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Targeting the vascular endothelial growth factor system to prevent ovarian hyperstimulation syndrome

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BACKGROUND: Ovarian hyperstimulation syndrome (OHSS) typically occurs when ovaries are primed with FSH/LH and subsequently exposed to hCG. The ultimate pathophysiological step underlying this clinical picture is increased vascular permeability (VP). METHODS: A search of the literature was carried out using PubMed and the authors' files. RESULTS: In rodents and humans, the expression of vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR-2) mRNA increases during ovarian stimulation. With the administration of hCG, the expression of each rises to a maximum. Expression of VEGF/VEGFR-2 mRNAs correlates with enhanced VP, with both peaking 48 h following an injection of hCG. Immunohistochemistry shows the presence of VEGF and VEGFR-2 proteins in the granulosa-lutein and endothelial cells of the entire corpus luteum. Increased VP may be mediated through adhesion molecules such as VE-cadherin, which is involved in the loosening of endothelial intercellular junctions. These findings regarding the pathophysiology of OHSS suggest that the syndrome can be prevented by inducing ovulation with LH or GnRH analogues, which prevent VEGF overexpression. Also, co-administration of a dopamine agonist inhibits phosphorylation of the receptor VEGFR-2. In a trial of 69 oocyte donors, the incidence of moderate OHSS was 20% with the dopamine agonist cabergoline and 44% with a placebo ($P = 0.04$). CONCLUSIONS: The pathophysiological mechanisms involved in OHSS suggest potential preventive approaches, but larger trials are necessary for evaluating the efficacy and safety of the pharmaco-prevention of OHSS.

Keywords: vascular endothelial growth factor; ovarian hyperstimulation syndrome; vascular permeability; dopamine agonist; pathophysiology

Introduction

Ovarian hyperstimulation syndrome (OHSS) is defined as the shift of serum from the intravascular space to the third space, and mainly to the abdominal cavity, that occurs when the ovaries become enlarged due to follicular stimulation. It is especially aggravated during gestation. This process leads, on the one hand, to haemoconcentration and the risk of thromboembolism and impaired general perfusion, and, on the other, to abdominal distension, which may produce abdominal discomfort and breathing difficulties (Mozes *et al*., 1965).

Typically, OHSS is a complication of ovarian stimulation with gonadotrophins followed by the administration of hCG to trigger the final steps of oocyte maturation (Mozes *et al*., 1965). It is more frequently seen when a strong ovarian response occurs, characterized by the development of a large number of follicles, high estradiol (E_2) values and enlarged ovaries (Rizk and Smitz, 1992).

The incidence of severe OHSS in IVF cycles in which ovarian stimulation is performed using gonadotrophins has been reported to be 0.5–2.0%, whereas in intrauterine insemination cycles, in which stimulation is performed with clomiphene citrate or aromatase inhibitors, the condition is rarely seen, with the exception of cases that show a particular susceptibility towards it (Rizk, 2006) (Table I).

Individual susceptibility may be the consequence of increased ovarian sensitivity to gonadotrophins or high levels of endogenous gonadotrophins (or gonadotrophin-like molecules) (Table I). Increased sensitivity has been well-documented in women with polycystic ovaries, of a young age, with low body mass index and with a history of allergies (Golan *et al*., 1989; Navot *et al*., 1992, 1996).

On occasions, OHSS may occur in the absence of exogenous gonadotrophin administration, in which cases the presence of endogenous hCG (spontaneous pregnancy) is the only determinant of hyperstimulation (Zalel *et al*., 1995; Özden *et al*., 2005).

Table I. Risk factors for the development of OHSS

The ovarian stimulation cycle
Use of gonadotrophins
High dose medication
hCG luteal support
Patient's susceptibility
Increased sensitivity to gonadotrophins
Young age
Low body mass index
PCOS
Allergy (immunoactivation)
FSH receptor mutation
High endogenous gonadotrophins production
Molar pregnancy
Hypothyroidism

PCOS, polycