms (Baird D. and Smith K, 1993). Inhibins are heterodimers composed of α and β subunits, which are linked by disulfide bridges. Inhibin-A consists of an α-subunit and a type-A β subunit, while inhibin-B is composed of an α subunit and a type-B β subunit. Both inhibin-A and inhibin-B are expressed by theca and GCs in follicles (Yamoto M, et al., 1992; Burns W, et al., 1990; Erämaa M, et al, 1993). Inhibin-B increases during the early follicular phase while inhibin-A stays at low concentrations (Groome N, et al., 1996; Schipper I, et al., 1998). Subsequently, the concentration of inhibin-B decreases and inhibin-A increases to a peak four days after the LH peak (LH+4). During the luteal phase inhibin-B decreases while

inhibin-A reaches its maximum concentration. Therefore, inhibin-B can be regarded primarily as a follicular hormone with a role in inhibiting FSH secretion during this phase, while inhibin-A is mainly a LH. Both inhibin-A and inhibin-B appear to be associated with follicular selection and therefore could be used to predict and monitor OHSS (Enskog A, et al., 2000).

Inhibin-A: Inhibin-A concentrations remain elevated after embryo transfer in OHSS patients but decrease in healthy patients which leads us to believe that inhibin-A production is elevated in OHSS ovaries (Enskog A, et al., 2000).

Inhibin-B: Inhibin-B concentrations are higher in FF obtained from gonadotropin stimulated cycles than those obtained from natural cycles (Magoffin D. and Jakimuk A, 1997). Therefore, inhibin-B is produced in antral follicles and especially in the dominant follicle in the natural cycle (Croome et al., 1996). However, the higher inhibin-B concentration in severe OHSS patients cannot be explained by the higher number of mature follicles, because it does not significantly differ with respect to the control group (Enskog A, et al., 1999), although enhanced GC hCG-sensitivity might explain this difference in OHSS patients (Humaidan P, et al., 2010).

Because there are no significant differences in inhibin-B levels at the beginning of OHSS between OHSS and control groups, it is unlikely that this hormone is responsible for the acute effects of the disease such as the formation of ascites. It has been suggested that the high concentrations of inhibin found before the onset of OHSS might prepare the follicles to produce large quantities of some factors which cause increased ovarian VP in response to hCG stimulation (Tollan A, et al., 1990).

1.8.2.8. Interleukins

Several studies have shown a correlation between inflammatory mediators such as plasma cytokines (interleukins 2, 6, and 8) and OHSS (Aboulghar M, et al., 1999).

Interleukin-2: Interleukin-2 (IL-2) is a glycoprotein with a half-life of 3 to 22 minutes which is produced by T cells and is released into the circulation. The release of this interleukin causes multiple toxic effects including the accumulation of extracellular fluid leading to ascites and pulmonary edema (Oppenheim J, et al., 1991). Its role in OHSS pathogenesis is debatable; several studies have shown that IL-2 concentrations in serum and peritoneal fluid are significantly higher in patients with OHSS (Aboulghar M, et al., 1999), and Orvieto et al. observed high levels of IL-2 in FF from OHSS patients versus control groups. Moreover, this group suggested that the concentration of IL-2 in FF can be used to predict OHSS, and that it may cause VP through systemic action because it passes directly into the peritoneal cavity (Orvieto et al, 1995). In contrast, another study found that IL-2 was undetectable in peritoneal fluid from severe OHSS patients (Revel A, et al., 1996). It is difficult to explain these conflicting results, although its short half-life may play a role. Although there is no evidence for a role for IL-2 in the pathogenesis of OHSS it is thought that it may be involved in the increase in VP.

Interleukin-6: Interleukin-6 (IL-6) is produced by monocytes, endothelial cells, T cells, and fibroblasts. It is involved in angiogenic processes which occur during ovarian follicle development and is linked to increased VP (Motro B, et al., 1990), and so it is thought that it could be involved in the development of OHSS, although exactly how remains unclear. Large amounts of IL-6 have been observed in ascitic fluid and serum from OHSS patients compared to control groups (Friedlander M, et al., 1993; Andus T, et al., 1992), supporting the hypothesis that it could serve as an OHSS marker (Aboulghar M, et al., 1999). IL-6 expression and its immunolocalization in OHSS patients has been studied in depth by Loret de Mola's group, who observed IL-6 immunostaining in corpus luteum and luteinized granulosa cells (LGCs; Loret de Mola J, et al., 1996a, b). The same group found that this cytokine is increased in OHSS and investigated whether its preovulatory concentration could predict OHSS. However they found that the preovulatory-state values during ovarian stimulation were similar between controls and OHSS patients (Loret de Mola J, et al., 1996a) indicating that IL-6 does not seem to be useful for

predicting the syndrome before hCG administration. In contrast Geva's group observed that high concentrations of IL-6 in FF at the time of oocyte aspiration, could predict early development of OHSS in high responders (Geva E, et al., 1997). Similarly, a study by Chen et al. confirmed that an increased IL-6 concentration in FF at oocyte recovery can be an early OHSS marker in patients undergoing IVF; additionally, IL-6 serum values on the day of hCG administration in IVF can distinguish patients with a high risk of OHSS (Chen C et al., 2000). These contradictory results have not yet allowed a relationship between IL-6 and OHSS to be firmly established.

Interleukin-8: This cytokine has neutrophil chemotactic activity and is an important angiogenic factor which has also been implicated as a possible OHSS marker. However, it is strongly correlated with E2 serum concentration and therefore it may not be directly involved in the development of OHSS (Chen C, et al., 2000). It is also known that interleukin-8 (IL-8) concentrations in peritoneal fluid in OHSS patients are significantly higher than in controls, but these differences are not reflected in serum levels (Revel A, et al., 1996). The importance of IL-8 in predicting OHSS has not yet been studied in depth, although a recent study has shown that the presence of IL-8 in the FF has an effect on the permeability of endothelial cells in culture, which may indicate a relationship between IL-8 and OHSS (Chen S, et al., 2010).

1.8.2.9. Vascular endothelial growth factor (VEGF)

Although the pathophysiology of OHSS remains to be elucidated, it is known that OHSS is caused by the presence of both pro- and anti-angiogenic factors in the FF. Due to its vasoactive properties, the pro-angiogenic cytokine VEGF is, very likely, the most important known mediator of OHSS (Pellicer A, et al., 1999; Garcia-Velasco J, et al., 2003). High levels of VEGF have been observed in the FF of patients suffering from OHSS, and several studies have highlighted the role of ovarian VEGF in mediating the development of OHSS (Doldi N, et al, 1997; Itskovitz-Eldor J, et al, 1997). Many studies have also shown that the concentration of VEGF in ascites fluid, serum, and plasma from OHSS patients is increased

(McClure N, et al, 1994 and Agrawal R, 1999). VEGF mRNA expression in LGCs is time and dose-dependent to the hCG (Neulen J, et al, 1995), and its high ovarian expression during OHSS development coincides with maximum VP. This relationship makes sense in the context of work establishing the close relationship between VEGF and hCG (Neulen J, et al, 1998), especially because the main factor that triggers OHSS is the administration of hCG which in turn stimulates VEGF secretion by GCs (Neulen J, et al, 1998; Fiedler K. and Ezcurra D, 2012), Figure 1. In fact, there are studies that clearly show how the GCs isolated from patients who later developed OHSS secrete larger amounts of VEGF in response to hCG than those collected from patients who did not develop OHSS after receiving hCG (Wang T, et al, 2002).

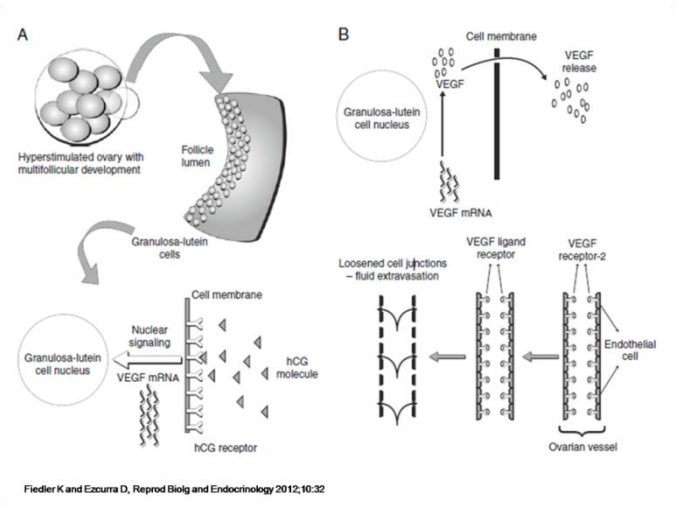

Figure 1. The pathogenesis of OHSS. A) hCG stimulates granulose-lutein cells, which increase their VEGF-mRNA production. The VEGF produced and released by granulose-lutein cells binds to VEGFR-2 on endothelial cell membranes and triggers an increase in VP.

There are two VEGF receptors produced by the endothelial cells (VEGFR-1 and VEGFR-2), and one of them exists in a soluble form in serum (sVEGFR-1), acting as a negative VEGF modulator. Excess VEGF increases the risk of OHSS, while excess sVEGFR-1 (and other anti-angiogenic factors) decreases the ovarian response and the risk of OHSS, although this is also accompanied by a decrease in the pregnancy rate (Pellicer A, et al, 1999). Total concentrations of serum VEGF have no value in OHSS risk assessment because of the natural variation in VEGF receptor-binding (Garcia-Velasco J, et al., 2003).

1.9. OVARIAN HYPERSTIMULATION SYNDROME PREVENTION STRATEGIES

It is not possible to completely prevent the occurrence of OHSS, but by identifying risk factors early on, and personalizing the ovarian stimulation protocol (La Marca A, et al, 2010) its incidence can be reduced. The COH protocol used should try to reduce the cycle cancellation rate and iatrogenic complications, including OHSS: a key factor in improving ART results (Nardo L, et al., 2009).

Before starting COH, patients with a high risk of OHSS can be identified based on their risk profile or biomarkers, and their stimulation protocol can be adapted to their needs via the COH. If COH is not applied correctly, patients are more likely to experience OHSS. To minimize the risk of severe OHSS complications during COH, secondary preventive measures are usually applied; several preventive protocols have been proposed to reduce or minimize these risks and are described in the following sections.

1.9.1. PRIMARY PREVENTION STRATEGIES

The different strategies known to reduce the risk of OHSS may be divided into two main types, *primary* prevention strategies and *secondary* prevention strategies. In primary prevention, the stimulation protocol is customized based on an initial assessment of the risk of developing OHSS, considering the different cases as *low*, *normal*, or *high responders*. Each of these is subjected to a different stimulation protocol in order to recruit a larger or smaller oocyte cohort depending on the type of responder they are.

1.9.1.1. Gonadotropin-releasing hormone (GnRH) antagonist protocol

In the 80s, GnRH agonists (GnRHas) were widely used for COH, which resulted in an increased incidence of OHSS (Humaidan P, et al., 2010). One possible explanation could be that pre-treatment to block endogenous gonadotropins requires an increased exogenous dose of FSH in order to promote ovarian stimulation. According to this hypothesis, unlike pretreatment with analoge agonits, GnRH antagonists (GnRHant) would quickly block the GnRH receptors therefore they would not need to be administered until just before the endogenous LH increase, typically at a follicle size of 12-14 mm. As follicle selection occurs at the beginning of the cycle, a small number of medium-sized follicles are produced. The lack of suppression of endogenous FSH during the early follicular phase means that a low dose of exogenous FSH is required for ovarian stimulation with GnRH antagonists (European Orgalutran Study Group, 2000; North American Ganirelix Study Group, 2001). Following on from this hypothesis, cycles in which antagonists are used would result in a lower incidence of OHSS compared with cycles in which agonists are used. This first emerged as a technique in 1988 (Itskovitz J, et al, 1988), and since then a large number of studies have investigated the safety and efficacy of the use of GnRH antagonists in COH.

Recent studies such as the Cochrane review (Al-Inany H, et al., 2006) have shown that the incidence of severe OHSS is significantly lower when using a protocol with agonists than when using antagonists (relative risk [RR] 0.61, confidence interval

[CI]: 95% (0.42 to 0.89), probability [*p*] = 0.01), and secondary prevention methods such as coasting and cycle cancellation were administered more frequently in patients where agonist protocols were used (RR: 0.44, CI: 95% (0.21 to 0.93), *p* = 0.03 [49]). Moreover, a second meta-analysis (Kolibianakis E, 2006) observed that the incidence of OHSS was significantly lower in antagonist cycles than in agonist cycles (RR: 0.46, CI: 95% (0.26 to 0.82), *p* = 0.01). Apart from reducing the risk of OHSS, E2 levels immediately reduced after the administration of GnRH antagonists, which was associated with a decrease in VEGF secretion, and therefore also of VP (Gustofson R, et al, 2006, Tong X, et al, 2008; Vrtačnik-Bokal et al, 2009; Giles J, et al, 2009).

However, despite these advantages, results from comparative studies have questioned the efficacy of GnRH antagonists with respect to results obtained from ART; for example, Humaidan et al. showed that the number of oocytes retrieved, fertilization rate, and embryo quality were similar in patients receiving GnRH versus those who received hCG (Humaidan P, et al, 2011), but there was a low pregnancy rate. The review by Cochrane concludes that, although shorter GnRH antagonist protocols significantly reduce the incidence of OHSS, they also lower the pregnancy and live birth rates compared to long standard protocols (Al-Inany H, et al, 2007). In contrast, other studies have found significant differences between both protocols when comparing live birth rates (Kolibianakis E, et al, 2006). Therefore based on current data, GnRH antagonists should be considered for use both in normal patients and high responders.

1.9.1.2. Luteal-phase support with progesterone

Higher steroid levels (estradiol and progesterone) present in the luteal phase after COH cause a impairment in this phase due to the negative feedback from the pituitary gland (Tavaniotou A, et al. 2001 and 2006), resulting in low levels of endogenous LH, which causes a reduction in implantation and pregnancy rates and an increase in the loss early pregnancies (Pritts E, et al., 2002). The use of hCG in luteal-phase support significantly counteracts these problems but is also

associated with an increased risk of OHSS and so it should be avoided when estradiol levels exceed 2700 pg/ml and when there are more than ten follicles (Fatemi H, et al, 2007). The use of progesterone (P4) seems to reduce this risk by half, and in addition improves pregnancy rates. Further evidence for the efficacy and safety of P4 comes from a prospective, multicenter, and randomized study of 1,211 IVF patients (Doody K, et al., 2008) who received P4 as a luteal-phase support; these patients had higher birth rates (35%-38%) and a lower incidence of OHSS.

1.9.1.3. *In vitro* oocyte maturation

An alternative technique to prevent OHSS could be *in vitro* maturation of immature oocytes collected during follicular puncture. Using this technique, the oocytes mature in a defined culture medium over 24-48 hours, after which they are fertilized *in vitro* and the embryo can subsequently be transferred. In a study of 56 patients with a high risk of OHSS occurrence during COH, hCG was given when the dominant follicle reached a diameter of 12-14 mm; 66% of the oocytes matured, resulting in a 46% rate of clinical pregnancy following fresh embryo transfer, and no cases of severe OHSS (Lim K, et al, 2005). In other clinical trials done on women with PCOS where oocyte maturation was used, the pregnancy rates per embryo transferred were adequate (20-54%) and implantation rates were acceptable (5.5-34.5%; Suikkari A, 2008). However, it should be highlighted that *in vitro* oocyte maturation is still an experimental procedure and that it is not yet considered an alternative fertility treatment. This is largely due to the technical difficulties in obtaining immature oocytes from unstimulated ovaries and cultivating them, because of the live birth rate is below that of conventional treatments, and as a result of a higher incidence of meiotic and chromosomal abnormalities when immature human oocytes are used (Lanzendorf S, 2006).

1.9.1.4. Insulin-sensitizing agents

Insulin resistance with compensatory hyperinsulinemia is thought to play a pathophysiological role in the ovarian dysfunction (Adashi E, et al., 1985; Barbieri

R, et al., 1986) and in hyperandrogenism (Dunaif A, et al., 1989; Nestler J, et al., 1991; Humaidan P, et al., 2010) associated with PCOS. It is known that insulin stimulates VEGF protein expression and secretion (Prakash A, et al., 2009), and that this stimulation is significantly higher in women with polycystic ovaries (PCOs) (Stanek M, et al., 2007). These insulin-sensitizing agents may reduce the incidence of OHSS by improving insulin sensitivity and reducing the hyperinsulinemia produced by these agents, thus, this strategy reduces both the symptoms of PCOS and the risk of OHSS. This could be explained by the fact that the GCs from women with PCOS are more sensitive to insulin and therefore, at least *in vitro,* they secrete more VEGF (Stanek M, et al., 2007) which normalizes hyperinsulinemia and could induce a decrease in GC VEGF secretion. Agents used for this purpose include the insulin sensitizer thiazolidinedione, although this is associated with increased cardiac morbidity and therefore may be contraindicative for use in healthy PCOS women (Dunaif A, et al., 2008). However, metfromin is an inexpensive, effective, and safe alternative which sensitizes cells to insulin, and which has been reported to reduce the risk of OHSS in women with PCOS (Costello M, et al., 2006).

1.9.2. SECONDARY PREVENTION STRATEGIES

Secondary methods of preventing OHSS are practiced when there is a risk factor resulting from excessive ovarian response after ART, and include the withdrawal, reduction, delay, or modification of elements of the stimulation protocol in order to prevent OHSS in these patients.

1.9.2.1. Coasting

Coasting, in order to prevent the OHSS, was first described in 1995 by Sher et al., and is a strategy in which hCG administration is postponed in women who respond to ovarian stimulation with high E2 plasma levels until those levels are considered to be safe. It is an alternative to cycle cancellation in situations where the serum E2 levels are higher than 3000 pg/ml and there are more than 20 follicles. Follicular size is correlated with the FSH threshold and therefore, large follicles (which are

more resistant to apoptosis and atresia) continue to grow when the FSH levels decrease, while the apoptosis rate of the smaller follicles (under 14 mm) increases. Coasting leads to selective regression of immature follicles, reducing the number of GCs available for luteinization, and resulting in a decrease in the vasoactive substances involved in the pathogenesis of OHSS, such as VEGF. Coasting has been used to reduce the incidence of OHSS in high-risk patients without affecting the cycle, as shown in several studies (Alper M, et al., 2009; Levinsohn-Tavor O, et al., 2003; García-Velasco J.A et al., 2004; Youssef M, et al., 2010).

Although it has been widely used, and retrospective studies have shown that coasting is effective, there is not currently sufficient evidence to recommend its use in routine practice. A careful review (Levinsohn-Tavor O, et al., 2003) concluded that coasting should be used in patients who present serum E2 levels higher than 3000 pg/ml and a follicle size between 15-18 mm, until E2 levels drop to safe levels below 3000 pg/ml. If it takes more than three days for these levels to drop it is advised that the embryos should be frozen because data shows that thereafter the pregnancy rate decreases within that cycle.

1.9.2.2. Reduced human chorionic gonadotropin (hCG) dose

HCG is used in ovarian stimulation treatments to trigger ovulation or to provoke oocyte maturation in IVF cycles. The most commonly used dose is 10,000 IU but it is known that these hCG doses may cause OHSS in certain patients. Decreasing the standard doses used to trigger oocyte maturation in these patients can prevent OHSS; 5,000 IU have been successfully used to trigger ovulation without impairing clinical outcome (Schmidt D, et al., 2004; Kolibianakis E, et al., 2007; Humaidan P, et al., 2010). Promising results have also been reported by Cornell using a low-dose protocol, who determined the hCG dosage according to serum E2 levels on the day of hCG administration. The levels of hCG administered are between 5,000 and 3,000 IU in women with E2 levels between 2,000 and 3,000 pg/ml. Women with levels of E2 higher than 3,000 pg/ml undergo coasting until E2 falls below 3,000 pg/ml (Humaidan P, et al., 2010).

A retrospective study of 250 cycles, which compared a dose of 5,000 IU versus 3,300 IU, observed that the latter dose is as equally effective at triggering oocyte maturation in ART as the former, and do not adversely affect the results of the cycle (Schmidt D, et al., 2004). However, other studies have shown that a dose of 2,000 IU is not sufficient to trigger oocyte maturation (Kashyap S, et al., 2010). Currently the benefits of low hCG doses for OHSS prevention are unclear, because data are scarce and the studies that have been conducted have small sample sizes and do not have a statistical power high enough to detect a significant difference in the rate of OHSS.

1.9.2.3. Cryopreservation of all embryos and oocytes

Cryopreservation is considered a traditional strategy to prevent OHSS when using COH techniques. This strategy consists of stimulating the normal cycle without transferring the embryos, instead cryopreserving them for transfer later when the patient's hormone levels are lower. Thus, although the early phases of OHSS can occur (Aboulghar M, et al., 2003; Wada I, et al., 1992; Queenan J, et al., 1997; D'Angelo A. and Amso N, 2002) later stages are prevented because the high levels of hCG associated with pregnancy are avoided (Wada I, et al., 1992; Amso N, et al., 1990; Salat-Baroux J, et al., 1990). Cryopreservation appears to reduce, but not eliminate, OHSS without adversely affecting pregnancy rates (Sills E, et al., 2008; Ferraretti A, et al., 1999), and in addition it has a higher pregnancy rate than coasting (Gera P, et al., 2010). However, this procedure does present risks, as shown by a retrospective study in which the maternal death rate due to OHSS was elevated in women whose embryos had been frozen due to previously observed OHSS symptoms (Braat D, et al., 2010).

Recently, interest in cryopreservation has increased due to the introduction of vitrification: an efficient cryopreservation method which improves survival after thawing. Because vitrification very quickly converts liquid to solid without forming ice crystals the damage inflicted upon embryos by freezing with traditional cryopreservation techniques is significantly reduced. Several studies have shown

that in ART vitrification is associated with better results than slow cooling (Busso C, et al, 2010). Embryo vitrification has proved very successful for preventing OHSS in women at a high risk of the syndrome (Selman H, et al., 2009). Moreover, one study showed a pregnancy rate of 36.9% when using vitrification, a rate three times higher than using traditional cryopreservation (Al-Hasani S, et al., 2007).

1.9.2.4. Cycle cancellation

hCG administration is one of the major OHSS risk factors. Cancelling the cycle is a strategy which is considered in patients whose scans show significant follicular development and/or have serum E2 levels that are too high. Although this prevention technique is the most effective, it is costly and psychologically demanding on patients. Therefore, this technique is generally reserved for patients at the highest risk of OHSS.

1.9.2.5. Alternative agents for triggering ovulation

hCG has been successfully used to trigger ovulation for over 60 years, however, the relatively long half-life of hCG in serum results in prolonged luteotropic effects (Damewood M, et al., 1989), the development of multiple corpus luteum, and increased levels of E2 and P4 in the serum throughout the luteal phase (Itskovitz J, et al., 1991), which increases the risk of OHSS (Humaidan P, et al., 2010). Therefore alternative agents for triggering final oocyte maturation and ovulation have been investigated.

Recombinant luteinizing hormone: Studies using recombinant LH (rLH) as a possible alternative to hCG for triggering ovulation showed that moderate OHSS was observed in 12% of patients who received two doses of recombinant LH compared to 12.4% of patients who received hCG, but did not observe moderate or severe OHSS in patients who received a single 30,000 IU dose of recombinant LH (The European Recombinant LH study Group, 2001). These results showed that a single dose of rLH (5,000 IU of hCG) is effective at inducing follicular maturation

and early luteinization, although despite its clear effectiveness at reducing OHSS its high cost makes it an unfeasible alternative.

Gonadotropin-releasing hormone: The GnRHas do not physiologically stimulate an endogenous LH peak when used to induce ovulation (Hoff J, et al., 1983; Itskovitz J, et al., 1991). Whereas hCG is detectable in serum for up to six days after intramuscular injection of (5,000 IU), a single injection of GnRHa results in a combination of FSH and LH peaks which lasts for 34 hours (Gonen et al., 1990). Therefore, the short duration of LH activity after GnRHa administration, as compared to hCG, may reduce ovarian stimulation in the luteal phase (Segal S. and Casper R, 1992).

Several studies have described a GnRHa 'flare up' effect in which the pituitary gland induces endogenous LH and FSH peaks (Bentick B, et al., 1988; Emperaire J. and Ruffie A, 1991). One or two GnRHa doses were administered to six patients, causing an increase in LH/FSH, and resulting in a moderate response in most patients, and an exaggerated response in eight subjects (Itskovitz J, et al., 1991). The levels of E2 and P4 were lower in patients who were administered GnRHas than in patients injected with hCG, and there were no signs of OHSS. These findings have since been backed up by similar results obtained by others (Imoedemhe D, et al., 1991.; Van der Meer S, 1993; Balasch J, et al., 1994; Shalev E, et al., 1994). In one study in which 179 women received 5,000 IU of either hCG or GnRHa to trigger follicle maturation, E2 concentrations were comparable in both groups and there were no cases of OHSS (Segal S. and Casper R, 1992).

1.9.2.6. Albumin and hydroxyethyl starch (HES) administration

Albumin is a protein present in plasma which can bind to the vasoactive agents responsible for the development of OHSS thus facilitating their removal from circulation. It has been proposed that intravenous administration of albumin during oocyte recovery could be used as a preventive measure in women at a high risk of the syndrome (Asch R, et al., 1993). The mechanism of albumin action is unknown but it is thought to block the vasoactive substances implicated in OHSS

pathogenesis and additionally, to increase the intravascular osmotic pressure, which prevents water loss from the intravascular compartment.

There are multiple studies with contradictory results about albumin use, but a careful meta-analysis (Aboulghar M, et al., 2002) reported that albumin use during follicular aspiration decreased the incidence of severe OHSS in high-risk women. However, the optimal dose of albumin and timing for its use are unclear, and it is unknown how it effects embryo implantation. There are some studies which do not agree with the use of intravenous albumin: a controlled study with 988 patients clearly concluded that the use of albumin at the time of oocyte recovery did not prevent OHSS (Bellver J, et al., 2003). The use of albumin also has side effects, such as fever, nausea, vomiting, and possible anaphylactic reactions. Furthermore, as it is a human biological product there is also the potential risk of the transmission of viruses and prions.

Hydroxyethyl starch (HES), a colloidal plasma substitute, has been used as an alternative to albumin as it causes an increase in intravascular volume and osmotic pressure. Its molecular weight is greater than that of albumin, and it has a half-life of about 10 hours. There are several studies which have published that HES is as effective as human albumin in preventing OHSS (Gokmen O, et al., 2001; Youssef M, et al., 2011) and moreover, it is free of the potential risks of albumin. Hence this method may be safer, cheaper, and more effective alternative to the use of albumin (Konig E, et al., 1998).

1.9.2.7. Dopaminergic agonist administration

Ovarian stimulation in PCOS patients leads a high risk of developing severe OHSS: prevention of the syndrome in these patients is difficult because there is a narrow margin between the dose required to produce a reasonable stimulation and the dose which induces OHSS. There is evidence that the release of LH has a dopaminergic component, so that pre-treatment with dopamine agonists, such as Cabergoline (Cb2), before inducing ovulation reduces the ovarian response to FSH, meaning that dopamine agonists could be used to prevent OHSS in PCOS

patients. Several studies have shown that if dopamine agonists, such as Cb2 and quinagolide, are given on the same day as hCG administration they may be able to reduce the incidence of OHSS by inhibiting VEGFR-2 phosphorylation in response to hCG (Gómez R, et al., 2010; Youssef M, et al., 2010), suggesting that Cb2 has a role in the secondary prevention of OHSS after ovarian stimulation. Two randomized trials have shown that Cb2 (0.5 mg/d) is more effective than intravenous albumin administration in preventing OHSS (Tehraninejad E, et al., 2010; Carizza C, et al., 2008). Recent studies have also demonstrated that Cb2 appears to reduce, but not eliminate, moderate OHSS and does not seem to affect the outcome of ART. Paradoxycally, dopamine agonists cannot prevent late OHSS (Gómez R, et al., 2010). Further study is still needed to confirm the efficacy and safety of Cb2 in primary and secondary prevention of OHSS during ovarian induction.

1.9.3. UNRECOMMENDED STRATEGIES

Follicular aspiration: The aspiration of GCs from an ovary, leading to intraovarian bleeding and limiting the production of OHSS mediators, allows the contralateral development of the other ovary. This strategy is not recommended because removal of the follicular aspirate is not easy, is costly, provokes patient discomfort, and is an invasive procedure which requires anesthesia.

Aromatase inhibitors: Aromatase is involved in estrogen production (Santen R, et al, 2003) and it has been suggested that using inhibitors of this enzyme could help to reduce excessive estradiol synthesis during ovarian stimulation and consequently reduce the risk of OHSS. Although the anti-estrogenic effects of these agents seems promising in preventing or reducing the risk of OHSS, there are not many studies which evaluate the impact of aromatase inhibitors on OHSS in women with the anovulatory infertility associated with PCOS, and so they cannot yet be recommended for clinical use (Humaidan P, et al., 2010).

1.10. THE VASCULAR ENDOTHELIAL GROWTH FACTOR / RECEPTOR -2 SYSTEM

1.10.1. Vascular Endothelial Growth Factor (VEGF)

Due to the important role of VEGF in OHSS, VEGF system must be described in detail. This growth factor was first described in the 80s as vascular permeability factor (Senger D, et al., 1983) and was later renamed vascular endothelial growth factor. VEGF plays an important role in regulating blood and lymph vesicle formation and in the maintenance of homeostasis. It is produced by endothelial, hematopoietic, and stromal cells in response to hypoxia and/or stimulation by other growth factors, such as interleukins or platelet growth factor. Specifically, VEGF interacts with hematopoietic cells, endothelial precursor cells, and differentiated mature endothelial cells. It is a heparin-binding homodimeric glycoprotein of approximately 40 kDa, which belongs to the PDGF (platelet-derived growth factor) family of growth factors. In mammals, the VEGF family consists in VEGF-A and its homologues VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PIGF). VEGF-A is the main member involved in angiogenic processes, while VEGF-B, VEGF-C, and VEGF-D are mainly involved in lymphangiogenesis. Recently, VEGF-C has been shown to be an important inducer of neurogenesis, without exerting angiogenic effects.

The human VEGF-A gene is located on chromosome 6 (ep12; Wei et al., 1996) and is composed of 8 exons (Figure 2; Harper S. and Bate D, 2008); alternative splicing of exons 6 and 7 generates six molecular variants which have a different number of amino acids. In humans, these variants correspond to: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183,}$ $VEGF_{189}$, and $VEGF_{206}$ (Tischer E, et al., 1991: Houck K, et al., 1991), of which the most common and well-studied isoforms are $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$.

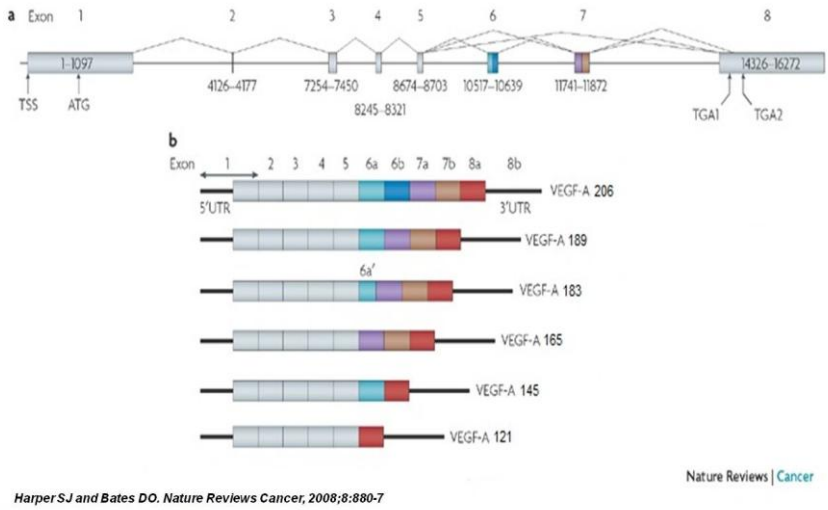

Figure 2. Schematic representation of the different isoforms of human VEGF-A. a) Gene structure of human VEGF. b) Alternative splicing of exons 6, 7 and 8 generates six isoforms. Inclusion or exclusion of exons 6 and 7 enhances the ability of each isoform to bind and activate the VEGF receptors (VEGFRs).

VEGF isoforms share the same N-terminal amino acid sequence, which contains the binding sites for VEGFR-1 and VEGFR-2 (contained by exons 3 and 4 respectively), but may or may not contain the sequences encoded by exons 6 and 7 in the C-terminus, due to the alternative splicing. This C-terminal region encodes two heparin-binding domains (HBDs), each of which confers the ability to bind to cell surfaces and extracellular matrix, thus determining the localization of the VEGF-A isoforms in the extracellular space. The VEGF-A_{145}, VEGF-A_{189}, and VEGF-A_{206} isoforms all contain two HBDs encoded by exon 6 and exon 7, and are therefore strongly sequestered in the extracellular matrix and on cell surfaces (Houck K, et al., 1992; Park et al., 1992), but are not secreted into the cytoplasm. Theses isoforms are detected in most tissues that express VEGF, except for VEGF-A_{206}, which is only detected in human fetal liver (Houck K, et al., 1991). However, the binding efficiency of the VEGF-A isotype varies with the presence of HBDs; for example VEGF-A_{165}, the predominant molecular form, only has one HBD

encoded by exon 7, meaning it has a reduced binding affinity and so while a significant proportion remains bound to the extracellular matrix, the other part is soluble (Houck K, et al., 1992). VEGF-A_{121} lacks the HBD and so it freely diffuses when it is secreted because does not bind to cell surfaces or extracellular matrix; it has been suggested that this isoform might form foci upon tissue injury, and may be required to form the new vessels required to repair tissues (Watkins R, et al., 1999).

1.10.2. The role of vascular endothelial growth factor and its receptors in angiogenesis and vascular permeability

The biological functions of VEGF polypeptides are mediated when they bind to type II receptor tyrosine kinases (RTKs), VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), or VEGFR-3 (Flt-4) (Ferrara N, 2004; Shibuya M, et al., 1990; Terman B, et al., 1991; Pajusola K, et al., 1992; Mattherws W, et al., 1991). These latter three consist of an extracellular domain comprising seven immunoglobulin (Ig)-like domains, a transmembrane domain, followed by a kinase domain divided into two parts by the insertion of a non-catalytic 100-amino acid residue sequence, and a C-terminal tail (Cross M. and Claesson-Welsh L, 2001). These receptors are expressed on the cell surface of many cells, including hematopoietic cells (Kabrun N, et al., 1997), macrophages, endothelial cells (Ferrara N. and Savis-Smyth T, 1997), some malignant cells (Bellamy W, 2002), and vascular smooth muscle cells (Ishida A, et al., 2001). VEGFs show distinct patterns of receptor specificity as indicated in Figure 3. The major signaling receptor for VEGF, mediating most of is biological activities in endothelial cells, is VEGFR-2, which seems to be more involved in the process of angiogenesis, vasculogenesis, and VP (Shalaby F, et al, 1995; Verheul H, et al., 2000).

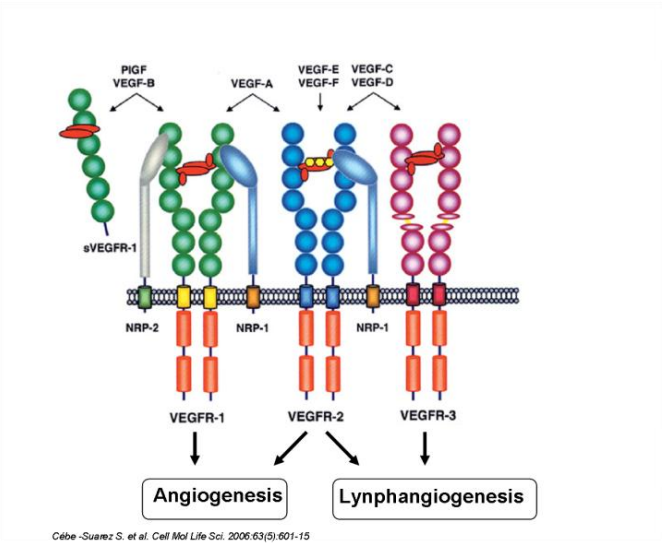

Figure 3. Schematic representation of VEGF ligands and their receptors. VEGF ligands are recognized by VEGFR receptors and carry out specific functions related to vascularization, such as angiogenesis and lymphangiogenesis. Placental growth factor (PlGF) can be recognized by VEGFR-1 and carries out a similar function in angiogenesis.

Angiogenesis is considered to be the formation of new blood vessels from pre-existing endothelium (Folkman J. and Klagsbrun M, 1987). It is required for both the development and differentiation of the endothelium, such as in the repair damaged organs (Schwartz S, et al., 1990; Klagsbrun M. and D'Amore P, 1991). Several angiogenic regulators have been identified but the most important regulator is VEGF, which has potent angiogenic activity in both *in vitro* and *in vivo* studies (Zimmermann R, et al., 2001; Bates D, et al., 1996; Ferrara N, et al., 1992 and 1997). VEGF facilitates angiogenesis in *in vitro* models, stimulating the formation of capillary-like structures and invasion of endothelial cells (Pepper M, et al., 1992). *In vivo* models have also demonstrated that VEGF has a strong angiogenic response (Folkman J. and Klagsbrun M, 1987: Klagsbrun and D'Amore, 1991; Folkman J. and Davis-Smith T, 1992) and is a potent mediator of VP (Keck P, et al, 1989).

VEGF regulates vasculogenesis and angiogenesis during embryonic development (Millauer B, et al., 1993) and in adult tissues which require cyclic angiogenesis such as endometrium (Charnock-Jones S, et al., 1993; Torry D, et al., 1996) and in corpus luteum formation in women of reproductive age (Ravindranath N, et al., 1992). This growth factor is also involved in diseases that are characterized by deregulated angiogenesis, and is elevated in the peritoneal fluid of women with endometriosis (McLaren J, et al., 1996). *In vitro* studies in which VEGF was activated in endothelial cells showed that this growth factor is able to induce changes in morphology (Senger D, et al., 1993), division, proliferation (Connolly D, et al., 1989; Whelan I. and Vlahos N, 2000), cell migration (Ferrara N, et al., 1992), increases in VP (Feng D, et al., 1996), angiogenesis (Leung D, et al., 1989; Ferrara N. and Davis-Smith T, 1997; Whelan I. and Vlahos N, 2000), and to promote the accumulation of cytoplasmic calcium (Brock T, et al, 1991).

VEGF expression is regulated by several factors: hypoxia, cytokines, NO, and hormones. Hypoxia is a condition in which tissues receive small amounts of oxygen, and is an important stimulus for angiogenic factors such as VEGF *in vitro* (Sandner P, et al., 1997), and *in vivo* (Shweiki D, et al., 1992). Hypoxia-induced VEGF production may be the driving force that stimulates the angiogenesis that accompanies organ formation during development (Neufeld G, et al., 1999). Some cytokines or growth factors such as TGF-β 1 (Frank S, et al., 1995), IL-1β (Li J, et al., 1995), IL-1 and PGE2 (Ben-Av P, et al., 1995), and IL-6 (Cohen T, et al., 1996) induce an increase in VEGF in different cell lines. Furthermore, the neovascular response, and many other related cellular mechanisms necessary for angiogenesis, occurs via the action of NO. NO can be triggered by vasoactive agents through autocrine and paracrine mechanisms (Davies M, et al., 1995) and VEGF increases NO in a dose-time dependent fashion in vascular endothelium (Kelly R, et al., 1996). Clear evidence that NO may mediate VEGF action is that the NO synthase inhibitors block the effect of VEGF in capillaries (Morbidelli L, et al, 1996; Whelan I. and Vlahos N, 2000).

Studies suggest that angiogenesis plays an active role in cyclic folliculogenesis because it is initiated in the early development of the follicle, and a complex vascular network is formed within the thecal cell layer during follicular growth (Koos R, et al, 1993; Zimmermann R, et al., 2001). VEGF mRNA is present in developing follicle GCs during the mid- and late-follicular phases, and mature follicles express higher amounts. Levels of VEGF mRNA are correlated with E2 and P4 levels (Doldi N, et al., 1997) indicating that angiogenesis triggered by VEGF is important in the growth and maintenance of ovarian follicles and corpus luteum. This theory is further supported by the fact that blocking VEGF with specific inhibitors causes inhibition of the ovulatory process (Zimmermann R, et al, 2001).

The relationship between VEGF and hCG (Neulen J, et al., 1998; Wang J, et al., 2002) is extremely important in understanding the development of OHSS because hCG administration is the main risk factor involved in the syndrome. In fact, recent studies show that GCs from patients who later develop OHSS secrete higher amounts of VEGF in response to hCG than those from patients who did not develop OHSS (Wang T, et al., 2002). McClure N, et al. (1994) studied the role of VEGF in OHSS and concluded that this growth factor is primarily involved in increasing VP.

Animals which lack one of the two VEGF alleles all die before birth because of cardiovascular system development defects (Ferrara N, et al., 1996; Carmeliet P, et al., 1996; Neufeld G, et al, 1999) thus highlighting the importance of VEGF as a central regulator of vasculogenesis and the cardiovascular system. Moreover, several studies have demonstrated that disrupting the genes encoding VEGFR-2 (Shalaby F., et al., 1995) and VEGFR-1 (Fong G, et al., 1995) results in severe blood vessel formation abnormalities in homozygous animals (Neufeld G, et al., 1999). It is known that VEGFR-1 expression is particularly prominent in arterial endothelium (Peters K, et al., 1993) whereas VEGFR-2 is increased in capillaries, veins, and venules (Millauer B, et al., 1993). VEGFR-1 plays an important role in the morphogenesis and structuring of endothelial cells (Fong G, et al., 1995) while VEGFR-2 is involved in mitogenesis (Shalaby F, et al., 1995; Gille H, et al., 2001).

Several studies have shown that VEGFR-2 is able to regulate angiogenesis and VP in endothelial cells whereas VEGFR-1 is not associated with increased VP (Waltenberger J, et al., 1994, Gille H, et al., 2001). For this reason it has been postulated that the effects of increased permeability are mediated by the binding of VEGF to VEGFR-2 and not to VEGFR-1 (Waltenberger J, et al., 1994, Gille H, et al., 2001).

1.10.3. Signal transduction mechanisms

Although the VEGF receptors are known, the specific endothelial responses mediated by VEGFR-1 and VEGFR-2, as well as their downstream signal transduction pathways, are not yet fully understood. Cultured endothelial cells express both VEGFR-1 and VEGFR-2 making it difficult to evaluate the contribution of these receptors to the responses induced by VEGF. However, based on studies which determined the enzymatic cascade originating from VEGFR2 signal transduction (Esser S, et al., 1998), the current view is that VEGFR-2 is the major endothelial cell receptor which transduces the effects of VEGF, as shown in Figure 4. VEGFR-2 is implicated in most, if not all, aspects of vascular endothelial cell biology, and importantly, during development VEGFR-2 is the first specific endothelial marker to be expressed on hematopoietic/endothelial progenitors.

Increases in VP associated with OHSS were significantly inhibited *in vivo* by blocking calcium channel transporters linked to the intracellular space (Bates D. and Curry F, 1997). Criscuolo G. and Balleduz J. (1996) hypothesized that calcium entry, promoted by VEGFR-2 signal transduction, induces changes in cell morphology by modifying endothelial cell cytoskeletal actin fibers, and that addition of VEGF caused the actin fibers in this endothelial monolayer to reorganize leading to the formation of gaps between cell junctions. Subsequently, it was determined that after VEGF addition, this reorganization occurs not only in cytoskeletal actin fibers, but also in intercellular junction proteins, such as occludin and zonula occludens-1 (ZO-1), which help to maintain tissue cohesion and stability (Kevil C, et al., 1998). These findings indicate that the cell junctions were missing, allowing

the formation of inter-cellular pores which facilitate leakage. Other reports support these findings, showing that the cell junction stability is dependent on the phosphorylation-dephosphorylation reactions promoted by VEGF (Antonetti D, et al., 1999).

Given the role of VEGF in modulating both VP and angiogenesis, there has been great scientific effort to develop and establish several strategies which can act on VEGFR-2 transduction pathways in order to inhibit VEGF secretion.

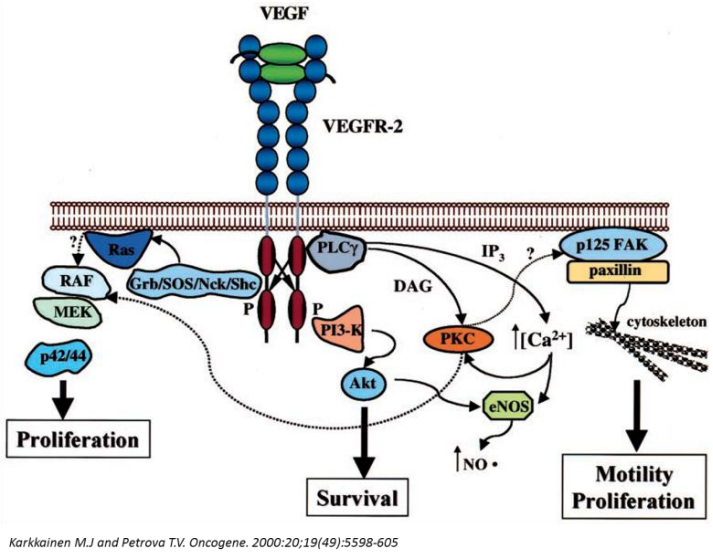

Figure 4. A simplified scheme showing the signaling events initiated in endothelial cells upon VEGFR-2 activation. VEGF binding to the VEFGR-2 in the endothelium requires VEGFR-2 autophosphorylation in order to activate it. Phospholipase C (PLC) is activated which releases diacylglycerol (DAG) and inositol triphosphate (IP3) as second messengers. These intermediaries activate different pathways, which leads to increased vascular permeability.

1.10.4. Vascular endothelial growth factor/receptor-2 pathway inhibitors

VEGF binding to VEGFR-2 leads the development of new blood vessels which often adhere to and nourish tumor cells. In order to block this union, different VEGF/VEGFR-2 pathway inhibitors have been studied. In 1995, rodent anti-VEGFR-2 neutralizing antibodies were generated, of which DC101 was the most used (Rockwell P, et al., 1995). Subsequently, both DC101 and cp1c11 (Zhu Z, et al., 1999) were modified for clinical use (humanized antibodies), marking the beginning of a new type of cancer treatment. Other strategies were used to generate peptides which would prevent VEGF from binding to the VEGFR-1 and VEGFR-2 receptors in the hope that these could lead to the development of pharmacologically useful VEGF antagonists (Wayne J, et al., 1998).

Although these antibodies were specifically designed to inhibit the growth of new blood vessels which could supply nutrients to tumor cells, their efficacy in specifically modifying VP has not been assessed. Suramin was originally used as an antitrypanosomal drug in 1916, but recently new properties, such as its ability to decrease VP have been uncovered, although its effect if altering mitochondrial genetic information makes it too dangerous to use as a therapeutic agent for pregnant women with OHSS. Furthermore, its permeability-blocking effects are not specific, and it tends to decrease endothelial mitogenicity. ZD4190 also represented a step forward in inhibitor development, not by increasing blocking ability but because the compound could be administered orally (Wedge S, et al., 2000). However, it is not a specific endothelial permeability inhibitor so its future use will depend on appropriately modifying it to be more specific.

Since then, a wide range of specific VEGFR-2 inhibitors have been developed (Strawn L, et al., 1996), such as SU5416, which inhibits VEGFR-2 phosphorylation, preventing signal transduction and therefore also the enzymatic cascade leading to significant increases in VP (Fong T, et al., 1997). This inhibitor was originally designed as an antitumor agent; however its most important effect is its ability to inhibit endothelial cell permeability in rodents harboring a tumor cell line. SU5416 is

an excellent candidate for blocking or inhibiting the *in vivo* VP which characterizes OHSS, because: 1) it decreases the endothelial permeability in rodents without apparent toxic effects; 2) it is specific for VEGFR-2 without cross reacting with other receptors in the same family such as VEGFR-1; 3) it has a high affinity for its receptor.

Gómez et al. demonstrated that the increase in VP brought on by hCG administration coincides with ovarian production and expression of VEGF and VEGFR-2, and used SU5416 in an animal OHSS model to counteract the increased VP caused by hCG administration (Gómez R, et al., 2002 and 2003). Despite this being the first study that established a specific OHSS therapy, there are two drawbacks which mean it is not suitable for humans, and is mutually exclusive to ART. First, the high toxicity of commercial VEGFR-2 inhibitors: for example, SU5416 brings on pulmonary hypertension and has thromboembolic effects (Taraseviciene-Stewart L, et al., 2001; Kuenen B, et al., 2003; Glade-Bender J, et al., 2003). Second, the absolute functional dependence of the ovary (Wulff C, et al., 2001; Pauli S, et al, 2005; Zimmermann R, et al, 2001 and 2003) and uterus (Rockwell L, et al., 2002; Heryanto B, et al, 2003) on VEGF secretion, and its requirement for maintaining pregnancy. Nevertheless, these studies demonstrated that blocking VEGFR-2 does decrease the excessive VP found in OHSS, and so based on this knowledge, new strategies which block increases in VP through VEGFR-2 or its signal transduction intermediates can be developed as effective and tolerable therapeutic treatments in humans.

1.11. ALTERNATIVE STRATEGIES FOR REPLACING COMMERCIAL VEGFR2 INHIBITORS TO PREVENT OHSS

In an attempt to avoid OHSS-associated angiogenesis, genes that were upregulated and downregulated in the ovaries of OHSS and control rats were studied (Gómez R, et al, 2003); only genes coding for target proteins that might have been involved in regulating VP were taken into account, resulting in the identification of 72 genes (including VEGF and prostaglandins) in the OHSS group which were possible OHSS mediators. From these genes, four were selected for further study based on a literature review: squalene epoxidase (SQE), tartrate-resistant acid phosphatase (TRAP), insulin-induced growth response protein (CL-6), and (TH). Interestingly TH, an enzyme present in dopaminergic cells and involved in dopamine (Dp) synthesis (Fujisawa H, et al, 1987; Zigmond M, et al., 1989), was downregulated in rat OHSS ovaries. Given that Dp inhibits the release of LH (Chang J, et al., 1990), which in turn is implicated in the development of OHSS when its expression is increased, a possible role for Dp in the development of OHSS via LH modulation was considered. However, a potential alternative mechanism for Dp in OHSS modulation was shown in which Dp might act as a modulator of VEGFR2 function (Basu S, et al., 2001). Given the paramount importance of VEGFR2 in the development of OHSS D2-ags were considered as possible candidates for OHSS treatment via VEGFR2.

The following section contains a detailed description of our current understanding of the dopaminergic system which will facilitate the subsequent comprehensive understanding of modulation of the VEGF/VEGFR2 system by Dp.

1.12. DOPAMINE AND DOPAMINERGIC RECEPTORS

Dopamine or 3,4-dihydroxyphenethylamine (Dp) is a neurotransmitter which acts as a chemical messenger that exerts its function by transmitting signals in the brain and other vital areas, and has been described in both humans and animals. Dopaminergic receptors belong to a class of G protein-coupled receptors, of which

Dp is the primary endogenous ligand. There are five subtypes: D1, D2, D3, D4, and D5, which are organized into two families (D_1-like or D_2-like); the D1 and D5 receptors are members of the D_1-like family, whereas the D2, D3, and D4 receptors are members of the D_2-like family. The biological function and location of the different Dp receptor subtypes still remains unclear (Vallone D, et al., 2000), although the presence of D2 receptors has been described on ECs (Basic F, et al., 1991), lymphocytes (Basu S. and Dasgupta, 2000), and platelets (Emerson M, et al., 1999).

Dp controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation (Missale C, et al, 1998). This catecholamine also exerts an inhibitory action on Prolactin (Prl) secretion (Lacasse P, et al., 2012; Freeman M, et al., 2000), which strongly inhibits lactation when it is suppressed (Flint D. and Gardner M, 1994; Taylor J. and Peaker M, 1975), therefore Dp administration has previously been used in the clinic to inhibit lactation.

1.13. THE DOPAMINERGIC SYSTEM IN THE OVARY

It is known that there are high levels of Dp both in FF and human ovarian biopsies (Bodis J, et al., 1992; Lara H, et al., 2001), for this reason its function within the ovary has been questioned: if Dp has a specific ovarian function, a requisite for its action is the presence of functional receptors. A study carried out by Rey Ares's group described the presence of D1 and D5 (D_1-like family) and D2, D4 (D_2-like family) but not D3 on human GCs (Rey-Ares V, et al., 2007), and of the Dp receptors found on them, D2 was had the highest expression.

Although GCs do not synthetize Dp (Mayerhofer A, et al., 1998), these cells are able to absorb it from the circulation, store it, and metabolize it (Greiner M, et al., 2008). Taking this knowledge and the findings provided by Rey Ares's study together, Dp might act through the D1, D2, D4, and D5 pathways which are co-expressed on GCs, therefore suggesting a complex role for Dp in the regulation of ovarian processes. Despite these data, the role of the dopaminergic system in the

human ovary remains unknown, although its potential role in modulating OHSS might perhaps be inferred by comparision of how the dopaminergic system regulates VEGF in other analogous organs, as described below.

1.14. MODULATION OF THE VEGF/VEGFR2 SYSTEM BY DOPAMINE

Basu et al. were the first to propose a relationship between the dopaminergic sytem and VEGF/VEGFR2 system after observing that angiogenesis was inhibited by Dp in several tumor types (Basu S. and Dasgupta, 2000). A year later, the same group confirmed this hypothesis by showing that Dp inhibited angiogeneis and VP induced by VEGF-secreting ovarian tumor cells injected into mice (Basu S, et al., 2001). This group reported that EC VEGFR2 phosphorylation was decreased by D2-ags which was very likely due to their induction of VEGFR2 internalization, presumably by endocytosis, thus leaving less cell surface-VEGFR2 able to interact with VEGF (Basu S, et al., 2001). In an attempt to improve the characterization of this molecular mechanism, a subsequent study carried out by Sarkar C et al. showed that inhibition of VEGFR2 phosphorylation by Dp altered MAPKs, src, and FAK (Sarkar C, et al., 2004). Finally, in 2009 an *in vitro* study by Sinha showed that D2-ags were able to decrease VEGFR2 phosporylation by inactivating specific tyrosine sites (Sinha S, et al., 2009).

Subsequently, Cristina C. et al. investigated the expression, localization, and function of VEGF in pituitary hyperplasia in D2-knockout female mice. The authors observed that the pituitary expresion of VEGF protein and mRNA were increased in these mice when compared to wild-type mice. Wild-type mice also showed an increase in pituitary VEGF expression and the levels of Prl released when they were treated with a D2-antagonist (Cristina C, et al., 2005). These observations suggested that the presence of D2 could inhibit or regulate VEGF production and secretion and thereby reduce the availability of VEGF. Therefore, this study suggested that two mechanisms by which the dopaminergic system (Dp/D2) inhibits the increases in VP at the root of OHSS may coexist. The first mechanism might involve direct action on VEGFR2 by D2 to inhibit its phosphorylation. The

second mechanism is likely to be an indirect interaction between D2 and VEGFR2 in such a way that D2 activation might inhibit VEGF secretion. In order to determine the potential role of these mechanisms further studies in human and animal models have been performed, the most relevant of which are detailed in the following section.

1.15. OHSS INHIBITION STUDIES WITH DOPAMINERGIC AGONISTS

1.15.1. Studies in animals

High doses of D2 agonists (D2-ags) have been demonstrated to simultaneously block tumor-related angiogenesis and VP in a mouse cancer model by interfering with VEGF/VEGFR2 signaling (Basu S, et al., 2001). Based on this finding Gómez et al. (2006) hypothesized that it might be possible to use the Dp/D2 pathway to interfere with VEGF/VEGFR2-mediated VP without interfering with angiogenesis. In order to corroborate this hypothesis, the authors conducted a study using Prl-supplemented OHSS rats which were treated with D2-ags; this demonstrated that administration of low doses of the D2-ag Cb2 in immature rats prevented increases in VP without affecting angiogenesis. Therefore, these findings suggested, for the first time, that low-dose Cb2 might provide a new, specific, and nontoxic approach to the treatment of diseases such as OHSS, which require VEGF/VEGFR2-mediated VP to be inhibited without affecting angiogenesis.

1.15.2. Studies in humans

Following on from these promising animal-study findings (Gómez R, et. al., 2006), and given that D2-ags have been widely used in the clinical treatment of human pathologies such as hyperprolactinemia (even in pregnant women) without toxicity or teratogenic effects, further studies were developed to evaluate the effectiveness of Dp as an OHSS treatment in humans. The first such study, aimed to prevent increases in VP and hemoconcentration (both signs of OHSS in humans), was a prospective trial carried out by our group in which MRI (magnetic resonance imaging) was used for the first time as a non-invasive method to quantify VP. In

this study oocyte donors received 0.5 mg/day of Cb2 for 8 days, starting on the same day that hCG administration was started (Álvarez C, et al., 2007a). Authors could confirm that D2-ags blocked the increases in hemoconcentration and VP triggered by hCG, thus reducing the incidence of moderate OHSS.

Although these findings were very promising because they showed that D2-ags had the same effect in humans as previously observed in animal OHSS models (Gómez R, et al., 2006), it was not possible to determine if the doses administered affected angiogenesis and would have therefore also attenuated reproductive function. Therefore a subsequent study was undertaken by Álvarez et al. to investigate the effects of Cb2 on implantation and related pregnancy rates in patients with a risk of OHSS treated with Cb2 or in a control group without a risk of OHSS, showing that there were no differences in the fertilization, implantation, or pregnancy rates between the two groups (Álvarez C, et al., 2007b) thus suggesting that Cb2 can prevent OHSS without affecting clinical outcomes.

Encouraged by both the efficacy and lack of toxic and/or teratogenic effects of D2-ags, subsequent studies with Cb2 were performed by several groups. Carizza et al. replicated the results obtained by Álvarez et al. in a prospective randomized study which evaluated the role of Cb2 in decreasing the incidence of OHSS in high-risk patients. They showed that women with a risk of OHSS did not develop early OHSS when were treated with Cb2 and neither implantation nor abortion rates were altered, corroborating that administration of D2-ags can affect VP without compromising angiogenesis. However, similar to our previous study, Cb2 administration also failed to prevent late OHSS (Carizza C, et al., 2008). Other recent studies have revealed similar findings in which treatment with Cb2 prevented the early-onset of OHSS in 50% of women at risk, but that Cb2 was ineffective at preventing the late-onset form of the syndrome (Youssef M, et al., 2010; Hosseini MA, et al, 2011; Tang H, et al., 2012; Seow KM, et al., 2013; Esinler I, et al., 2013; Leitao VM, et al 2013). Moreover, most studies using D2-ags other than Cb2, such as bromocriptine (Sherwal V, et al., 2010; Spitzer D, et al., 2011) and quinagolide (Busso C, et al., 2010) also found similar results.

In spite of the potential benefits of Cb2 in preventing early OHSS, there is no consensus on the choice of the best agonist dose to use. One study using Cb2 stated that at lower doses it reduces the incidence of OHSS as much as at higher doses (Shaltout A, 2012). However, a study with a different D2-ag (Quinagolide) clearly showed a dose dependent effect on OHSS prevention (Busso C, et al., 2010). In this study 182 women with a risk of OHSS were distributed into 4 groups: the control group (placebo) and the study groups treated with 50 mg/day, 100 mg/day, or 200 mg/day of quinagolide. The results showed that the incidence of OHSS was 23% in the placebo group, whereas it was significantly lower at 12%, 13%, and 4% respectively in the treated groups. Moreover, at the highest dose there were no incidences of severe OHSS.

1.16. IMPLICATIONS OF THE DOPAMINERGIC AND VEGF SYSTEMS ON CORPUS LUTEUM REGULATION IN PHYSIOLOGICAL AND PATHOLOGICAL CONDITIONS

It is known that women suffering from PCOS overexpress and oversecrete VEGF during the luteal phase after the induction of ovulation (Kamat B, et al., 1995; Agrawal R. et al., 2002; Neulen J, et al., 1995), indicating that VEGF is deregulated in these patients. Moreover, elevated Prl levels (Hernandez I, et al., 2000) have also been observed in PCOS which could indicate a reduced dopaminergic tone in these patients. In the light that the Dp/D2 pathway modulates VEGF production and secretion and is therefore implicated in PCOS, this thesis hypothesized that deregulation of the VEGF/VEGFR2 pathway in women with PCOS might be at least be partially due to abnormalities in the Dp/D2 system. To test this hypothesis our main goal was to ascertain whether activation of the dopaminergic system can modulate the VEGF/VEGFR2 system in human GCs and ECs. We also sought to evaluate whether there were significant differences in the dopaminergic tone in healthy vs. PCOS patients which could explain their susceptibility to developing OHSS.

Firstly it was necessary to isolate GCs and ECs in order to perform our *in vitro* studies. However, we did not have an optimized isolation protocol. There was only one previous publication about EC isolation (Ratcliffe K, et al., 1999), but several GC isolation methods had been described. Therefore, the efficiency of these different GC isolation methods were compared in order to determine the optimal method for obtaining the highest number of GCs without compromising cell purity or integrity. The process of attaining a GC isolation method and the evaluation of the interaction of the dopaminergic system (Dp/D2) and VEGF/VEGFR2 system in physiological and pathological conditions on these isolated cells constitutes the main body of this thesis.

2. HYPOTHESIS

2. HYPOTHESIS

It is known that development of OHSS is caused by increased VP which is mediated by the VEGF/VEGFR2 system. Regulation of ovarian VEGF production, secretion, and action is dependent not only on gonadotropins but also on other factors, among them D2.

Therefore we hypothesized that D2-ags can inhibit increases in VP by inhibiting either VEGFR2 phosphorilation or VEGF production. This hypothesis suggests the existence of a dopaminergic system inside the ovary which would determine each woman's individual susceptibility to changes in the VP of their ovaries caused by the VEGF/VEGFR2 regulation system. Considering this hyphotesis, we postulated that difference or deficiencies in expression or regulation of dopaminergic tone may be associated with the deregulation of the VEGF/VEGFR2 system in the ovary and thereby also with an individual's susceptibility to developing ovarian-dependent pathologies such as OHSS.

In this context, given the importance of VEGF in OHSS, and since its regulation is mediated by Dp, we hypothesized that in patients susceptible to OHSS, such as PCOS patients, VEGF/VEGFR2 is deregulated because these patients have a deregulated dopaminergic tone.

3. OBJECTIVES

3. OBJECTIVES

To ascertain whether activation of the dopaminergic system (Dp/D2) is able to modulate the VEGF/VEGFR2 system in GCs and ECs and to evaluate whether there were significant differences in the dopaminergic tone in healthy vs. PCOS patients which could explain their susceptibility to developing OHSS.

3.1. SPECIFIC OBJECTIVES

1. To identify ovarian populations with an active dopaminergic system by immunolocalizing D2 in these cells.

2. To compare the efficiency of different FF-derived cell isolation methods in order to determine which method allows the highest number of interested cells to be obtained for *in vitro* culture without compromising their cellular purity.

3. To determine whether the dopaminergic system modulates the secretion of VEGF and VEGFR2 activation in *in vitro* cultured FF-derived D2 expressing cells.

4. To compare the D2 expression pattern in ovarian cell populations over the different phases of the menstrual cycle in both physiological and pathological conditions.

5. To asses the Dp/D2 and the VEGF/VEGFR2 system in FF-derived cells from healthy vs. PCOS patients in order to analyze whether there are differences in the dopaminergic tone between them.

4. MANUSCRIPTS

4.1. MANUSCRIPT-I

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Efficiency and purity provided by the existing methods for the isolation of luteinized granulosa cells: a comparative study

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background: Several protocols for the isolation of luteinized granulosa cells (LGCs) contained in follicular fluid have been described but no previously published study has compared the relative efficiency of these protocols. Our objective is to obtain conclusive scientific evidence for the superiority of one method over another.

methods: Different purification methods for LGCs based on the recognition of specific cell markers, aggregates, differential adhesion and LGC size were evaluated. We compared the levels of CD45 cell contamination and the percentage of total cell viability in paired aliquots of cells (before and after purification) derived from the follicular fluid obtained from women who were donating oocytes (n ¼ 72). Each of the six purification methods was performed six times using pooled follicular fluids from two women.

results: Samples processed by means of recognition of specific cell markers were characterized by their greater purity (0.1–1.33% CD45+) but low rate of LGC recovery (17.13–25.4%) when compared with the other methods (3.29–12% CD45+, P , 0.05 and 51.67–73.20% LGC, P , 0.05). It is noteworthy that the filter method, which is based on the LGC size, combined one of the highest rates of LGC recovery (×70%) with acceptable low levels of contamination (, 5%).

conclusions: There is currently no gold standard method for the isolation of LGCs, and protocols should be chosen depending on the purpose in question. We conclude that fluorescence-activated cell sorting is the best protocol for isolating LGCs when purity is the principal criterion and magnetic separation when both purity and viability are essential. However, cell straining (filter) is probably the least laborious and, overall, the most efficient method to isolate LGCs.

Key words: isolation method / granulosa cells / immune cells / purity /viability

Introduction

Granulosa cells (GCs) are somatic cells that exist within the develop ing oocyte in the ovarian follicle (Brů čková et al., 2008) and form a multilayer that surrounds the oocyte. The functions of GCs include the production of a myriad of growth factors that are vital for oocyte development, and of sex steroids, which regulate ovarian function.

Following ovulation, GCs are luteinized and produce progesterone in order to maintain the viability of the endometrium during a potential pregnancy (Perry et al., 2005). Given the importance of luteinized GCs (LGCs) to the development of the oocyte and the maintenance of pregnancy, they are often used as a model for studying multiple aspects of ovarian function. Moreover, LGCs are identified as a valu- able tool for studies in reproductive biology, given the ease of access during assisted reproduction techniques (Greenseid et al., 2011).

Human primary LGCs employed for in vitro studies are obtained during oocyte retrieval from woman undergoing controlled ovarian stimulation (COS) protocols. The oocytes are collected by an invasive method employing transvaginal ultrasound-guided aspiration, which requires penetration of the uterine wall, ovarian membranes and connective tissue. Given that these structures are vascularized, the cells obtained from the follicular fluid are contaminated with vascular components (Levay et al., 1997; Jozwik and Wolczynski, 1998), thus resulting in a heterogeneous cell population in which LGCs are mixed with leukocytes (15 – 17%; Beckmann et al., 1991; Figenschau et al., 1997) and erythrocytes as well as other cell types, such as endothelial and epithelial cells.

The presence of immune cells in the follicular fluid is especially critical, as these directly influence LGC function. Indeed, the results of molecular and cell culture studies (Quinn et al., 2006) of follicular aspirates in which LGCs have not been purified must be interpreted with caution. Isolation techniques are necessary to establish a pure or almost pure, in vitro culture of LGCs if we want to determine the specific role of these cells in follicle development and ovarian function.

The gold standard method for isolation of pure cell populations is considered to be fluorescence-activated cell sorting (FACS), a specialized method of flow cytometry by which cell populations expressing a specific marker on the cell surface are selected. However, to our knowledge, no previous report has described a single marker, or combination of multiple membrane markers, that are positively expressed in LGCs, although there have been descriptions of said markers in contaminating cells found in the follicular fluid, such as cells deriving from the myeloid lineage, allowing a positive discrimination of these cells.

Sorting methods for isolating LGCs and purifying this specific cell type are based on the negative selection of LGCs through the depletion of contaminating immune cells.

Currently, employed strategies for the depletion of unwanted cells can be classified in two main groups, according to a criterion of restrictiveness: (i) highly specific strategies of depletion based on immunological recognition of specific cell markers of contaminating cells (Neurauter et al., 2007) and (ii) non-specific strategies of depletion based on the differential physical properties of contaminating cells and desired cells.

(i) The methods based on the recognition of specific cell markers in contaminating immune cells are expected to be more restrictive and thus render LGCs of purity ~ 90%. However, the number of LGCs lost during the application of these methods is usually very high, which rules out their subsequent use for the purpose of in vitro culture.

(ii) On the other hand, methods based on the differential physical properties of contaminating immune cells and LGCs should lead to a greater number of LGCs being obtained than with the above method. However, because of the less restrictive nature of this isolation process, a higher percentage of immune cells will probably 'contaminate' the resulting cell suspension.

Our objective in this work was to compare the efficiency of the depletion methods described for the isolation of LGCs and determine which of them allows the highest number of LGCs to be obtained for culture without compromising their purity. For this purpose, we estimated the percentage of LGCs recovered, evaluated the purity of the cell suspension obtained and established the rate of total cell viability after LGC isolation using the six isolation protocols described below.

Materials and Methods

Collection of LGCs

In order to isolate LGCs, the follicular fluid was collected via transvaginal ultrasound-guided aspiration in egg donors (n = 72) undergoing a 'classic' protocol of COS (Fedorcsak et al., 2004) in our institution (patient characteristics in Table I). The COS protocol consisted of pituitary down-regulation with an GnRH agonist, ovarian stimulation with recombinant FSH and ovulation induction with hCG. Women gave their written informed consent, and the study protocol was approved by the institutional ethical committee (CEIC-IVI Valencia).

Experimental design

Each of the LGCs purification methods evaluated in this study was repeated six times. As some of the methods are subject to the skills, and selection and evaluation criteria of the person manipulating the samples, three individuals performed each of the purification methods twice. LGCs were collected and two samples were pooled, therefore six samples were used for each of the purification methods. The efficiency of each method was evaluated by pooling and separating the follicular fluid into two aliquots named 'C' (control) and 'E' (experimental). The 'C' aliquot was used as an internal standard control against which the 'E' aliquots were compared, after a specific isolation method was completed.

Table I Characteristics and clinical features of oocyte donors recruited for the comparative study of methods for isolating

Method	Age (years) (n = 12)	No. oocytes retrieved	Serum level of E2 (pg/ml)	BMI (kg/m^2)
FACS	24 ± 4.47	15 ± 4.24	1985 ± 1120.42	21.35 ± 2.35
Dynabeads	27 ± 3.52	15 ± 3.45	2874 ± 1135.10	22.58 ± 2.58
MACS	26 ± 2.42	13 ± 2.47	2185 ± 1042.41	21.34 ± 2.87
40-mm-filter method	23 ± 2.81	17 ± 3.51	1710 ± 1074.15	22.21 ± 2.48
Cells aggregates method	25 ± 4.01	16 ± 4.13	2746 ± 987.25	20.4 ± 3.42
Flask method	24 ± 3.75	13 ± 4.10	1340 ± 1104.24	23.65 ± 3.89

E2, estradiol; MACS, magnetic-activated cell sorting; FACS, fluorescence-activated cell sorting. Data are mean ± SD. No significant differences were found between groups. Data are mean ± SD of the six pools from egg donors patients who were recruited for the assessment of each method (n = 12).

Follicular fluid-derived cells in the 'C' aliquots were separated from erythrocytes by density gradient centrifugation (Kossowska et al., 2009) on 5 ml of Ficoll (v/v, Sigma-Aldrich, St. Louis, USA) for 20 min at 400g. Following centrifugation, three layers could be distinguished: a top layer containing the follicular fluid, a bottom layer containing erythrocytes and, in the middle, a ring-like layer containing the cells-derived from the follicular fluid. This middle layer was collected, washed by centrifugation for 5 min at 600g and resuspended in 1-ml phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA).

In order to break up aggregates mechanically, Pasteur pipettes were heated and stretched to obtain different diameters, and these were used sequentially, from wide to narrower bore, to disperse the follicular fluid-derived cells. In order to reduce aggregate size homogeneously during the whole study, we took special care in maintaining pipette diam- eter uniformity between groups (Figs 1–3).

'E' aliquots were processed in the following.

(i) For assessment of the methods based on recognition of specific cell markers, such as FACS, Dynabeads and MACS (magnetic-activated cell sorting; Fig. 1), 'E' aliquots were first processed in exactly the same way as 'C' aliquots. Subsequently, they were subjected to the isolation methods in question and the recovered cells were resuspended in 1-ml PBS with BSA 1%.

(ii) For assessment of the methods that are based on the physical properties of LGCs and adhesion properties of immune cells (with the exception of the methodology employing cell strainers, which required a unique pre-isolation protocol, described below) erythrocytes were depleted from 'E' aliquots by means of density gradient centrifugation before performing the isolation method to be assayed (cell aggregates and Flask; Fig. 2). After the isolation methods, the LGCs were washed, resuspended in 1-ml PBS with 1% BSA and mechanically dispersed using pipettes stretched to different diameters.

(iii) In 'E' aliquots which were designated to be processed by the cell strainer (filter) method (Fig. 3), erythrocytes were not first depleted by density gradient. Instead, 'E' aliquots were passed directly through cell strainers following the procedures for the isolation of LGCs described below. The obtained cell suspension was washed, resuspended in 1-ml PBS with 1% BSA and mechanically dispersed.

Isolation methods tested

The FACS method

The FACS method (Neubourg et al., 1996; Evagelatou et al., 1997), described in countless works, is a method for sorting a heterogeneous mixture of cells into two or more containers, one cell at a time, and is based upon the specific light scattering and fluorescent characteristics of each type cell.

In order to isolate LGCs by means of FACS, cells obtained from the follicular fluid were selected against CD45, a common marker of cells deriving from the myeloid lineage, thereby allowing the depletion of immune cells in a suspension. To do this, the monoclonal mouse anti-human CD45 antibody (Sigma-Aldrich) was added to the cell suspension (diluted 1:20 in PBS) for 30 min at room temperature. Following incubation, the primary antibody was eliminated by centrifugation for 5 min at 600g and the pellet was then resuspended in PBS. This cell suspension was incubated on ice for 1 h in the dark with a secondary goat anti-mouse immunoglobulin (Ig)G antibody-labelled with fluorescein isothiocyanate (FITC; Anti-Mouse IgG-FITC antibody, Sigma-Aldrich) at a dilution of 1:100. Finally, LGCs were washed as described previously, resuspended in a volume of 500 ml PBS and exposed to 5-mg/ml propidium iodide (PI; Sigma-Aldrich) to exclude cells damaged during the process, which were separated with a FACS calibur instrument (Beckman Coulter, Brea, CA, USA) and CellQuest software (Beckman Coulter).

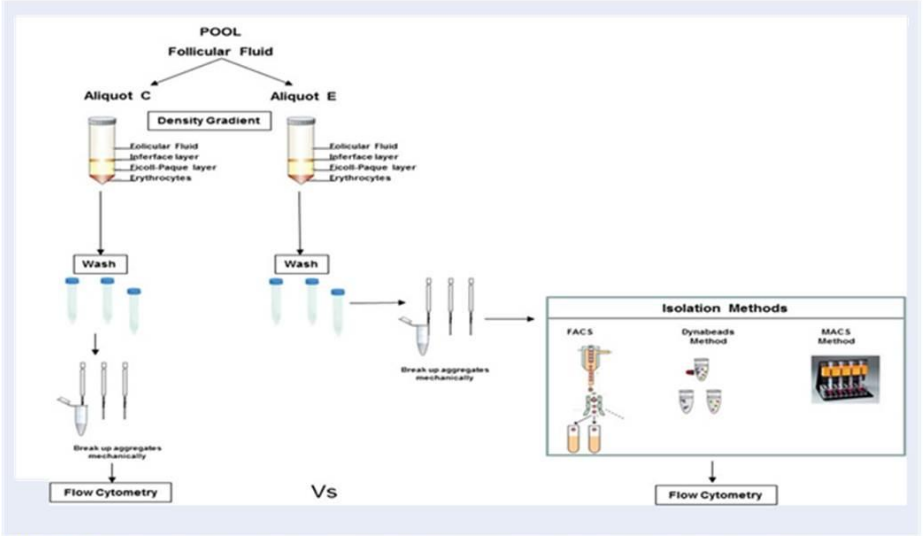

Figure 1 Experimental design for the evaluation of the efficiency of the methods based on the recognition of specific cell markers in the isolation of LGC.

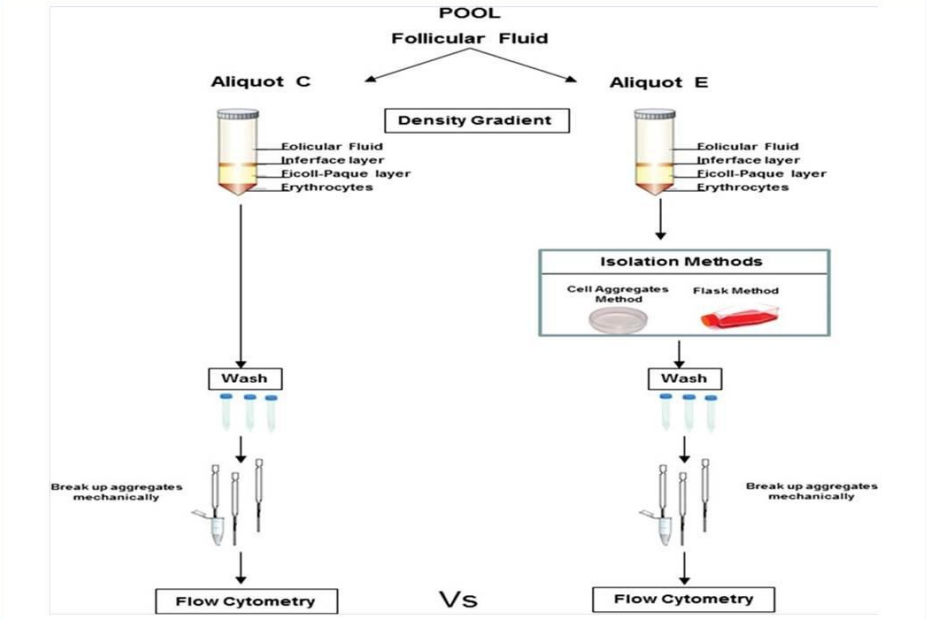

Figure 2 Experimental designs for the evaluation of the efficiency of the methods based on the differential adhesive/aggregative properties of LGC and immune cells in the isolation of LGC.

Dynabeads method

As described previously (Smith et al., 1997; Enien et al., 1998; Salmassi et al., 2005; Bencomo et al., 2006; Acosta et al., 2009), including one report by our own group (Garrido et al., 2002), the Dynabeads methodology has been used for the isolation of LGCs, and is based on the depltion of unwanted cells using magnetic beads covalently bound to antibodies that recognize specific markers present on the cell surface. We employed this technology to deplete immune cells by incubating samples with a monoclonal mouse anti-human CD45 antibody (CD45 clone BRA-55, Sigma-Aldrich) diluted 1:20 in PBS, for 30 min at room temperature. Following incubation, the excess primary antibody was eliminated through centrifugation (5 min, 600g). The cell pellet was subsequently resuspended and incubated with magnetic beads coated with secondary antibody (Dynabeads pan mouse IgG, Dilution: 1:20; Invitrogen, Carlsbad, CA, USA) for 30 min at 48C while being agitated at 160 g. The plastic tube containing the mixture was then placed next to a fixed magnet for 2 min. The unlabelled cells of interest were collected by decanting while the immune cells associated with the beads remained attracted and retained to the plastic wall closest to the magnet.

MACS method

MACS method (Miltenyi Biotec, Germany), previously described by other authors (Pesce and De Felici, 1995; Kodaman and Behrman, 2001). In a similar fashion to Dynabeads, the MACs methodology is based on the depletion of unwanted cells using magnetic forces. The main differences between the two technologies are the smaller size of the MACS microbeads employed for separation and the fact that in the MACS procedure the magnetic separation is performed in a column filled with magnetic beads rather than using a classic magnet.

Following the manufacture's recommendations, cells obtained after mechanical cell dispersion were washed and resuspended in 80 ml of MACS buffer (Miltenyi Biotec S.L., Germany), to which 20 ml of CD45 monoclonal antibody covalently bound to magnetic microbeads (CD45 MicroBeads, human; Miltenyi Biotec S.L., Germany) were added. The mixture was incubated for 15 min in a refrigerator (2–8°C), the cell suspension was washed and the pellet was resuspended in 500 ml MACS buffer.

Finally, the cell suspension was passed through the MACS magnetic column, which retained the cells labelled with CD45 microbeads and the unlabelled cells were collected.

Cell aggregation method

LGCs from the follicular fluid form aggregates of a considerably larger size than those formed by immune cells, a characteristic that can be employed to physically isolate this cell type. The follicular fluid-derived cells collected in the middle layer following density gradient were carefully aspirated, transferred into a Petri dish and observed under a dissecting microscope. All structures which, according to the criteria of the observer, showed that ≥20 aggregated cells were collected manually with a Pasteur pipette and subsequently washed and dispersed, as described above (Figenschau et al., 1997).

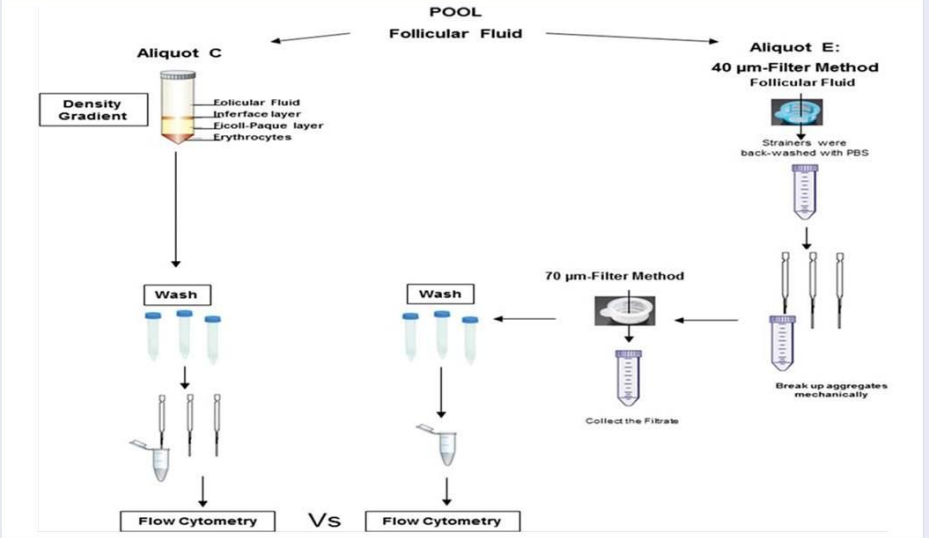

Figure 3 Experimental design for the evaluation of the efficiency of the method based on the LGC size: 'cell strainer' (filter) in the isolation of LGC.

Flask method

This method takes advantage of the fast and selective adherence of leukocytes to the surface of cell culture plastic ware. In order to deplete the immune cells from the cell suspension, follicular fluid-derived cells obtained by the density gradient were collected from the interface, incubated in 20-ml PBS and poured into a T75 Flask (BD Biosciences, Oxford, UK), where they were maintained at 37°C for 15 min. It was expected that, while white cells would adhere to the plastic during this period, LGCs would not, thus allowing the easy segregation of the two cell types according to this physical property. To test this possibility, the flask was decanted to recover the non-adhering, putative LGCs (Fedorcsák et al., 2007).

40-mm-filter method

The cell strainer methodology is based on the fact that LGCs form large cell aggregates in such a way that clusters can be retained by 40-mm pore cell strainers while erythrocytes and other blood contaminants (such as single cells and aggregates of a smaller size) pass through the strainer.

Following the cell strainer methodology (Carlberg et al., 2000; Richardson et al., 2009) but with some modifications (Fig. 3), the aspirates were first filtered through a 40-mm cell strainer (BD Biosciences) and the hypothetical clusters of LGCs were retained. The strainer was rinsed with 12-ml PBS in order to remove the last traces of blood contaminants and the strainer was then back washed with PBS to collect the 'hypothetical' LGCs. The acquired suspension was incubated for several minutes and repeatedly aspirated through Pasteur pipettes stretched to different diameters to break up aggregates mechanically. The suspension was then filtered through a 70-mm cell strainer (BD Biosciences) to remove unwanted undispersed material, which was retained in the filter. The cell suspension, collected after filtering the sample, was subsequently washed by centrifugation for 5 min at 600*g*, after which the supernatant was removed and the pellet was resuspended in 1-ml PBS.

Determination of absolute count, cell viability and percentage of CD45 contaminating cells by flow cytometry

Absolute count by flow cytometry

After assaying each specific isolation method in 'E' aliquots, the total number of cells was determined through cytometric analysis in both 'C' and 'E' aliquots using flow-count fluorospheres (Beckman Coulter) of uniform size and containing a dye with a fluorescent emission range of 525–700 nm when excited at 488 nm. Following the manufacturer's instructions, 100 µl flow-count fluorospheres (1025 fluorospheres per µl) were added per 100 µl cell suspension and the mix was passed through the cytometer. The total cell number was calculated using the following formula:

$$\text{Absolute count (cell/ml)} = \frac{\text{Total number of cells counted}}{\text{Total number of fluorospheres counted}} \times \text{Flow-count fluorospheres assayed concentration}$$

Determination of viability and percentage of contaminating CD45+ population in the aliquots

After performing each specific isolation method, two equal fractions were employed for the determination of cell viability and the percentage of

contaminating CD45 cells in each of the original 1-ml 'C' and 'E' aliquots. In one fraction of each 'C' and 'E' aliquot, an antibody specific for CD45 directly labelled with PE (anti-CD45 phycoerythrin-conjugated; Beckman Coulter) was added at a dilution of 1:10. The other fraction was incubated with a similar concentration of human IgG1 isotype labelled with PE (IgG1-PE, Beckman Coulter) as a negative control for CD45 antibodies, and to establish a baseline for positive CD45 counting. All fractions were subsequently incubated on ice for 1 h in the dark, after which they were centrifuged to remove excess primary unlabelled antibodies. The pellets were finally resuspended in 500-ml PBS, to which 5-mg/ml PI was added (Sigma-Aldrich) with the aim of being able to exclude damaged cells during the cytometric analysis. The total cell viability (PI + cells) of the cell suspension after the purification procedure and the percentage of the CD45+population were determined using Cytomics FC500 (Beckman Coulter), which counted 10 000 events per sample.

Formulas used to determinate the parameters of interest

In order to assess the percentage of LGCs recovered, we first estimated the LGC count in the 'C' and the corresponding 'E' aliquots after each isolation method. To do this, the absolute count after flow cytometry was corrected by subtracting the percentage of contaminating CD45 cells according to the following formula:

$$\text{Number of LGCs} = \text{Absolute count} \times \frac{100 - \text{percentage of CD45+ cells}}{100}$$

The percentage of recovered LGCs was subsequently estimated by the formula:

$$\frac{\text{Number of LGCs in aliquot 'E'}}{\text{Number of LGCs in aliquot 'C'}} \times 100$$

In order to evaluate the efficiency of the depletion of immune cells (percentage reduction in CD45+cells) by each method, the following formula was used:

$$\%\ \text{reduction in CD45} + = 100 - \frac{\%\ \text{CD45} + \text{in aliquot E x 100}}{\%\ \text{CD45} + \text{in aliquot C}}$$

The percentage cell viability preserved by each method was estimated according to the formula:

$$\%\ \text{Cell viability} = \frac{\%\ \text{Cell viability in 'E' aliquot}}{\%\ \text{Cell viability in 'C' aliquot}} \times 100$$

Statistical analysis

Data are expressed as mean + SD. As the data were normally distributed, ANOVA test was performed. To adjust for multiple comparisons, post-hoc Bonferroni and Scheffé test were applied. Significance was defined as $P \leq 0.05$. Statistical analysis was performed using the Statistical Package for Social Sciences 17.0 (SPSS, Chicago, IL, USA).

Results

Methods based on the recognition of specific cell markers: FACS, Dynabeads and MACS

Flow cytometric analysis in 'C' control aliquots showed that the amount of CD45+contaminating cells pre-purification was quite similar in all groups (ranging from 17.35 to 19.33%). Similarly, a small but slight variation in cell viability (ranging from 98.35 to 96.85%) was observed from group to group.

After purification, analysis of the flow cytometry data revealed that the CD45+contaminating cells in 'E' aliquots had been reduced by 90–100% by the depletion techniques, based on the recognition of specific cell markers (Table II).

As expected, FACS was the most effective of these methods in reducing the immune cell population, with nearly 0.1% of CD45+ contaminating cells detected in the 'E' aliquots. However, the Dynabeads and MACS were also highly efficient in reducing CD45+ contamination, with no significant differences between them.

Despite the high percentage purity of the LGCs obtained, we observed a drastic reduction in the number of LGCs recovered by the MACS, Dynabeads and FACS technologies when compared with the other methods. Indeed, the highest number of unrecovered (lost) LGCs occurred in the Dynabeads method. Although we expected that the FACS technology would give the highest number

Table II **Data obtained from the E aliquots of the follicular fluid for viability, efficiency of depleting contaminating CD45+ cells and of recovering LGCs, for each isolation method.**

Method	Viability (%)	%CD45 reduction	%CD45 1cells	%LGCs recovered
FACS	$52.26 + 8.23^{a}$	$99.1 + 2.9^{a}$	$0.1 + 0.42^{a}$	$17.13 + 3.2^{a}$
Dynabeads	$87.36 + 2.22^{a}$	$93.12 + 2.1^{b}$	$1.33 + 0.23^{b}$	$21.1 + 3.9^{b}$
MACS	$93.26 + 1.93^{a}$	$96.12 + 0.3^{c}$	$0.74 + 0.3^{c}$	$25.4 + 2.3^{c}$
40 mm-filter method	$92.75 + 0.65^{a}$	$82 + 7.6^{a,b,c,d}$	$3.29 + 0.46^{a,b,c,d}$	$70 + 4.9^{a,b,c,d}$
Cell aggregate method	$92.35 + 0.73^{a}$	$27.79 + 7.1^{a,b,c,d}$	$8.42 + 1^{a,b,c,d}$	$73.20 + 6.2^{a,b,c}$
Flask method	$90.14 + 1.15^{a}$	$40.69 + 8.9^{a,b,c,d}$	$12.04 + 3^{a,b,c,d}$	$51.63 + 12.6^{a,b,c,d}$

Results represent flow cytometry analysis, expressed as mean + SD (n¼ 6, as samples were pooled from two patients). Identical superscripts within the same column denote significant differences ($P = 0.05$) between the first category and the remaining, from top to bottom. Viability: percentage of total cells which retained viability after performing each purification method. CD45+cells: final concentration of CD45+ contaminating cells after performing the purification method. CD45 reduction: efficiency of each method in depleting (CD45+) contaminating cells. LGCs recovered: percentage of viable LGCs which could be recovered by each purification method.

of recovered cells, the data obtained in flow cytometric analysis showed that recovery of LGCs barely reached 20%, as almost 50% of cells died during the sorting process.

In this way, FACS proved to be a less efficient technology in terms of recovering viable LGCs than the methods based on the differential adhesive/aggregative properties of immune cells.

Methods based on the differential adhesive/aggregative properties of LGC and immune cells

Cell aggregation and flask methods

In agreement with the similar processing performed in all the 'C' aliquots in all groups, the mean percentage of CD45+ contaminating cells pre-purification in these groups (15.98 ± 4.9) was similar to data observed for the above methods based on the recognition of specific cell markers (18.32 ± 2.47). Similar slight variations in cell viability (range: 99.65–97.01%) were observed pre-purification in these groups.

Both the aggregation and Flask methods provided good cell viability and a high percentage (average ×65%) of recovered LGCs (Table II). Indeed, these methodologies gave the highest percentage of recovered LGCs of all the protocols tested, though it should be pointed out that a high variability in recovered LGCs (aggregation: 73.20 ± 6.2; flask: 51.63 ± 12.6) was observed among samples. In particular, the aggregation method produced the highest variability, probably because it involved the subjective criteria of the person performing the separation. Aside from the high variability in the results obtained, the main inconvenience of these methodologies seems to be their inability to discriminate between different cell populations. As a result, they were less effective in selectively depleting white cells, as demonstrated by the low percentage reduction in contaminating CD45+cells compared with the other methods.

LGCs isolation involving removal of blood contaminants by a 'cell strainer' (filter)

Analysis of viability and CD45 contamination pre-purification showed values of 1.80 ± 0.3 and 18.23 ± 5.3, respectively, in agreement with all the 'C' aliquots. The cell strainer technology gave a high percentage of recovered LGCs. Flow cytometry revealed that barely 3.29% of the cells in the suspension belonged to the myeloid linage, and an 82% reduction was observed in the percentage of cells labelled with CD45 (Table II). Overall, filtering was the most efficient method, as it resulted in the highest percentage of recovered LGCs with acceptable levels of CD45 contamination.

Discussion

To our knowledge, this is the first work that compares the efficiency of the most widely employed methods of isolating LGCs in terms of total number, viability of LGCs recovered and percentage of contaminating cells remaining. In terms of contamination, we focused our study only on the number of CD45+ (immune cells), although leukocytes from peripheral blood are the most abundant contaminating population in the follicular fluid, once the erythrocytes have been depleted. We did not evaluate other minor contaminating cell populations from the vascular system, such as endothelial cells or other steroidogenic cells of the follicle wall and ovarian stroma, owing to the low concentration of these components in the follicular fluid (Fedorcsák et al., 2007). Moreover, because of their high secretory activity, white cells are expected to interfere strongly with the activity/secretion profile of GCs. Our results demonstrate that the isolation of LGCs using magnetic devices is a powerful technique for obtaining a pure and viable population of LGCs. Therefore, these technologies should be the method of choice when a study uses a technique such as quantitative fluorescence PCR, differential display or array analysis, which is not limited by the amount of biological material available but which does require pure, uncontaminated samples if the results obtained are to be considered reliable. However, such techniques would not seem to be appropriate for studies designed to include diverse in vitro assays involving LGCs. The reason for this is that LGCs are terminal cells, and the primary culture of this cell type produces a very slow rate of replication, or none. Therefore, the hypothetical number of in vitro assays that can be performed will be limited by the amount of biological material initially available. This obstacle is especially critical if pools of LGCs cannot be employed, in order to preserve the individual characteristics of each patient sample. Based on our results, the most appropriate technique in such a scenario would be the strain-filter method, which provides higher number of LGCs with acceptable levels of purity, without compromising the viability of cells in subsequent culture. In fact the present study has arisen as a consequence of attempts in our laboratory to implement a more efficient LGCs isolation protocol than the Dynabeads methodology we have used in the past, with the aim of ensuring a sufficient number of LGCs with which to perform the in vitro culture assays required for our objectives (Gómez et al., 2011). Before carrying out the experiments described herein, we reviewed the literature in order to study the different isolation methods currently available and to identify which of them were most commonly used by researchers. Surprisingly, after reproducing the methods in our laboratory, we have found that FACS, the gold standard technique for isolation of pure cell populations, is not as efficient as we expected. Despite providing the highest percentage of pure LGCs, FACS led to a low viability rate during the process, which suggests that LGCs are very sensitive to cell sorting. As a consequence, at least in our hands, FACS resulted in a lower number of recovered LGCs than the other methods and, therefore, would not appear to be an appropriate technique for the isolation of LGCs prior to in vitro culture. The methodologies based on the magnetic separation of LGCs provided acceptable levels of purity. Thus, pragmatism is likely to tip the balance in favour of selecting magnetic methods over FACS when purity of the cell population is the main criterion. The flask and aggregation methods seem to be the least appropriate methods for isolating LGCs, principally because of the high numbers of contaminating immune cells present following isolation. We cannot envisage a scenario in which flask or aggregation methods might provide advantages over magnetic or cell strainer methods when LGCs are subsequently to be employed in the context of molecular biology or in vitro culture. Naturally, groups that employ the individual methods assayed herein routinely, specifically those involving flask or aggregated methodology, must generally obtain more efficient results than those we report here. However, we believe the present work can serve as a useful guide for newcomers who have never isolated LGCs and who wish to choose the methodology which best fits the individual aims and characteristics

of their research projects. We also believe that more experienced researchers in the field might benefit from our data by improving the methodology they currently use or by adopting a new approach which overcomes the obstacles and inconveniences they may experi- ence during the isolation of LGCs.

In summary, after assaying the most commonly used methods of LGC isolation, we conclude that FACS is the best protocol for isolating LGCs when purity is the principal criterion. Magnetic separation is the best option when both purity and viability are essential for subsequent experimentation. However, cell straining is probably the least labori- ous, most pragmatic and, when considered in global terms, most effi- cient technology with which to isolate LGCs, as it offers the best combination of purity, viability and total number of cells recovered from all the methodologies currently available.

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Authors' roles

H.F. and F.D.R. were involved with study design, execution and statistical analyses. H.F. also wrote and edited the manuscript. R.G. and A.P. devised and supervised the study and contributed to interpret- ation of the data and drafting of the manuscript. M.M. and C.G.P. were involved in execution of flow cytometry. R.C.Z. and C.S. coor- dinated statistical analyses, contributed to interpretation of the data. All authors reviewed the manuscript and provided critical feedback and discussion.

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Conflict of interest

None declared.

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4.2. MANUSCRIPT-II

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To Whom It May Concern:

The manuscript, "Dopamine receptor 2 activation inhibits ovarian vascular endothelial growth factor secretion in vitro: implications for treatment of ovarian hyperstimulation syndrome with dopamine receptor 2 agonists," by Hortensia Ferrero et al. was accepted by *Fertility and Sterility* on January 22, 2014. It is scheduled to be published in the May issue of our journal, and is currently available online at http://download.journals.elsevierhealth.com/pdfs/journals/0015-0282/PIIS001502821400079X.pdf.

Please feel free to contact me if you have any concerns.

Best regards,

Nancy Kucik
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ORIGINAL ARTICLE: REPRODUCTIVE ENDOCRINOLOGY

Dopamine receptor 2 activation inhibits ovarian vascular endothelial growth factor secretion in vitro: implications for treatment of ovarian hyperstimulation syndrome with dopamine receptor 2 agonists

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Objective: To ascertain whether vascular endothelial growth factor (VEGF) secretion by luteinized granulosa cells (GCs) is modulated by the dopaminergic system in a dose-dependent fashion and how this is related to the differential efficacy of dopamine receptor 2 (D2)-agonists (D2-ag) in preventing ovarian hyperstimulation syndrome (OHSS).
Design: The relationship between the dopaminergic system and VEGF secretion in luteinized GCs was evaluated. Archived human ovaries were immunostained to characterize D2 expression.
Setting: University affiliated infertility center.
Patient(s): Premenopausal women and egg donors.
Intervention(s): Luteinized GCs were cultured with the D2-ag cabergoline. Human ovarian sections were immunostained for D2.
Main Outcome Measure(s): The VEGF was measured by ELISA and D2 expression was evaluated by In-Cell ELISA. The D2 expression throughout the luteal phase was characterized by immunohistochemistry.
Result(s): The VEGF secretion was decreased by the D2-ag in a dose-dependent fashion. The efficiency of this process was correlated with the amount of D2 expressed by luteinized GCs. A decrease in D2 expression in ovarian sections was observed during the late luteal phase.
Conclusion(s): The efficacy of D2-ags in preventing OHSS might rely on their capacity to inhibit VEGF secretion by luteinized GCs. Because this capacity is dose-dependent, increasing the intraovarian concentration of D2-ags should be explored as a means of increasing the efficacy of these drugs in preventing OHSS. (Fertil Steril® 2014;■:■–■. ©2014 by American Society for Reproductive Medicine.)
Key Words: OHSS, VEGF, granulosa cells, dopamine receptor 2, dopamine receptor 2 agonist

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Hortensia Ferrero and Carmen M. García-Pascual should be considered similar in author order in this work.
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Ovarian hyperstimulation syndrome (OHSS) is an exaggerated response to the induction of ovulation, which is associated with the use of hCG for triggering oocyte release (1). There are several plausible theories for the clinical origin of OHSS (2), although it is thought to be mediated by vasoactive cytokines (3), which are secreted in excess by the corpora

lutea (CL) from hyperstimulated ovaries (4–7). Among these cytokines vascular endothelial growth factor (VEGF) has emerged as one of the factors most likely involved in the pathophysiology of OHSS (8, 9). In humans hCG administration increases VEGF messenger RNA (mRNA) expression in luteinized granulosa cells (GCs) (10, 11). Oversecreted VEGF makes local capillaries leaky by binding to and phosphorylating VEGF receptor 2 (VEGFR2) on endothelial cells (12). The increase in vascular permeability (VP) leads to fluid leakage from the vascular compartment that accumulates in the third space, and causes intravascular dehydration (13). In the most severe cases the patient becomes hypovolemic because of the excessive fluid loss and risks respiratory, circulatory, and renal problems (14). Administration of SU-5416, a compound that blocks VEGFR2 phosphorylation, was shown to prevent increases in VP in an OHSS rat model (15), which highlights the importance of the VEGF/VEGFR2 pathway as a therapeutic target for OHSS. However, this drug cannot be used in humans to treat OHSS because of its side effects that include thromboembolism and vomiting (16). Dopamine receptor 2 (D2) agonists (D2-ags) are drugs with benign side effect profiles that are already clinically used to treat hyperprolactinemia (17–21). These compounds were shown to reduce VP in an OHSS rat model (22) by inhibiting endothelial cell VEGFR2 activity (23, 24). A human proof-of-concept study showed that the D2-ag cabergoline (Cb2) reduces early onset OHSS by reducing ovarian VP in women at risk of OHSS (25). Randomized clinical trials (26, 27) later confirmed that different commercial D2-ags exert similar effects (28). Paradoxically, these drugs impede the onset of early onset OHSS in 50% of women at risk of the syndrome, but did not prevent late onset OHSS (28).

A recent clinical trial from Busso et al. (26) showed that the efficacy of D2-ag-related therapies in preventing early onset OHSS was dose dependent. In contrast, a study by Shaltout et al. (29) challenged this idea by showing that low D2-ag doses are equally effective in preventing OHSS. There is currently no scientific rationale for favoring one approach over the other because the molecular mechanisms by which D2-ags prevent OHSS have not yet been thoroughly explored in humans. Improving our understanding of these mechanisms might provide us with clues as to why D2-ags are ineffective in preventing late onset OHSS, which is especially puzzling because the only overt difference between the two forms is its appearance at different luteal stages.

Given that D2 activation can modulate VEGF secretion in different organs and that luteinized GCs both express D2 and secrete high amounts of VEGF (22, 30), we hypothesized that D2-ags might prevent OHSS by inhibiting VEGF secretion by luteinized GCs. To test this theory we performed a series of functional in vitro experiments to assess [1] whether VEGF inhibition in luteinized GCs by D2-ags is dose dependent, and [2] whether the abundance of surface D2 on luteinized GCs was also an important factor. We also performed immunohistochemical studies in human ovarian sections throughout the luteal phase to assess stage-dependent changes on the expression of D2 by luteinized GCs. With such an approach we aimed to support the hypothesis that D2-ags might not prevent late onset OHSS because D2 expression is decreased in the late luteal phase.

MATERIALS AND METHODS

This study comprised two parts: [1] we cultured and studied human luteinized GCs derived from follicular fluid (FF), and [2] we examined human ovarian sections obtained during the luteal phase. Women gave informed written consent, and our protocols were approved by the ethical committee at the IVI.

Drugs and Chemicals

The ABC staining kits for immunolabeling human ovarian sections was obtained from Vector Laboratories. The anti-CD68 and anti-D2 primary antibodies were from DakoCytomation. The hCG (Profasi) was obtained from Serono Laboratories and the D2 agonist Cb2 (Dostinex) was obtained from Pharmacia & Upjohn. The D2 antagonist (L-741.626) was obtained from Sigma-Aldrich. The Quantikine kit used to detect human VEGF by ELISA was obtained from R&D Systems. The Apoptag ISOL Dual Fluorescence kit to detect apoptosis, and the primary rabbit anti-human antibody to detect D2 in vitro were obtained from Chemicon, Millipore. The In-Cell ELISA kit used for in vitro D2 quantification was obtained from Thermo Scientific. Quinck-RNA MicroPrep for RNA extraction was obtained from Zymo Research and the Advantage real time (RT)-for-polymerase chain reaction (PCR) kit was from Clontech. Taqman assays for human VEGF (Hs00900055_m1) and β-actin (Hs99999903_m1) were obtained from Applied Biosystems.

In Vitro Studies on Luteinized GCs

Tissue collection. Follicular fluid-derived luteinized GCs were obtained at the time of oocyte retrieval from 84 egg donors aged 25–30 years, who had normal response profiles to controlled ovarian hyperstimulation (COH) protocols (oocytes retrieved, 10–20; E_2 <2000 pg/mL; body mass index [BMI] <30).

Luteinized GC isolation and culture. Luteinized GCs were isolated using a previously published protocol (31) with minor modifications (32). Isolated cells were seeded in 96- or 24-well culture plates at a density of 10,000 or 50,000 cells/well, respectively, and left overnight (37°C, 5% CO_2) to attach. Subsequently the cells were washed and cultured in M199 with 10% fetal bovine serum and 5 IU/mL hCG (unless otherwise indicated). Time course and dose response experiments were performed to assay the effects of a dopamine agonist and/or antagonist on VEGF secretion. The conditioned culture medium and cells were collected at the end point for further analysis.

Enzyme immunoassays on cultured luteinized GCs. Levels of VEGF-A in the supernatant were measured using ELISA kits. The intra-assay and interassay coefficients of variation (CV) were 8.9% and 11.1%, respectively.

IN-Cell ELISA. Luteinized GCs were fixed using 4% formaldehyde, washed with phosphate-buffered saline (PBS), incubated with blocking buffer, and then with anti-D2 primary antibody for 1 hour at room temperature. The raw levels of D2 expression were determined by measuring the absorbance

of the secondary horseradish peroxidase conjugate at 450 nm. Subsequently, luteinized CGs were stained with Janus green and the cell number was determined by measuring the absorbance at 615 nm. Raw D2 expression levels were then normalized to the total cell number to adjust for differences in cell plating. Results were expressed as the ratio of absorbance 450/absorbance 615.

VEGF quantitative fluorescent real time PCR. Vascular endothelial growth factor and β-actin mRNA expression levels in each luteinized GCs samples was estimated after real time quantitative fluorescent PCR amplification of 1 μg RNA equivalent using TaqMan assays. The VEGF levels were normalized to the housekeeping gene β-actin and its expression in each of the luteinized GC samples was quantified using the $\Delta\Delta$Ct method and expressed as arbitrary fluorescence units.

Studies on Human Ovarian Sections

To characterize the dynamics of ovarian D2 expression throughout the luteal phase, immunohistochemical analysis was carried out on sections of paraffin-embedded human ovaries obtained from the department of pathology at the University of Cordoba. The sections were derived from substantial portions of 45 disease-free ovaries from premenopausal women (aged 30–45 years) who had undergone ovariectomy and hysterectomy, mainly due to uterus/cervix-related benign pathologies. Of these ovaries 5 were in the follicular phase, and 20, 12, and 8 were in the early, mid, and late luteal phases, respectively, as diagnosed by histopathological and hormonal profile analyses.

Dopamine receptor 2 immunohistochemical detection in human ovaries. Human ovary sections were immunostained for D2 using previously described methodology (33). Because macrophages are not easily distinguished from luteinized GCs or theca lutein cells, adjacent sections were incubated with a primary antibody against the macrophage marker CD68 (1:500 dilution).

Quantitative analysis of immunohistochemical D2 staining in luteinized GCs. A total of 16 photographs (four sections of four fields) were analyzed to quantify D2 in each ovary sample. The TIFF images were opened with Photoshop (Adobe) and each pixel was assigned a constant area to calibrate the instruments. Regions of interest (i.e., the area occupied by luteinized GCs) were outlined using the marquee tools and subsequently segmented and saved. Photoshop-segmented TIFF images were opened in Matlab (Math Works) and processed following previously described commands and algorithms (34). The amount of "energy" (staining) present per pixel was determined by taking the square root of the difference of each file's energy squared and dividing it by the number of pixels. The D2 staining intensity on luteinized GCs in the early, mid, and late luteal phases were expressed as energy units per pixel.

Statistical Analysis

Statistical analysis was performed using SPSS 15.0, and the data were expressed as mean ± SD. Nonparametric methods (Mann-Whitney *U* test) were used for the VEGF ELISA assays, as the data were not normally distributed. Linear regression was used to estimate the relationship between D2 expression and the percentage of VEGF inhibition by luteinized GCs at different Cb2 doses. A probability of $<.05$ ($P<.05$) was considered statistically significant.

RESULTS

In Vitro Studies with Luteinized GCs

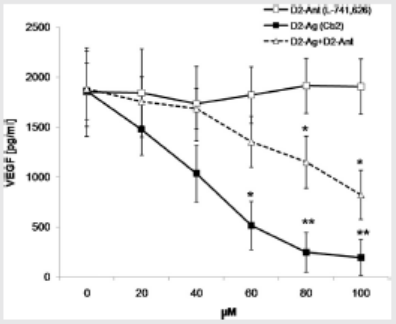
Pilot experiments. Before starting our functional experiments we performed a series of pilot experiments to set up optimal culture conditions. For such purposes we determined the hCG dose and the optimum timing of administration when luteinized GCs secrete the highest amounts of VEGF (Supplemental Fig. 1, available online) and also the maximum doses at which D2 agonists and antagonists can be added to the culture medium without exerting toxic effects (Supplemental Fig. 2, available online).

Experiment 1: D2 activation inhibits VEGF secretion by luteinized GCs in a dose-dependent fashion. We first determined whether VEGF production by luteinized GCs might be inhibited by D2-ags. Luteinized GCs were cultured in the presence or absence of different doses of the D2-ag Cb2 or the D2 antagonist (D2-ant) L-741,625. When assayed alone Cb2 inhibited VEGF secretion in a dose-dependent fashion. In contrast, none of the doses of the D2-ant modified VEGF secretion when compared with controls. Interestingly, the decrease in VEGF initiated by D2-ags was partially abated when equal doses of a D2-ant were administered concomitantly (Fig. 1). Therefore, it is likely that the dose-dependent inhibition of VEGF exerted by D2-ags in luteinized GCs was meditated by D2.

Experiment 2: the capacity of luteinized GCs to inhibit VEGF in response D2-ag is related to the cell surface abundance D2. We assessed whether the capacity of luteinized GCs to inhibit VEGF secretion in response to a D2-ag depends on the amount of D2 expressed on the surface of luteinized GCs in a given patient. Therefore, we determined the expression of D2 on the surface of the luteinized GCs used in experiment 1. A clear correlation between the extent of VEGF inhibition and the amount of D2 on the surface of luteinized GCs was observed when these cells were exposed to Cb2 at 20- to 60-μM doses. However, this correlation diminished doses of D2-ags increased and completely disappeared at 80–100 μM (Fig. 2). These results suggest that at low D2-ag doses the luteinized GCs with higher D2 cell surface expression levels are more efficient at inhibiting VEGF secretion than luteinized GCs with a low cell surface D2 expression density.

Experiment 3: D2 activation inhibits VEGF secretion post-transcriptionally. We explored whether the action of D2-ags on VEGF protein secretion might have been indirectly mediated by interfering with hCG's capacity to increase VEGF transcription. To test this we simultaneously measured VEGF mRNA expression and VEGF protein secretion in luteinized GCs cultured with or without hCG and in the presence or absence of Cb2 for 24, 48, or 72 hours. There was a clear reduction in VEGF protein secretion, but not in mRNA levels

FIGURE 1



The effects of dopamine receptor 2 agonists (D2-Ag) and antagonists (D2-Ant) on vascular endothelial growth factor (VEGF) secretion by luteinized granulosa cells (GCs). Graph shows a dose-response experiment in which luteinized GCs (n = 13) were cultured in the presence of 5 IU hCG with 0 (vehicle control), 20, 40, 60, 80, or 100 μM doses of the D2-ag cabergoline, the D2-ant L-741.626, or a combination of both compounds for 72 hours. The amounts of VEGF in the supernatant were measured at the end point. Each point on the graph represents the mean of 13 patients at each of the D2-Ag and/or D2-Ant doses assayed. A VEGF dose-response inhibition is observed in luteinized GCs cultured with D2-ag alone. The decreases in VEGF secretion initiated by cabergoline were partially prevented when similar doses of L-741.625 were administered concomitantly. *$P<.05$, **$P<.01$, compared with the vehicle.

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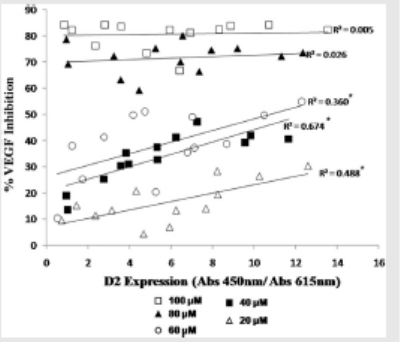
FIGURE 2



Linear regression between the amount of dopamine receptor 2 (D2) and the percentage of luteinized granulosa cell (GC) vascular endothelial growth factor (VEGF) inhibition in response to increasing doses of D2 agonists. Graph shows the correlation curves between D2 luteinized GC-surface expression (n = 13; cultured with 0 [vehicle control], 20, 40, 60, 80, or 100 μM doses of D2 agonists [cabergoline] in the presence of 5 IU/mL hCG for 72 hours; X-axis) and the extent of VEGF secretion inhibition (the presence of VEGF in the supernatant; Y-axis). The percentage of VEGF inhibition accomplished with each of the D2 agonist doses assayed was determined by using the formula: 100 − [(VEGF measured in cabergoline treated condition × 100)/VEGF measured in cabergoline untreated conditions]. Each different symbol (△, ■, ○, ▲, □) represents each of the five D2 agonist doses at which the same luteinized GCs (n = 13) were assayed. When cultured with 20–60 μM cabergoline the efficiency of VEGF secretion inhibition by D2 agonists depends on the luteinized GC-surface D2 density, as shown by the correlation between these parameters. When cultured with 80–100 μM cabergoline the efficiency of VEGF secretion inhibition by D2 agonists does not depend on the amount of D2 expressed by luteinized GCs as shown by the lack of correlation between these parameters. *$R^2 > 0.3$ was considered a positive correlation. Abs = absorbance.

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in Cb2-treated versus nontreated luteinized GCs (Fig. 3), and this pattern was similar in the presence or absence of hCG in the culture media. These results suggest that inhibition of VEGF secretion by D2-ags is exerted post-transcriptionally, making it likely that D2-ags decrease VEGF protein secretion without interfering with hCG-mediated pathways.

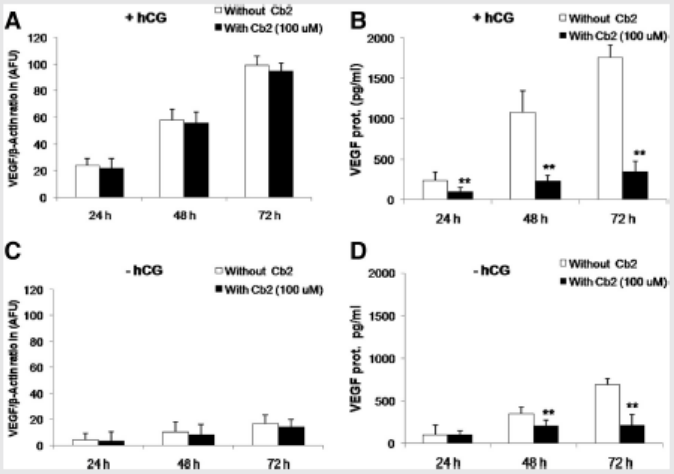
Studies with Human Ovarian Sections

D2 expression declines in luteinized GCs as the age of the CL increases. The D2 expression was not detected on the surface of luteal or stromal blood vessel endothelial cells (Fig. 4) as we had expected. Its expression was, however, restricted to macrophages, theca lutein cells, and luteinized GCs (Fig. 4A–4C). The D2-positive macrophage staining was very low in early and late CL (Fig. 4A), and significant amounts were found only in remnant or degenerating CL (data not shown). The strongest D2 staining in CL was observed in theca-lutein cells (Fig. 4A–4C), with no apparent variation in the signal intensity throughout the luteal phase (data not shown). In contrast, we observed cyclical variations in D2 intensity and distribution in the GC layer. Staining was weak in the preovulatory phase (data not shown), but substantially increased and peaked in the early phases (Fig. 4A). Luteinized GC staining subsequently decreased as the age of the CL increased (Fig. 4B), to the point where expression was almost abolished in the later stages (Fig. 4C).

DISCUSSION

The VEGF overproduction by luteal cells and its subsequent binding to the VEGFR2 expressed on endothelial cells is widely accepted as the principal cause of early onset OHSS. Because the dopaminergic system has been shown alter VEGF secretion (30), we hypothesized that the efficacy of D2-ag therapies in preventing early onset OHSS might rely on their capacity to inhibit VEGF release by luteinized GCs. To test this hypothesis we used an in vitro model of luteinized GCs that, to some extent, recreates in vivo CL formation. Because the severity of OHSS seems to correlate with the amount of VEGF released by luteinized GCs in vivo (10, 11), we explored whether D2-ags might inhibit the release of VEGF by luteinized GCs in a dose-dependent fashion. The

FIGURE 3



The effects of the dopamine receptor 2 agonist cabergoline (Cb2) on vascular endothelial growth factor (VEGF) messenger RNA (mRNA) production and protein secretion by luteinized granulosa cell (GC) over a time course. The VEGF mRNA expression (expressed as arbitrary fluorescence units [AFU]) in the presence (A) or absence (C) of 5 IU/mL hCG, and VEGF protein secretion levels (expressed as picograms per milliliter) in the presence (B) or absence (D) of 5 IU/mL hCG in luteinized GCs (n = 8) cultured with/without Cb2 (100 μM) at different time points (24, 48, or 72 hours). Cabergoline affected VEGF protein levels in treated versus control luteinized GCs but not VEGF mRNA expression. *$P<.05$, **$P<.01$, compared with Cb2 untreated conditions.

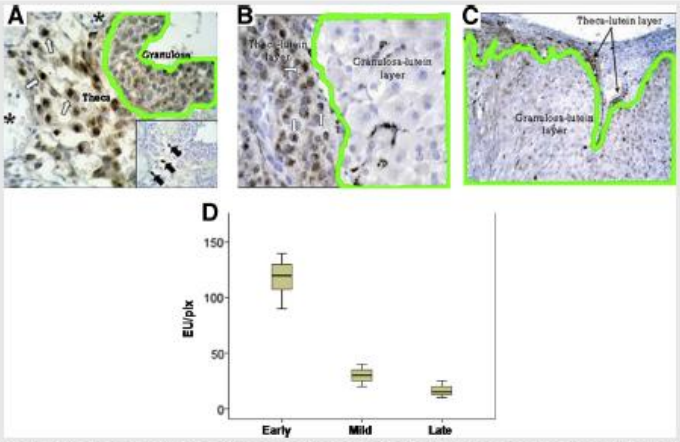
Ferrero. D2-ag (Cb2) inhibits VEGF secretion by LGCs. Fertil Steril 2014.

rationale was to support the hypothetical convenience of using higher (26) instead of lower (29) doses of D2-ags to improve the prevention of OHSS. Our observations that Cb2 inhibits VEGF secretion in a dose-dependent fashion (experiment 1) concur with the findings of Busso et al. (26) that high doses (200 μg) of the D2-ag quinagolide are significantly more effective than lower ones (50 μg) at preventing early onset OHSS. Although our in vitro observations may not reflect the behavior of Cb2 in vivo, we do have further compelling evidence from recent experiments in a validated rat OHSS showing that D2-ags prevent increases in VP and decreases ovarian VEGF production in a dose-dependent fashion (unpublished data). Nevertheless it remains clear that larger studies in humans would still be required to support our hypothesis clinically.

Clinical observation that administering a fixed dose of D2-ag only prevents early onset OHSS in 50% of patients (18, 28, 35) has remained a major puzzle. One explanation might be that some patient's luteinized GCs produce dopaminergic signals in response to D2-ags more efficiently than others. If the amount of dopaminergic signaling depends on the abundance of luteinized GC-surface D2 expression, interpatient variation in this parameter would mean that these cells also have a differential capacity to decrease VEGF. To test this possibility we explored whether VEGF inhibition in response to fixed doses of Cb2 correlated with the amount of D2 expressed by luteinized GCs in vitro. We observed a clear correlation between D2 and VEGF inhibition with 20- to 60-μM doses of Cb2, meaning that luteinized GCs with a higher D2 density elicited more VEGF inhibition. In contrast, at high D2-ag doses (80–100 μM) the correlation between D2 and VEGF inhibition disappeared. Therefore luteinized GCs with a low D2 density were as efficient at decreasing VEGF secretion as those that expressed higher amounts of D2.

Our observations were consistent with classic responses for receptors in the presence of saturating and nonsaturating agonist doses (36, 37). We expected the dopaminergic response to be proportional to the quantity/density of luteinized GC-surface D2 receptors, until the maximal dopaminergic response is reached (when increasing D2-ag ligands or D2 density cannot increase the dopaminergic response further). Therefore, in the presence of equal, nonsaturating (20–60 μM) Cb2 doses, the differential luteinized GC-surface D2 density seems to determine the differential luteinized GC dopaminergic response, explaining the correlation between D2 and VEGF inhibition. In the presence of equal, saturating (80–100 μM) Cb2 concentrations, the maximal dopaminergic response is reached because of the high

FIGURE 4



Immunohistochemical detection and quantification of dopamine receptor 2 (D2) in ovarian luteinized granulosa cell (GC) sections throughout the luteal phase. Images represent immunohistochemical detection of D2 (*brown*) in ovarian sections obtained from women undergoing surgery during the early (A), mid (B), or late (C) luteal phases. Intense D2 immunostaining was present in theca layer (*white arrows*) throughout the luteal phase. Macrophages were observed in the theca layer, by immunostaining of parallel sections with the macrophage marker CD68 (*black arrows* in the inset in A). Neither stromal nor thecal ovarian blood vessels (*asterisks* in A) stained positive for D2. Variation in luteinized GC D2 staining intensity (tissue outlined in *green*) was observed throughout the luteal phase. The D2 immunostaining was clearly positive in luteinized GCs during early luteal phase (A) but became faint during the midluteal phase (B), and disappeared in GC-lutein cells during the late luteal phase (C). Graph below the images (D) corresponds to quantitative image analysis of D2 staining throughout the luteal phase (expressed as energy units/pixel [eu/pix]) in the green area of outlined luteinized GCs.

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probability that D2 is bound by the excess D2-ag. Because the cell surface density of D2 is no longer a limiting factor in determining the dopaminergic stimuli, the correlation between D2 and dopaminergic response (i.e., extent of VEGF inhibition) disappears.

On the basis of our in vitro observations we speculate that the reported differential efficacy of D2-ag prevention of early onset OHSS can be explained if the patients were "underdosed" with "nonsaturating" doses of D2-ags. Our in vitro experiments showed that when luteinized GCs are exposed to similar nonsaturating D2-ag doses, there is a three- to fivefold difference in their reduction of VEGF secretion between the cells with the highest and lowest cell-surface D2 density. It remains to be seen if or how our in vitro experiments reflect the in vivo situation, but assuming they do, we speculate that the differences caused by interpatient variation in luteinized GC-surface D2 expression determines which patients successfully respond to fixed D2-ag doses and which ones develop early onset OHSS. It is also likely that higher doses of D2-ags are required to accomplish a similar reduction in early onset OHSS in patients who express lower levels of D2.

Our in vitro experiments suggest that the efficiency of D2-ag-mediated inhibition of VEGF secretion is lower in luteinized GCs with lower cell-surface D2 expression. Quantification of this expression in human luteal phase ovaries suggests that D2-ags might fail to prevent late onset OHSS because the cell-surface density of D2 dramatically decreases during the late luteal phase (i.e., decreased ovarian dopaminergic tone coincides with low luteinized GC-surface D2 expression). It is unclear whether our characterization of ovarian luteal phase D2 expression in unstimulated patients reflects the situation occurring in hyperstimulated ovaries. But if so we speculate that D2-ags have previously failed to prevent late onset OHSS because low luteal phase luteinized GC-surface D2 expression means that the dopaminergic response they produce is not sufficient to decrease VEGF secretion to the extent required to prevent significant increases in VP (38).

In our experiments the concomitant administration of a D2-ant minored the decreases in VEGF exerted by the D2-ag; however, the single administration of the D2-ant had no effect. These observations are in agreement with the classic definition of receptor antagonist (i.e., a type of receptor ligand or drug that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses) (37).

Although our experiments do clearly reflect the involvement of D2/D2-ag in the regulation of VEGF, those have only

provided modest clues to decipher the molecular mechanism through which this process is given. In this regard our attempts to reverse the inhibitory effects of D2-ag on VEGF secretion by previously inactivating the classic D2 (pertussis toxin sensitive and insensitive) transduction pathways (37) were unsuccessful (unpublished observations). Therefore we speculate that another mediator rather than a component of the classic D2 transduction pathway might be responsible for the phenomenon observed. Our experiments suggest that modulation of VEGF by D2-ag is exerted at the translational rather than at the transcriptional level. Indeed D2-ag affected VEGF protein but not VEGF mRNA levels or the stability of transcripts (data not shown). The number of plausible mechanism regulating VEGF at the post-transcriptional and translational level is diverse and increasing (39). We hope our observations might be of significant value to those researchers trying to characterize the molecular mechanism through which D2-ag modulates VEGF secretion.

Although this article describes in vitro findings that may differ in vivo, we provide evidence to support a hypothetical mechanism explaining why D2-ags only partially prevent OHSS. We suggest that D2-ags do not inhibit increases in ovarian VP by binding to D2 on endothelial cells as previously suggested (18) but rather by decreasing VEGF production and secretion by luteinized GCs. Furthermore, we speculate that failure of D2-ag to prevent OHSS in some women and circumstances D2-ags might be explained by the current use of low D2-ag doses and low luteinized GC-surface D2 expression, such as those that characterize the late luteal phase, also exist in some patients in the early luteal phase. Even if our speculations regarding the role of D2-ag in OHSS are wrong, our studies are still of interest for research purposes as they show a new link between the dopaminergic and VEGF system. Assuming that our speculations are correct, we believe that OHSS might be better treated by increasing the intraovarian D2-ag concentration with higher oral or vaginal doses of D2-ags, or intravenously if high dose toxicity becomes an issue.

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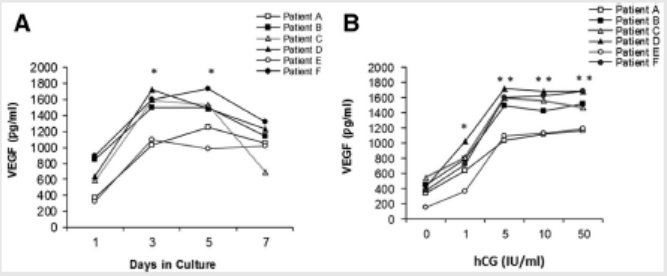
SUPPLEMENTAL FIGURE 1



Time course and dose-response effects of hCG on vascular endothelial growth factor secretion and apoptosis in luteinized granulosa cells (GCs). Graph in (A) shows time course experiments in which luteinized GCs (n = 6) were cultured for 1, 3, 5, or 7 days, in the presence of 10 IU/mL of hCG, to define the peak in vascular endothelial growth factor (VEGF) secretion. Graph in (B) shows a dose-response experiment in which luteinized GCs (n = 6) were cultured with 0, 1, 5, 10, or 50 IU/mL of hCG to identify which dose induced the highest level of VEGF secretion after 72 hours. The VEGF secretion peaked after 72 hours. The VEGF levels increased in response to hCG in a dose-dependent fashion, reaching a plateau at 5 IU/mL. *$P<.05$, **$P<.01$, compared with control conditions (day 1, hCG at 0 IU).

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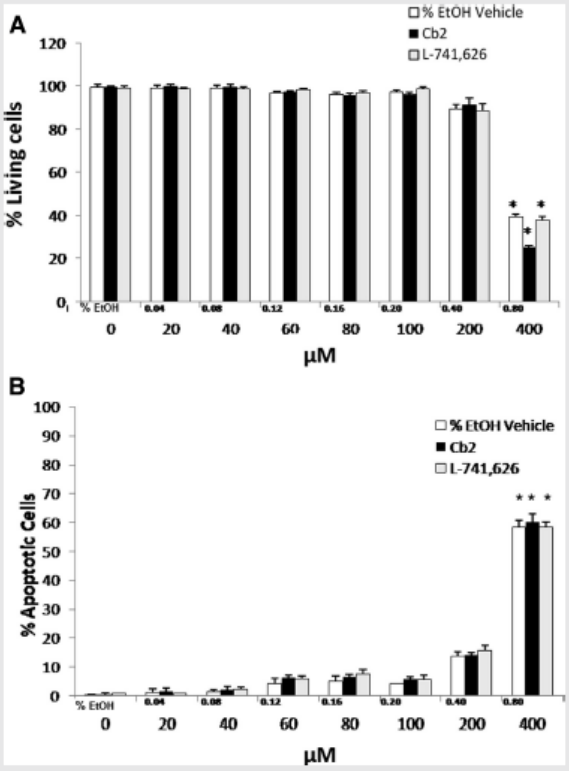
SUPPLEMENTAL FIGURE 2



Dose-response evaluation of the toxic effects exerted by dopamine receptor 2 agonists and antagonists in luteinized granulosa cells (GCs). Luteinized GCs (n = 8) were cultured with 5 IU/mL hCG plus cabergoline (Cb2) (dopamine receptor 2 agonist) or L-741,626 (dopamine receptor 2 antagonist) at 0, 20, 40, 60, 80, 100, 200, 400 μM, or an ethanol (EtOH) vehicle, for 72 hour. (A) Cell viability: luteinized GCs were incubated with Hoechst 33342 and propidium iodide and the specific absorbance of each dye was assessed with an In-Cell analyzer to provide automatic quantification of cell viability. (B) Apoptosis (as the percentage of the total number of cells): luteinized GCs were fixed using 4% formaldehyde and apoptosis was assessed using the ApopTag ISOL Dual Fluorescence kit. The amount of vehicle required to dilute each drug dose is expressed as the final percentage (vol:vol) of EtOH in the cultures within its corresponding bar. There was a slight but insignificant decrease in cell viability observed starting at 200 μM, but dramatic decreases were observed at 400 μM doses of both dopamine receptor 2 agonist and dopamine receptor 2 antagonist, and this decrease was paralleled by similar decreases when corresponding amounts of the vehicle alone was used. A similar pattern to that observed for cell mortality was seen during the analysis of apoptosis. A one-way analysis of variance (ANOVA) followed by Tukey post-hoc analysis was used to compare apoptosis and viability rates in treated luteinized GCs. *$P<.05$, compared with the corresponding control group (0 μM, 0 EtOH).

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4.3. MANUSCRIPT-III

J Clin Endocrin Metab. First published ahead of print June 6, 2011 as doi:10.1210/jc.2011-0075

ORIGINAL ARTICLE

Endocrine Research

Evidences for the Existence of a Low Dopaminergic Tone in Polycystic Ovarian Syndrome: Implications for OHSS Development and Treatment

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Context: The dopamine/dopamine receptor 2 (D2/Drd2) pathways modulates vascular endothelial growth factor (VEGF)-dependent vascular permeability and angiogenesis in the ovary. Deregulation of the VEGF/VEGF receptor (VEGFR)-2 pathway leading to increased risk of ovarian hyperstimulation syn-drome has been described in the ovary of patients suffering from polycystic ovarian syndrome (PCOS).

Objective: The objective of the study was to ascertain whether deregulation of the VEGF/VEGFR-2 might a least be partially due to abnormalities of the D2/Drd2 pathway in PCOS women.

Design: Dated, archived ovaries from PCOs and control group patients as well as human chorionic gonadotropin-stimulated luteinized granulosa cells form PCOS and non-PCOS oocyte patients were used.

Setting: The study was conducted at a private research center.

Patients or Other Participants: PCOS and nonpolycystic ovarian patients and oocyte patients par-ticipated in the study.

Intervention(s): Human ovarian sections were stained against the Drd2 antibody. Human chorionic gonadotropin-stimulated luteinized granulosa cells (LGC) were cultured in the presence/absence and the Drd2 agonist cabergoline.

Main Outcome Measure(s): Drd2 and vascularized stained area in the theca layer of antral (§8 mm) and luteinized follicles was quantified. VEGF, D2, and its related metabolites were measured in the supernatant of cultured LGC by ELISA and HPLC, respectively. VEGFR-2 and Drd2 expressed by LGC was quantified through an In-Cell ELISA.

Results: Decreased Drd2 expression and increased vascularization in the theca layer of antral and luteinized follicles of PCOS ovaries was observed. A lower dopamine production and reduced efficacy of cabergoline in inhibiting VEGF secretion was uncovered in LGC from PCOS.

Conclusions: Decreased dopaminergic tone as well as deregulated Drd2 signaling might explain higher VEGF and vascularization leading to increased ovarian hyperstimulation syndrome risk in PCOS. (***J Clin Endocrinol Metab* 96: 2484 – 2492, 2011**)

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* R.G. and H.F. contributed equally to this work.

Abbreviations: D2, Dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; Drd2, D2 receptor 2; Drd2-A, Drd2 agonist; Cb2, cabergoline; GC, granulosa cell; hCG, human chorionic gonadotropin; LGC, luteinized granulosa cell; OHSS, ovarian hyperstimulation syndrome; PCOS, polycystic ovary syndrome; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor 2; VP, vascular permeability

The polycystic ovary syndrome (PCOS) is a disorder associated with chronic oligoanovulation and typical polycystic ovarian morphology (1, 2). Increased produc-tion and secretion of ovarian vascular endothelial growth factor (VEGF) has been commonly observed in women suffering from this disease (3, 4) For example, increased expression of VEGF can be found in the dense hyperthe-cotic stroma (3), which induces increased stromal vascu-larity (5, 6). Also, follicular expression of VEGF is in-creased. Hypersecretion of follicular VEGF in response to gonadotropin stimulation has been considered one of the factors responsible for the inhibited growth of all cohort follicles as commonly seen during infertility treatment (7). Lastly, overproduction of VEGF during the luteal phase after gonadotropin stimulation is thought to be responsi-ble for the development of ovarian hyperstimulation syn-drome (OHSS) frequently seen in this group of patients (8).

Therefore, it has been suggested that deregulation of the VEGF/VEGF receptor 2 (VEGFR-2) plays a role in the pathophysiology of PCOS (7). Regulation of ovarian VEGF production, secretion, and action is dependent not only on gonadotropins but also other factors among them dopamine (D2). In support of such a role for D2 is the finding that in D2 receptor 2 (Drd2) knockout mice mod-els, both VEGF/VEGF receptor 2 (VEGFR2) mediated vascular permeability (VP) and angiogenesis were aug-mented by 10 fold compared with wild-type (9). Prelim-inary evidence in the human for an ovarian modulating role of the D2/Drd2 pathway in the regulation of the VEGF/VEGFR-2 is derived from the finding that admin-istration of the Drd2 agonist (Drd2-A) cabergoline (Cb2) ameliorates early-onset OHSS (10 – 12) by reducing VP (10, 11).

In addition to the findings of high levels of D2 in the ovary (13) as well as identification of dopamine receptors in luteinized granulosa cells (LGC) (11, 13), the expression of Drd2 in cycling human normal ovaries has been recently described by our group (14). Briefly, normal cycling ova-ries showed strong Drd2 immunostaining in the theca layer, whereas immunostaining was barely detectable in the granulosa layer of growing and preovulatory follicles. In corpora lutea, intense immunostaining was found in the theca-lutein layer, and a fainter expression were found in the granulosa-lutein layer. All those findings have lent further support for an intraovarian regulatory role of the D2/Drd2 pathway. Indeed, we recently demonstrated that Cb2 inhibits, in a dose-dependent fashion, VEGF produc-tion and secretion in LGC in non-PCOS patients under-going *in vitro* fertilization (14). All these findings suggest that the D2/Drd2 pathway modulates VEGF-dependent VP and angiogenesis in the ovary under physiological con-ditions. The clinical observation that a high percentage of women suffering from PCOS have elevated prolactin lev-els (15) makes it likely that the dopaminergic tone is de-regulated in women suffering from this condition. Based on these findings, we hypothesize that the deregulation of the VEGF/VEGFR2 pathway in PCOS women might a least be partially due to abnormalities of the D2/Drd2 pathway. To test our hypothesis, we performed Drd2 ex-pression studies on dated, archived PCOs and control group ovaries obtained during different stages of the men-strual cycle. Subsequently we performed functional stud-ies on human chorionic gonadotropin (hCG)-stimulated LGC, imitating within certain limits corpus luteum func-tion. In this setting we analyzed the D2/Drd2 and the VEGF/VEGFR2 pathway in the absence and presence of the Drd2-A Cb2 in PCOS and non-PCOS patients.

Materials and Methods

The present study consisted of two parts: human ovarian sections and follicular fluid derived cultured LGC. Women gave written informed consent, and the protocol of this study was approved by the ethical committee from IVI.

Studies with ovarian sections

To compare the dynamics of Drd2 expression and ovarian vascularization between PCOS and control conditions, immu-nohistochemical studies against Drd2 and CD34 (a specific en-dothelial cell marker) were carried out based on a retrospective analysis, using paraffin blocks from the archives of the Depart-ment of Pathology at the University of Cordoba. A total of 40 ovaries corresponding to normal cycling women (10 in the fol-licular phase, and 10, 12, and eight in the early, mid-, and late luteal phase, respectively) were selected from a larger series.

TABLE 1. Subject characteristics and clinical features of patients recruited for the study of the dopaminergic system in LGC

	Mean ± SD (range)		
	Non-PCOS	PCOS	Significance
Age	27.37 ± 4.47 (22–32)	30.91 ± 5.22 (24 –36)	NS
BMI	22.6 ± 2.35 (20.4 –27.87)	22.22 ± 4 (19.48 –30)	NS
Oocytes	15.4 ± 4.57 (12–20)	16.5 ± 6.19 (9 –27)	NS
E2 (pg/ml)	2466.71 ± 1120 (1340 – 4016)	2244.25 ± 921.18 (173–3087)	NS

BMI, Body mass index; E2, estradiol; NS, not significant.

Ovarian samples were obtained from women, who underwent oophorectomy due to benign gynecological disease and were not receiving hormonal therapy during the 3 months prior to surgery (age range 21–35 yr). Fifteen PCOS ovarian samples were obtained from women aged 20 –30 yr undergoing ovarian wedge resections as a treatment modality for infertility. All these women had been diagnosed with PCOS (Stein-Leventhal syn-drome) and had not received any medication for this condition during the 3 months prior to surgery. All ovarian samples were reevaluated, confirming the phase of the cycle (in control sam-ples) or that they corresponded to nonovulatory PCOS. All tis-sues had been fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin. Sections (5 0m thick) were cut, placed on poly-L-lysine-coated slides and used for immunohistochemistry. Additional sections were stained with hematoxylin and eosin for morphological analysis.

Immunohistochemical detection of Drd2 and CD34

For Drd2 immunostaining, sections were immersed in 10 mM citrate buffer and submitted to antigen retrieval in a microwave oven (twice, 5 min each, 700 W). Thereafter slides were incu-bated overnight with a 1:500 dilution of the primary anti-Drd2 (Dako, Glostrup, Denmark). Because macrophages are not easily distinguished from LGC and theca lutein morphologically, adjacent sections were incubated with a diluted 1:500 primary antibody against the macrophage marker CD68 (Dako) Specific blood vessel immunostaining was carried out with monoclonal CD34 antibodies (NCL-END clone QBEND/10; Novocastra Laboratories Ltd., Barcelona, Spain), following previously described methods (6). Sections were then processed according to the avidin-biotin-peroxidase complex technique following the manufacturer's instructions. Negative controls were run in par-allel by replacing the primary antibody with preimmune serum or PBS.

Quantification by immunohistochemistry

Quantitative analysis ofDrd2 and CD34 was performed using an image analysis system linked to a Nikon Eclipse E800 camera (Tokyo, Japan), and the data were processed using ImagePro Plus version 6.03 (Media Cybernetics, Silver Spring, MD). Fol-licles of interest were identified, and the theca cell compartment was outlined and analyzed separately. Subsequently the total endothelial cell (CD34)- or Drd2-immunostained area located within this compartment were identified, and the area was ex-

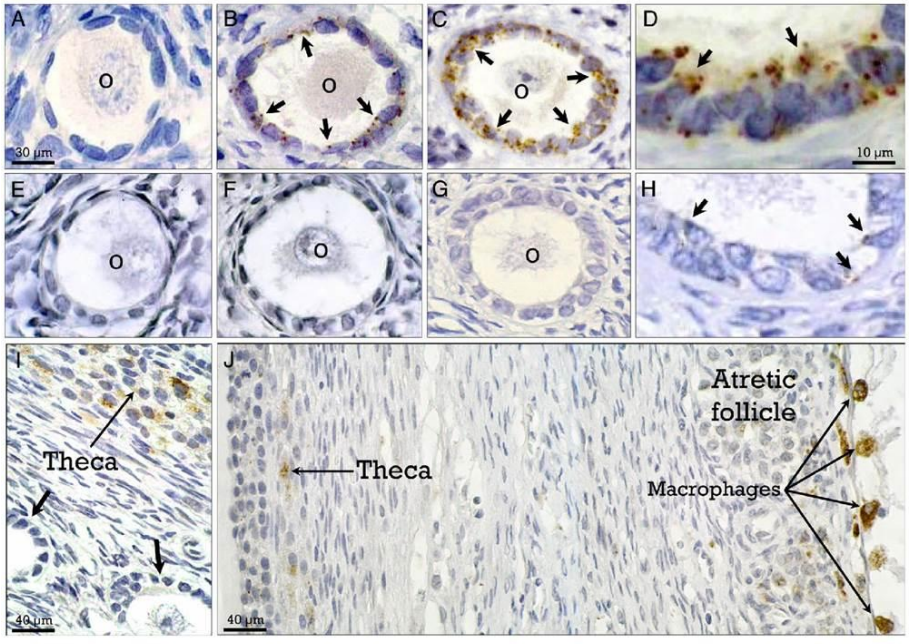

FIG. 1. Expression of Drd2 in cycling (A–D) and PCOS (E–J) human ovaries. In control ovaries, immunostaining was absent in primordial and transitional (A) follicles and showed a punctate immunostaining pattern in the GC (*arrows*) of primary (B) and secondary (C and D) follicles. In contrast, in PCOS ovaries, immunostaining was absent or barely detectable (*arrows*) in the GC of transitional (E) and most primary (F) and secondary (G and H) follicles. The absence of immunostaining in small follicles (*arrows* in I) and the decreased expression in the theca of antral follicles in PCOS ovaries was specific for the follicles because Drd2 immunostaining was strong in the macrophages present in the central cavity of atretic follicles (J) in the same sections. o, Oocyte.

pressed as micrometers squared. Vascular and Drd2 density expressed as percentage was calculated by forming a ratio of Drd2- and CD34-positive area divided by the total theca layer area multiplied by 100. At least three different areas from each follicle were analyzed. Antral follicles (2– 8 mm in diameter), showing normal features and absence of pyknotic (*i.e.* apoptotic) granu-losa cells, as a sign of atresia, were analyzed. This follicle size was selected because larger healthy follicles were not found in PCOS ovaries. For Drd2 expression, a total number of 23 and 24 fol-licles (for cycling and PCOS ovaries, respectively) were studied. Similarly, a total of 28 and 27 follicles (for cycling and PCOS ovaries, respectively) were used for blood vessel quantification.

Due to the anovulatory condition of PCOS ovaries, the Drd2 receptor expression and the vascularization of corpora lutea in these women, which is relevant for their possible implication in OHSS, cannot be analyzed in ovarian sections. However, ar-rested follicles showing luteinization of the theca layer (*i.e.* the so-called theca-lutein cysts) were abundant in PCOS ovaries and occasionally found in normal ovaries. Changes in the theca layer of these follicles are reminiscent of changes that happen in the theca-lutein layer of corpora lutea. In this context we decided to analyze also Drd2 expression and vascularization in five and seven luteinized follicles from cycling ovaries, and eight and 17 luteinized follicles from PCOS ovaries, for Drd2 and CD34 immunostaining, respectively.

Studies with LGC

To compare the dopaminergic tone and the regulation of the VEGF/VEGFR2 by the dopaminergic system, LGC were isolated at the time of oocyte pickup from non-PCOS (n ¼ 11) and PCOS (n ¼11) [attending to Rotterdam criteria (1, 2)] patients showing similar profiles to controlled ovarian hyperstimulation protocols (Table 1).

LGC isolation and culture

The LGC were isolated using a previously described methodology by our group (16). The granulosa cells (GC) were seeded in 96-well cell culture plates at a density of 10,000 cells/well with M-199 medium containing 10% fetal bovine serum, 0.1 mg/ml gentamicin, and 0.55 μg/ml Fungizone-amphotericin B. After an overnight incubation (37 C, CO_2 5%) that allowed attachment, cells were washed to remove remaining red blood cells because these did not adhere to the plastic surface and subsequently cultured with 5 IU hCG and the Drd2-A Cb2 at 0, 20, 40, 60, 80, and 100 μM or Cb2 vehicle (ethanol) during 72 h as an end point.

The culture conditioned medium was collected and stored at ¼ 20 C for subsequent ELISA measurements of VEGF and HPLC determination of D2 and its related metabolites. The cells were washed and fixed in paraformaldehyde for subsequent In-Cell ELISA colorimetric detection and quantification of Drd2 and VEGFR2 protein expression levels.

Enzyme immunoassays

Levels of VEGF-A in the supernatant were measured through a competitive enzyme immunoassay (Chemicon, Temecula, CA). The intra- and interassay coefficients of variation were 8.9 and 11.1%, respectively.

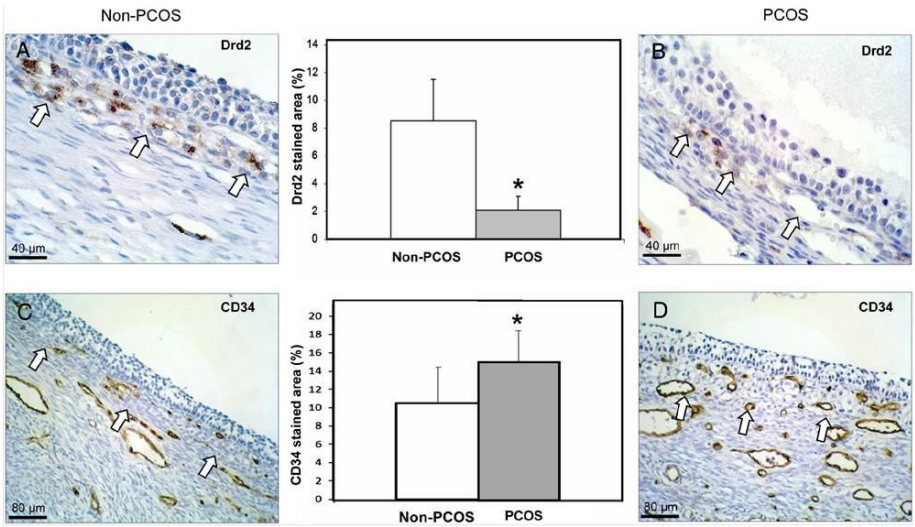

FIG. 2. A and B, Immunohistochemical detection (*brown color*) of Drd2 in the theca of antral follicles from cycling (A) and PCOS (B) human ovarian sections. Graphs between pictures shows the quantitative comparative analysis of Drd2 expression between both conditions. C and D, Vascularization (*brown color*) in the theca of antral follicles from cycling (C) and PCOS (D) human ovarian sections through immunohistochemical detection of CD34 (endothelial cell marker). Graphs between pictures shows quantitative comparative analysis of CD34 expression between both conditions. Note the decreased Drd2 staining and increased vascularization in the theca of antral PCOS follicles. *, P § 0.05 compared with nonpolycystic ovaries.

HPLC

Levels of D2 and their related metabolites homovanillic acid and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured using techniques previously described (17).

In-Cell *ELISA*

The relative amount of specific Drd2 and VEGFR2 protein was determined using target-specific primary antibodies (Up-state Biotechnologies, Lake Placid, NY) and a horseradish per-oxidase-conjugated detection reagent. The results were analyzed by normalizing the absorbance at 450 nm (horseradish peroxi-dase activity) values to 615 nm (Janus Green dye absorbance to estimate cell number.

Statistical analyses

The statistical analysis was performed using the Statistical Package for Social Sciences 12.0 (SPSS, Inc., Chicago, IL). A Student *t* test was used to determine significant mean differences between groups for all the parameters evaluated except for ELISA experiments in which nonparametric a Mann-Whitney *U* test was performed, given the fact that data were not normally distributed. *P* § 0.05 was considered significant.

Results

Expression of Drd2 and vascularization in cycling and PCOS human ovaries

In the current study, we focused on the differential Drd2 expression in PCOS *vs.* non-PCOS ovaries. In small follicles, a punctuate Drd2 immunostaining pattern was present in the granulosa layer of transitional, primary, and preantral follicles (Fig. 1, A–D) in non-PCOS, whereas most small follicles in PCOS ovaries lacked, or showed barely detectable, immunostaining (Fig. 1, E–I). In the theca layer of antral follicles, Drd2 immunostaining was fainter in PCOS than in normal ovaries (Fig. 2). Decreased expression of Drd2 in PCOS ovaries seemed to be specific for the granulosa (in small follicles) and for the theca layer in antral follicles because macrophages present in atretic follicles showed intense immunostaining, similar to that found in cycling ovaries (Fig. 1J). In general, whole PCOS ovaries showed a higher vascularization than control ova-ries, which was more prominent in the theca layer of antral follicles in PCOS ovaries (Fig. 2). Quantitative data indi-cated that antral follicles in PCOS ovaries showed a sig-nificantly lower expression of Drd2 and a higher vascu-larization than normal cycling ovaries (Fig. 2). These differences were particularly prominent in luteinized fol-licles (Fig. 3).

Studies with cultured GC

In the absence of Cb2, both groups secreted similar amounts of VEGF (Fig. 4A) in response to solely hCG.

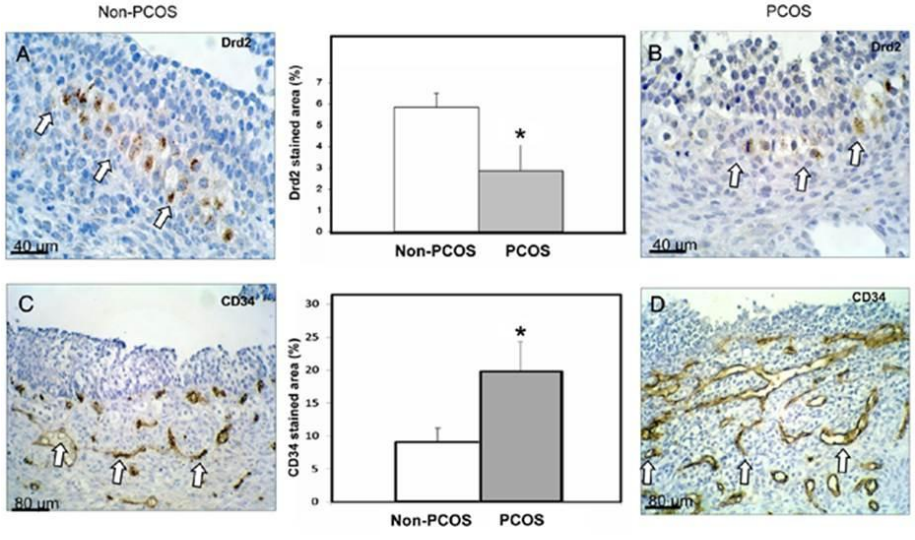

FIG. 3. A and B, Immunohistochemical detection (*brown color*) of Drd2 in the theca of luteinized cysts from cycling (A) and PCOS (B) human ovarian sections. Graphs between pictures shows the quantitative comparative analysis of Drd2 expression between both conditions. C and D, Vascularization (*brown color*) in the theca of luteinized cysts from cycling (C) and PCOS (D) human ovarian sections through immunohistochemical detection of CD34 (endothelial cell marker). Graphs between pictures shows the quantitative comparative analysis of CD34 expression between both conditions. Note the decreased Drd2 staining and increased vascularization in the theca of antral PCOS cysts. *, *P* § 0.05 compared with nonpolycystic ovaries.

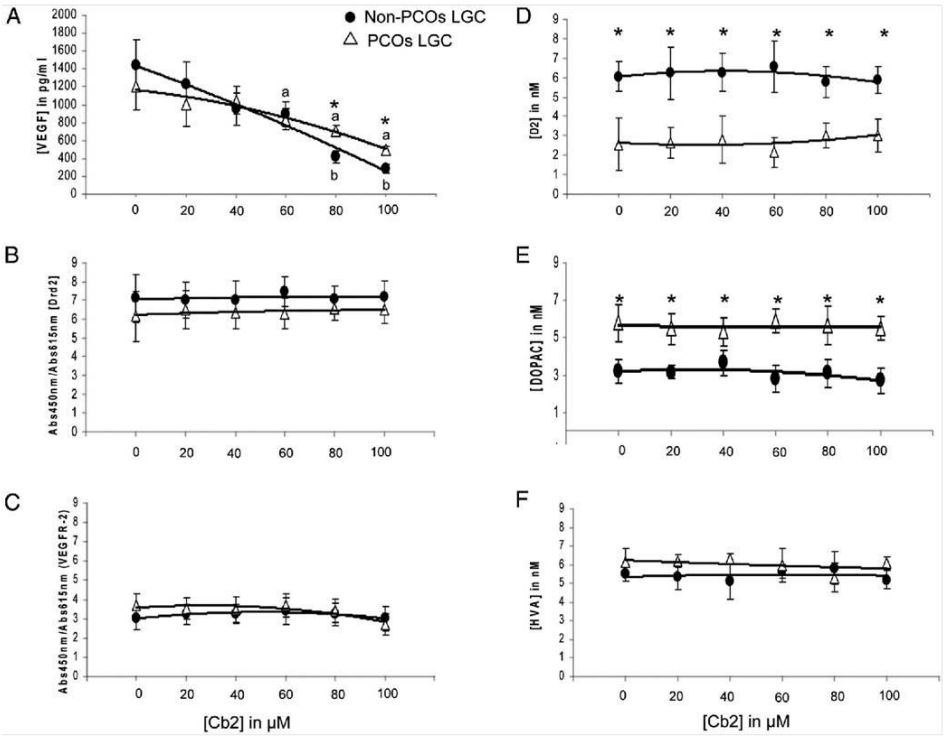

FIG. 4. Secretion levels of VEGF (A), D2 (D), and related metabolites DOPAC (E) and homovanillic acid (F) and protein expression levels of Drd2 (B) and VEGFR2 (C) in non-PCOS and PCOS lutein GC cultured with 5 IU hCG plus Cb2 at 0, 20, 40, 60, 80, and 100 *0*M during 72 h. *, *P* § 0.05, comparison of the control *vs.* PCO group at a specific Cb2 dose; a, *P* § 0.05 compared with the Cb2 0 *0*M in the control group; b, *P* § 0.05, compared with the Cb2 0 *0*M in the PCOS group

Addition of Cb2 to the culture media decreased VEGF in a dose-dependent fashion with maximal inhibition at the highest Cb2 dose (100 *0*M) assayed in both groups. Inhi-bition of VEGF, however, was more prominent in the con-trol than the PCOS, reaching statistical significance be-tween groups at a 100-*0*M Cb2 dose (Fig. 4A). We suspected that lack of significant differences in VEGF se-cretion between groups with the lower-than-100-*0*M Cb2 doses assayed might obey to the high interpatient vari-ability existing among patients for this parameter. The parameter percentage of VEGF inhibition increased also in a dose-dependent fashion with the Cb2 doses assayed but unlike VEGF showed a little variation between indi-viduals at any of the doses assayed. By using this former parameter, we uncovered a lower efficiency of LGC from PCOS in decreasing VEGF because the percentage of VEGF inhibition was detected to be lower in PCOS LGC *vs.* control at all but the 20-*0*M doses assayed, reaching statistical significance at Cb2 40, 80, and 100 *0*M doses (Fig. 5A). Accounting for a decreased dopaminergic tone in PCOS, we interestingly observed that LGC from PCOS secreted lower amounts of D2 than those from control ovaries (Fig. 4D). In contrast, the secretion of DOPAC, the primary metabolite of D2, was significantly increased in PCOS (Fig. 4E). These findings suggested that the metab-olism of D2 was faster in PCOS than in the control group.

Otherwise, we did not detect differences in the expression of Drd2 and VEGFR2 between groups whether in the pres-ence or absence of Cb2 (Fig. 4, B and C), suggesting the activation of Drd2 did not play a role in the regulation of those parameters.

Discussion

The dopaminergic tone has never been assessed in poly-cystic ovaries. In this study, we describe for the first time

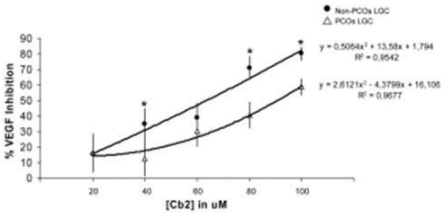

FIG. 5. Percentage of VEGF inhibition, as determined by the formula 100 - [(VEGF levels in Cb2 treated conditions/VEGF levels in vehicle treated conditions) x 100], in non-PCOS and PCOS lutein GC cultured with 5 IU hCG plus Cb2 at 20, 40, 60, 80, and 100 _M during 72 h. Equations and degree of the correlationship arising from the polynomial adjustment side of the correlationship curves obtained in each group. *, $P < 0.05$, comparison of the control *vs.* polycystic ovary group at a specific Cb2 dose.

that expression of Drd2 receptors is decreased in PCOS ovaries when compared with a control group consisting of regularly cycling women. Despite the fact that we did not detect differences in the amount of Drd2 expressed by LGC from both groups in our *in vitro* culture, both qualitative and quantitative immunohistochemical analysis of ovarian sections demonstrated that expression of Drd2 was lower in theGCof small follicles as well as in the theca layer of secondary and tertiary follicles from PCOS *vs.* control ovaries. The decrease in Drd2 expression in PCOS ovaries was restricted to follicular structures because immunostaining was similar in macrophages found in PCOS and control ovaries. The possible physiological relevance of very low or even absent expression of Drd2 in the granulosa cells of small follicles seen in PCOS patients remains to be determined. However, the bulk of Drd2 expression in the ovary was restricted to the theca layer of antral follicles. Decreased Drd2 expression in the theca of growing follicles was associated with an increased vascularization in the theca of PCOS follicles. In this sense, the inverse relationship between Drd2 expression and vascularization was even more prominent in luteinized follicles that are reminiscent of the changes that happen during luteogenesis.

Agreeing with previous studies (3, 6), we also observed an increased stromal vascular density in the ovaries of PCOS. Previous studies in other systems have demonstrated that VEGF is highly expressed in the theca and stroma of the PCOS ovaries *vs.* control (3, 6) and that the D2/Drd2 system acts as a negative regulator of vascularization (18), very probably through the modulation of the VEGF secretion (9). It is therefore conceivable that as with LGC, D2 might play a similar role in inhibiting VEGF secretion in other Drd2-expressing ovarian cell populations like the theca. In such a scenario, a decreased density or a deregulated function of Drd2 in PCOS ovaries might have a permissive effect, allowing higher local VEGF production and secretion when compared with non-PCOS patients, which in turn would drive increased thecal and stromal ovarian vascularization commonly observed in the affected patients. Due to unavailability of stromal or thecal tissue, we performed functional studies with available VEGF-secreting/Drd2-expressing LGC to evaluate whether Drd2 signaling might be deregulated in PCOS.

Unlike previous findings from Agrawal *et al.* (4) but in agreement with results reported by Stanek *et al.* (19), VEGF production and secretion by LGC solely stimulated with hCG was similar in the PCOS and control groups in our study. Apart from the higher variability amount of VEGF secreted by our patients, the experimental design in the work by Agrawal *et al.* differed from ours. They compared PCOS conditions consisting of high-responder (double the number of follicles) PCOS patients developing OHSS (40% of the cases) *vs.* normal-responder women not showing OHSS at all. Because LGC from patients developing OHSS secrete higher amounts of VEGF than LGCfrom patientswhodo not develop the syndrome (20), it was not unexpected that Agrawal *et al.* found differences in VEGF secretion between groups in their study. In addition, they also incubated the LGC with insulin, which was reported by Stanek *et al.* (19) to be more sensitive in increasing VEGF in LGC from polycystic ovaries than in controls.

Our results do not exclude the possibility of LGC from PCOS being more susceptible to secrete higher VEGF than non-PCOS under *in vivo* condition (4) but may merely reflect our unavailability to mimic *in vitro* the different ovarian environment existing under PCOS (*i.e.* lower D2 levels, higher levels of insulin, *etc.*) and control conditions.

It must be kept in mind that the absence of corpora lutea in anovulatory PCOS patients does not allow the study of the expression of Drd2 receptors in this tissue that is responsible for VEGF release upon hCG stimulation related to OHSS. In addition, theca-lutein cells, which show the higher density of dopaminergic receptors, were not studied *in vitro* due to the unavailability of this tissue, and subsequently the possible paracrine interactions between theca-lutein and granulosa-lutein cells in the corpus luteum cannot be analyzed in our culture system.

On the other hand, our *in vitro* results might merely reflect the fact that gonadotropin-dependent VEGF stimulation might not be altered in LGC from PCOS patients.

In such a scenario, it is expected that the addition of factors with deregulated signaling pathways like dopamine, as shown in this work, or insulin and IGF as shown by works from others (4, 19), might play a more important role for the increased VEGF production for PCOS as reported *in vitro* and as suspected *in vivo*. Even with the limitations of the *in vitro* approaches performed, the significantly lower.

percentage of VEGF inhibition in response to similar doses of Cb2 observed in PCOS *vs.* control points to the existence of a decreased efficiency of Drd2 to decrease VEGF in LGC from these patients. The fact that higher doses of Cb2 were required to reach similar levels of VEGF inhibition in the PCOS than controls (Fig. 5) might be related to a lower affinity of Drd2 for its ligand and subsequently the higher VEGF production in response to Drd2 activation in these patients. This statement is supported by the finding that the whole amount of VEGF secreted by LGC from PCOS was significantly higher than controls when stimulated by Cb2 at a 100-_M dose. In addition, the finding of decreased amounts of D2 released by polycystic ovaries in culture also accounts for the existence of a deregulated dopaminergic tone in PCOS. Although LGC do not synthetize (21), these cells are able to absorb from the circulation, store (21), and metabolize D2 (22). In this context, a decreased secretion of D2 and an augmented release of DOPAC, the first product derived from the metabolism of D2 by LGC, suggest an accelerated D2 metabolism in the corpora lutea of PCOS patients. Thus, it is tempting to speculate that if D2 released by LGC activates Drd2 to regulate their own secretion of VEGF in an autocrine fashion, the ability of LGC from PCOS to regulate VEGF production might be limited because of the lower amounts of D2 secreted in these cells when compared with control.

Thus, we postulate that the probably lower ovarian production of D2 as well as the inefficiency of Drd2 in LGC from PCOS to inhibit VEGF when activated by dopamine might account for the decreased ability of these patients to decrease VEGF overproduction when stimulated with hCG, explaining why PCOS women are at an increased risk of developing OHSS. For this reason we suspect that the addition of exogenous Cb2 is expected to be less effective in preventing OHSS in PCOS women. A second factor possibly contributing to the higher OHSS risk in PCOS patients is related to ovarian hypervascularization, which increases the vascular surface area in the ovary. Because the number of ovarian endothelial cell VEGFR2 targets for VEGF becomes increased, proportionate rise blood vessel permeability is expected.Wespeculate that decreased ovarian Drd2 expression as demonstrated in this study might be related to the higher ovarian VEGF production (3, 4) and subsequently the higher vascularization observed in PCOS (6).

In summary we have shown for the first time that the dopaminergic tone in the ovaries from women suffering from PCOS is decreased when compared with regularly cycling females. It is likely that D2-modulated changes in the VEGF/VEGFR2 pathway contribute to the typical vascular changes seen in PCOS ovaries and could explain. why this group of women is at a higher risk of developing OHSS.

Acknowledgments

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5. RESULTS SUMMARY

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I. Efficiency and purity provided by the existing methods for the isolation of luteinized granulosa cells: a comparative study.

Human Reproduction 2012; 27(6):1781-9

***Ferrero H**, Delgado-Rosas F, García-Pascual CM, Monterde M, Zimmermann RC, Simón C, Pellicer A, Gómez R.*

In order to determine the dopaminergic effects on the production of VEGF in physiological and pathological conditions, the individual characteristics of each patient had to be preserved and therefore, pools of LGCs could not be used. Hence the highest possible number of LGCs needed to be obtained from each patient for our *in vitro* cultures without compromising their purity.

Several LGC isolation methods were described and among them were methods which allowed a very pure population to be obtained, however it yielded very few cells; other methods yielded a large number of cells but compromised the purity of the LGC population. In this first study, we aimed to ascertain which of the existing methods allowed the highest number of LGCs to be obtained without compromising their purity or viability for our subsequent *in vitro* cultures.

LGCs which were destined for use in our *in vitro* studies had to be obtained during the oocyte retrieval process, an invasive method that may penetrate vascularized structures. Hence, the cells obtained from the FF obtained in these retrievals are contaminated with vascular components, resulting in a heterogeneous cell population containing LGCs, CD45+ immune cells, erythrocytes, and lower quantities of other cell types such as ECs and epithelial cells. The presence of immune cells in the FF is especially critical, as these cells can directly influence the results obtained in subsequent *in vitro* studies with LGCs. Therefore, isolation techniques had to be applied in order to establish pure, or almost pure, *in vitro* LGC cultures so that the specific role of these cells in follicle development and ovarian

function could be inferred. Given that GCs do not express a specific marker which allows their isolation by positive discrimination we instead compared the efficiency of the different depletion methods described for the LGC isolation.

The main stratergies currently used for the depletion of unwanted cells can be classified into two main groups according their restrictiveness: (i) highly specific depletion strategies based on immunological recognition of specific contaminating cell markers and (ii) non-specific depletion strategies based on the differential physical properties of the contaminating and desired cells. In order to determine the best isolation method, each of the FF samples was pooled into two aliquots: named ‘C’ (Control) or ‘E’ (Experimental). The control aliquot was used as an internal standard control against which the experimental aliquots were compared after the specific isolation method was complete. The different methods used in this comparative study were described in manuscript-I. Both control and experimental aliquots were analyzed by flow cytometry to evaluate the contaminating cell population, which was identified as CD45+ cells.

The analysis of the flow cytometry data revealed that depletion techniques based on the recognition of specific cell markers reduced the CD45+ contaminating cells by 90-100%. Dynabeads and MACS (magnetic-activated cell sorting) methods were highly efficient at reducing CD45+ cell contamination, with no significant differences between them, as shown in Table II in manuscript-I. However, as expected, FACS (fluorescence-activated cell sorting) was the most effective of these methods at reducing the immune cell population, with just under 0.1% CD45+ contaminating cells detected in the experimental aliquots. However, despite the high purity of the LGCs obtained, we observed a drastic reduction in the number of LGCs recovered by these depletion techniques when compared with other methods. Although we expected that FACS would give the highest recovery of viable cells, the data obtained from flow cytometric analysis showed that LGC recovery was less than 20% as almost 50% of cells died during the sorting process. Therefore, despite providing the highest percentage of pure LGCs, the

FACS process led to a low cell viability, which suggests that LGCs are very sensitive to cell sorting. As a consequence FACS resulted in a lower LGC recovery rate than the other methods.

Regarding the aggregation and flask methods (which are usually based on the differential adhesive and aggregative properties of immune cells and LGCs respectively and so require very little manipulaton), a high percentage of viable LGCs was obtained, as shown in Table II in manuscript-I. Therefore, unexpectedly these methodologies gave the highest viable LGC recovery when compared to all of the other protocols tested (aggregation: 73.20% ± 6.2; flask 51.63% ± 12.6). The main concern with these techniques was the purity of the cell population obtained from them. Unfortunately for our purposes the high percentage of CD45+ cells observed in samples obtained by these methods meant that they were not suitable for our purposes. In particular, the aggregation method provides the lowest percentage of CD45+ reduction, probably because it involved the subjective judgement of the person performing the separation. These methodologies were not able to sufficiently discriminate between different cell populations and as result they are less effective than the other methods at selecting out contaminating CD45+ white cells.

Therefore, depletion techniques based on the recognition of specific cell markers could not be used for our purposes due to the low number of cells obtained, and neither could techniques based on the physical/chemical characteristics of the cells because of their poor ability to discriminative out contaminanting cells. We found an alternative cell strainer method for LGC isolation method in the literature (Richardson MC, et al., 2009). This methodology is based on the fact that LGCs form large cell aggregates in such a way that clusters can be retained by 40 μm cell strainers while erythocytes and other blood contaminants pass through the membrane. We assayed this methodology but with some modifications to improve it: the LGC suspension was not incubated at 37º C for 3-4 h before being aspirated them through pipettes and they were not subsequently centrifuged after break up.

Our results showed that the cell strainer method gave a high percentage of LGCs recovery (70%) with a viability of 92.75%. Moreover, flow cytometry revealed that only 3.29% of the cells in the suspension were CD45+ cells, as shown Table II in manuscript-I. Since any isolation method which obtains a less than 5% contamination rate is considered acceptable, strainer method was considered to be the best for obtaining the highest number of LGCs without compromising their purity, and therefore it was used in all the studies described in this thesis.

II. Dopamine receptor 2 activation inhibits ovarian Vascular Endothelial Growth Factor secretion *in vitro*: implications for treatment of Ovarian Hyperstimulation Syndrome with Dopamine receptor 2 agonists

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In a previous study from our group (Gómez R, et. al., 2006) we observed that the administration of D2-ags to a prl-supplemented OHSS rat model blocked the OHSS-associated increase in VP and was associated with a decrease in ovarian VEGFR2 phosphorylation levels. Subsequently, studies from our group verified that the D2-ag Cb2 reduced the incidence of moderate OHSS in humans by inhibiting the increase of ovarian VP (Álvarez C. et al., 2007). Given that the presence of D2 on human LGCs seemed restricted to some patients (Álvarez C, et al 2007) this study originally aimed to ascertain whether this phenomenon was associated with the differential efficacy of these drugs in preventing OHSS in some patients. The efficacy of treatments seemed to be restricted to 50% of patients for early OHSS with a total inefficacy in preventing OHSS during the late stage of CL but not late OHSS. Hence, the main initial goals of this study were:

a) To evaluate whether D2-ags modulate the VEGF/VEGFR2 system in human ovarian populations which express D2.
b) To ascertain whether some patients express ovarian D2 while others do not, and whether this could be a plausible explanation for the apparently restricted efficacy of these drugs in preventing early OHSS in some patients.
c) To ascertain whether ovarian D2 expression abruptly declines or totally vanishes during the late-lutal phase as a plausible explantation for the total inefficacy of D2-ags in preventing OHSS during the late CL stage.

To pursue these goals we immunolocalized D2 in human ovarian tissue samples taken from different cycle phases under physiological conditions. We observed that, although there were different levels of staining, D2 was consistently expressed in all of our human ovarian samples. Therefore, it was more likely that differential D2 expression between patients (some patients expressing it while others not at all) was related to the differential efficacy of these drugs in preventing early OHSS.

To our suprise we observed that D2 expression was not detected in ovarian ECs as we had expected. This meant that the effects of D2-ags on reducing ovarian VP were not mediated directly by acting on VEGFR2 expressed on ovarian ECs but rather via an alternative mechanism. When trying to establish a link between the dopaminergic system and VEGFR/VEGFR2 system in the ovary we observed that D2 staining was restricted to: macrophages, LGCs, and theca cells, as shown in Figure 4 A-C in manuscript-II. Due to ovarian ECs do not express D2 and the number of theca lutein cells obtained from FF was not sufficient for *in vitro* culture, we had to limit our subsequent *in vitro* studies to LGCs as the only D2-expressing cell population which we could easily and consistently obtain from FF.

In order to determine whether (and how) VEGF production by LGCs might be modulated by the dopaminergic system, LGCs were cultured in the presence/absence of different doses of Cb2 and the D2-antagonist L-741.625 for three days. We observed that when Cb2 was assayed alone it inhibited VEGF secretion in a dose-dependent fashion whilst L-741.625 had no effect when assayed under similar conditions. Interestingly, the decrease in VEGF initiated by D2-ags was partially abated when equal doses of the D2-ant were administered concomitantly, as shown Figure 1 in manuscript-II. Therefore, it is likely that the VEGF dose-dependent inhibition exerted by D2-ags in LGCs was due to a D2-mediated effect.

Subsequently we assessed whether the capacity of LGCs to inhibit VEGF secretion in response to D2-ags depends on the amount of D2 specifically expressed on the cell surface of each patient. Therefore, we determined the amount of D2 expression on the surface of LGCs treated with different D2-ag concentrations. We observed that at lower Cb2 concentrations (20-60 μM) there was a clear correlation between the percentage of VEGF inhibition and LGC surface D2 expression. However, this correlation tended to be extinguisted with increasing doses of the D2-ag to the point of extinction at the highest doses (80-100 μM). In this latter scenario even LGCs with very low levels of D2 expression showed VEGF inhibition similar to that shown by samples with high levels of D2 expression (Figure 2 in manuscript-II). These results suggest that at low D2-ag doses the LGCs expressing a higher amount of D2 on their surface are more efficient than LGCs with a low D2 density at inhibiting VEGF secretion. In contrast, this D2-dependent ability shown by LGCs could be counteracted by administering higher quantities of D2-ag. Overall this experiement suggested that perhaps patients with higher LGC surface D2 expression might be more likely to respond successfully to D2-ag therapies aimed at preventing OHSS than those with a low D2 LGC surface expression.

In our subsequent experiment we wanted to determine the molecular mechanism by which Dp exerts its action on VEGF secretion in LGCs. Unfortunately, we were unable to identify the D2 pathway involved in VEGF secretion, but we discovered that inhibition of VEGF secretion by Cb2 was exerted postranscriptionally and not at the transcriptional level. Moreover, because we observed the same results both in presence and absence of hCG we concluded that D2-ags decrese VEGF protein secretion without interfering with hCG-mediated pathways.

To reach these conclusions we measured VEGF mRNA expression and VEGF protein secretion simultaneously in LGCs cultured with or without hCG in the presence/absence of Cb2 for 24, 48 or 72 hours. In presence of this D2-ag there was a clear reduction in VEGF protein secretion; in contrast, this reduction was not observed in VEGF mRNA levels in D2-ag-treated vs. non-treated LGCs over this

time course, as shown Figure 3 in manuscript-II. The same results were observed in the presence or absence of hCG.

As we mentioned in our hypothesis, the inefficacy of D2-ags in preventing late OHSS might be due to the abrupt decrease in D2 expresion in CL during the late stage, in which appears OHSS. We characterized and quantified D2 expression in human ovarian sections over the whole ovarian cycle, observing that D2 expression was the strongest in theca cells and remained unchanged throughout the luteal phase. In contrast, we observed cyclical variations in D2 intensity and distribution in GCs: staining was weak at the preovulatory phase but substantially increased and peaked during the early luteal phase, as shown in Figure 4 A in manuscript-II. Subsequently, LGC staining started to decrease as the age of the CL advanced, until its expression was nearly abolished at the later CL stages, as shown in Figure 4 B-C in manuscript-II. On the basis of these findings we suggest that D2-ags might not prevent late OHSS because LGC surface D2-expression at this stage of the luteal phase is decreased and therefore is not sufficient to inhibit VEGF secretion to the extent by which the increase in VP is prevented.

III. Evidences for the existence of a low dopaminergic tone in Polycystic Ovarian Syndrome: Implications for OHSS development and treatment.
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The finding that D2 is expressed in theca and GCs throughout the luteal phase in normal human ovaries (manuscript-II) lent further support for an intraovarian regulatory role for the Dp/D2 pathway in OHSS. Moreover, the dose-dependent inhibition of LGC VEGF production and secretion mediated by Cb2 (manuscript-II) suggested that the ovarian VEGF/VEGFR2 system can be modulated by the ovarian dopaminergic system in the ovary, and that this inhibition also directly correlates with the amount of D2 expressed by LGCs. Given that the dopaminergic tone in women with PCOS might be deregulated, as shown by the high percentage of them with elevated prl levels, and that the VEGF/VEGFR2 system also seems to be altered in PCOS patients, we hypothesized that deregulation of the VEGF/VEGFR2 pathway in PCOS women might at least be partially due to Dp/D2 pathway abnormalities.

To test this hypothesis we analyzed the expression of D2 on dated, archived, PCOS and non-PCOS ovaries obtained at different stages of the menstrual cycle. We observed D2 expression in the granulosa layer of primordial, primary, and preantral follicles in non-PCOS ovaries, as shown in Figure 1 A-D in manuscript-III, whereas most small follicles in PCOS ovaries lacked, or showed barely detectable, D2 expression, as shown in Figure 1 E-I in manuscript-III. Decreased D2 expression in PCOS ovaries seemed to be specific to GCs in small follicles and to the theca cell layer in antral follicles; because macrophages present in atretic follicles showed intense expression, similar to that found in cycling ovaries, as shown in Figure 1 J in manuscript-III. The theca layer of the antral follicles in PCOS ovaries showed significantly lower D2 expression and higher vascularization

(CD34+) than normal cycling ovaries, as shown in Figure 2 in manuscript-III. These differences were particularly prominent in luteinized follicles, as shown in Figure 3 in manuscript-III.

We subsequently aimed to ascertain whether VEGF/VEGFR2 pathway deregulation might at least be partially due to abnormalities in the Dp/D2 pathway in PCOS women. Therefore, we performed functional studies by culturing hCG-stimulated LGCs isolated from PCOS and non-PCOS patients with Cb2 at concentrations of 0, 20, 40, 60, 80, or 100 µM for 72 h. In the absence of the D2-ag LGCs from both groups secreted similar amounts of VEGF in response to hCG, as shown in Figure 4A in manuscript-III. However, when Cb2 was added to the culture media the VEGF secretion decreased in a dose-dependent fashion with maximal inhibition in both groups at the highest Cb2 dose (100 µM). However, the most interesting finding was that the inhibition of VEGF secretion seemed to be more prominent in non-PCOS than PCOS patients.

These results suggested that LGCs from non-PCOS patients are more efficient than those from PCOS women at decreasing VEGF secretion in response to D2 activacion. In order to confirm our suspicions, we analyzed the percentage of VEGF inhibition in these samples, showing that LCGs from PCOS were less efficient at decreasing VEGF, as shown in Figure 5 in manuscript-III. Additionally, after compensating for decreased dopaminergic tone in PCOS patients, we observed that LGCs from PCOS samples secreted lower amounts of Dp than those from non-PCOs ovaries, as shown in Figure 4D in manuscript-III. Interestingly, the secretion of 3,4-Dihydroxyphenylacetic acid (DOPAC; the first product derived from Dp metabolism) was significantly increased in PCOS sample cells, as shown in Figure 4E in manuscript-III. These findings suggested that not only is the amount of Dp reduced but in addition this metabolite is also more quickly metabolized in PCOS patients, supporting the notion that dopaminergic tone and metabolism are deregulated in PCOS women.

Overall, the evidence complied in thesis suggests that the dopaminergic tone is deregulated in PCOS patients and that this factor might explain, at least in part, the deregulated expression of VEGF in these women and thus also the higher susceptibility of these patients to developing OHSS.

6. DISCUSSION

OHSS affects a wide range of women of reproductive age; it is mainly characterized by an increase in VP which is mediated by the VEGF/VEGFR2 system. Regulation of ovarian VEGF production, secretion, and action is dependent not only on exogenous gonadotropins but also on other factors, among them the D2 receptor.

Research in murine tumor models (Basu S, et al, 2001) and *in vitro* studies in HUVECs (*Human umbilical vein endotheial cells*) (Sinha S, et al., 2009) have shown that administration of D2-ags can drastically reduce VP and angiogenesis. These studies suggested that activation of D2 on ECs exerts its effects on VEGFR2, inhibiting VEGF secretion and consequently reducing VP. Based on these findings, we hypothesized that the efficacy of D2-ags in decreasing VP may rely on the capacity of these drugs to inhibit VEGFR2 phosphorylation or VEGF secretion. This hypothesis suggests the existence of a dopaminergic system inside the ovary which determines the susceptibility of each woman to developing OHSS via VEGF/VEGFR2 system regulation.

In this context, given the importance of VEGF in OHSS and because its regulation is mediated by Dp, we hypothesized that deregulation of the VEGF/VEGFR2 pathway in women susceptible to OHSS, such as PCOS patients, might at least be partially due to abnormalities in the Dp/D2 system. In order to test the hypothesis outlined in this thesis, our main goal was to ascertain whether activation of the dopaminergic system is able to modulate the VEGF/VEGFR2 system in human LGCs, and to evaluate whether there were significant differences in the dopaminergic tone in healthy vs. PCOS patients which could explain their susceptibility to developing OHSS.

We used an *in vitro* model consisting of LGCs isolated from FF in order to similate the *in vivo* CL luteinization process. Given that the FF is comprised of a heterogeneous cell population, it was necessary to establish an optised LGC isolation method in order to obtain adequate purity to culture these cells *in vitro*.

To do this firstly we compared the efficiency of the different LGC cellular depletion and isolation methods already published. We noted that depletion strategies based on the physical properties of the contaminating and desired cells yeilded the highest percentage of viable LGCs but with high number of contaminating cells compared to other protocols. Methodologies based on immunological recognition of specific cell markers on contaminating cells provided acceptable levels of cellular purity and viability but a low number of LGCs, an obstacle which is especially critical when FF cannot be pooled because individual patients' characteristics must be preserved. Therefore the number of *in vitro* assays which could be performed would have been unacceptably limited by the low number of LGCs yielded by this method. Finally, we decided to use a strain-filter isolation technique because it provided the highest number of LGCs and gave acceptable levels of purity without compromising the viability of the cells. Naturally, researchers that routinely use the aforementioned methods must generally obtain a more efficient recovery than ours. However we chose the strain-filter method to achieve our objectives in the *in vitro* studies.

Once we found the best LGC isolation method, we focused our efforts in ascertain the potential role of the dopaminergic system in modulating angiogenesis through the VEGF/VEGFR2 pathway.

Previous studies from our group hypothesized that the Dp/D2 system might be responsible for the decreases in ovarian VP observed in hyperstimulated rats (Gómez R, et al., 2006) as well as in women with a high risk of OHSS, when treated with D2-ags (Álvarez C, et al., 2007), and that this decrease might be due to a D2-ag mediated VEGFR2 phosphorylation blockade. Morevoer, this previous study in women with a risk of OHSS detected that they differentially express D2 on their ovarian ECs, leding us to hypothesize that this difference might explain the differences in the efficacy of D2-ags in preventing OHSS in some patients.

To date there is no consensus on the best dosage of D2-ags, however our study reported that VEGF secretion was decreased by D2-ags in a dose-dependent fashion. This result led us to think that previous observations by Shaltout A. et al., in a study with Cb2 in women with OHSS risk, in which low D2-ag doses can reduce the incidence of OHSS equally efficiently as high doses (Shaltout A, 2012) can probably be explained by the fact they only used low D2-ag doses; this would mean that they did not observe significant differences in VEGF secretion because low D2-ag doses are not as effective as high ones at inhibiting VEGF. Therefore, it is likely that the amount of dopaminergic signaling elicited in LGCs is the factor that determines VEGF inhibition. This observation supports the hypothesis that prevention of early OHSS might be improved by increasing the does of D2-ags administered to at-risk patients. This is in agreement with the findings of Busso et al. who demonstrated that high D2-ag doses were significantly more effective than lower doses in preventing early OHSS (Busso C, et al., 2010).

Subsequently, we studied D2 modulation in order to evaluate the dopaminergic tone, establishing that the effect of D2-ags is mediated by the D2 receptor. Therefore, it is likely that dopaminergic signaling depends on D2 expression in LGCs. This finding led us to think that D2 expression in LGCs might be a factor determining the decrease in VEGF, hence, LGCs from some patients would be more efficient at inhibiting VEGF than others in response to the same D2-ag dose, depending on the amount of D2 expressed by these cells.

To test this hyphotesis we explored if VEGF inhibition in response to fixed doses of D2-ags correlated with the amount of D2 expressed by LGCs *in vitro*. We observed a positive correlation between D2 and VEGF inhibition at low D2-ag doses because D2 expression on LGCs is the factor that determines differential VEGF secretion. In contrast, at high doses the excess D2-ag increases the probability of its binding to D2 so much that maximal VEGF inhibition is reached. Therefore, as D2 expression on the cell surface stops being a limiting factor in determining the dopaminergic stimuli, the correlation between D2 and the dopaminergic response disappears.

The immunohistochemical data we obtained that D2 expression was decreased during the late luteal phase. Assuming that our *in vitro* findings do indeed reflect the *in vivo* situation, we suggest that a low or decreased dopaminergic tone in human ovary, coinciding with the timing of the late luteal phase, causes the ineffectiveness of D2-ags in preventing OHSS. We speculated that due to low D2 expression during late luteal phase, D2-ag doses administered to our patients in the past could not prevent late OHSS because these LGCs were not capable of producing the dopaminergic response necessary to sufficiently decrease VEGF. Therefore, we reasoned that the prevention and/or treatment of the onset of OHSS might be improved by increasing the intra-ovarian concentration of D2-ags by administering higher oral or vaginal doses. Moreover, intravenous treatment could be used if toxicity to high D2-ag doses becomes an issue, even in patients for whom the administrarion of low doses of D2-ags is insufficient to impede the early onset of OHSS.

Once the role of the dopaminergic system in modulating angiogenesis through the VEGF/VEGFR2 system was ascertained, we aimed to understand whether there were significant differences in the dopaminergic tone between physiological and pathological conditions which could explain the particular susceptibility that PCOS patients have to developing OHSS.

The findings presented in this thesis showed that PCOS women exhibit significantly lower D2 expression in theca and GCs and higher vascularization in the theca layer of antral and luteinized follicles compared to a non-PCOS group, consisting of women with a regular menstrual cycle. This observation agrees with the finding that VEGF is highly expressed in ovaries from PCOS patients (Kamat B, et al., 1995; Neulen J, et al., 1995; Agrawal R. et al., 2002) leading us to hypothesize that PCOS patients may express less D2 or that their D2 function might be deregulated which could induce elevated ovarian VEGF production and secretion compared to non-PCOS patients.

We expected our observations to show that PCOS women secrete more VEGF than non-PCOS women. Nevertheless, the results obtained suggested that the VEGF production and secretion by LGCs was similar in PCOS and non-PCOS patients. However, we observed less VEGF inhibition in PCOS vs. non-PCOS women in response to similar doses of D2-ags. This indicated that D2 was less efficient at decreasing VEGF in LGCs from PCOS than non-PCOS patients, so that higher doses of D2-ags are required to reach similar levels of VEGF inhibition in these patients. These results might be related to a low affinity of D2 to its ligand which would subsequently trigger high VEGF production in response to D2 activation in PCOS patients. This idea is supported by the finding that the overall amount of VEGF secreted by LCGs from PCOS women is significantly higher than in non-PCOS women when they are stimulated with a higher D2-ag dose.

In addition, our *in vitro* study reported that the Dp released by PCOS patient LGCs cultured with D2-ags was lower than that of non-PCOS women, indicating the existence of a deregulated dopaminergic tone in PCOS patients. Although LGCs do not synthetize Dp (Mayerhofer A, et al., 1998), these cells may be able to absorb Dp from the circulation, and store and metabolize it (Greiner M, et al., 2008). Therefore the observed decrease in Dp secretion and an increase in the amout of DOPAC released is suggestive of an accelerated Dp metabolism in the CL of PCOS patients. The lower amount of Dp released by LGCs derived from PCOS led us to speculate that VEGF secretion could be regulated by the Dp-mediated activation of D2, and that the ability of LGCs to reduce VEGF secretion might be limited in PCOS women.

Based on the findings obtained in this study, we suggest that an accelerated Dp metabolism, as well as inefficient LGC D2 function leading to the poor inhibition of VEGF in PCOS patients, are the main causal factors in their increased susceptibility to develop OHSS. This is because the ovarian D2 present in these patients has an attenuated ability to inhibit VEGF secretion after D2-ag

administration. This would also explain why the addition of exogenous D2-ags is expected to be less effective in preventing OHSS in PCOS women.

The *in vitro* findings presented in this thesis suggested that the efficacy of D2-ags in preventing OHSS might be improved by increasing the intraovarian concentration of D2-ags. We are aware that results obtained from *in vitro* cultured LGCs can be criticized because they might not represent the *in vivo* ovarian environment; the validity of our data might also be criticized because of the high doses of D2-ags used in our *in vitro* study. Therefore, the application and therapeutic utility of these findings for the clinical management of patient disease must be further corroborated in *in vivo* studies.

Although not included in this thesis, it is worth mentioning that we have also assayed the effect of D2-ags on ovarian VEGF secretion in our well-established and validated prl-supplemented OHSS animal model (Gómez R, et al., 2006). Although this work has not yet been published we can confirm that D2-ags inhibited VEGF protein production in the ovary in this model, thus supporting the assertations set out in this thesis.

In summary, we sincerly hope that our findings will be helpful in improving knowlege of the regulation of VEGF/VEGFR2 by dopaminergic tone, as well as to help devise ways to better treat OHSS or related pathologies in which deregulated dopaminergic tone might lead to VEGF/VEGFR2-dependent pathologies. The findings obtained in the *in vitro* studies presented in this thesis led us to suggest the main conclusions detailed below.

7. CONCLUSIONS

1. FACS is the best protocol for isolating LGCs when purity is the principal requirement. Magnetic separation is the best option when both purity and viability are essential for subsequent experimentation. However, cell straining is probably the most efficient method with which to isolate LGCs as it offers the best combination of purity, viability, and cell recovery from all the methodologies currently available.

2. D2 is consistently expressed in granulosa, theca, and immune cells throughout the luteal phase in the ovaries of all women. Therefore, the absence of this receptor in women who develop OHSS is not a plausible explanation for the differential efficacy of these drugs in preventing OHSS as had been previously hypothesized.

3. D2 is not present on ovarian ECs, therefore, it is likely that D2-ags do not inhibit increases in ovarian VP by binding to D2 on ovarian ECs as we had previously hypothesized.

4. The D2 staining intensity on LGCs decreases with the age of the CL to the point of nearly vanishing during the late luteal phase in all women.

5. D2-ags decrease VEGF production and secretion by LGCs *in vitro* in a D2-ag/D2-dependent manner.

6. Unresponsiveness to the administration of D2-ags may be explained by the fact that low doses of D2-ags were used and because low levels of D2 expression similar to those that characterize the late luteal phase also exist in the early luteal phase in some patients.

7. The dopaminergic tone in the ovaries from patients suffering from PCOS is decreased when compared to regularly cycling women.

8. It is likely that D2-modulated changes in the VEGF/VEGFR2 pathway contribute to the typical vascular changes seen in PCOS ovaries and could explain why this group of women is at a higher risk of developing OHSS.

9. We infer that the efficiency of dopaminergic therapies for OHSS treatment is likely to be improved by increasing the intra-ovarian concentration of D2-ags by administering higher oral or vaginal doses of these compounds.

8. REFERENCES

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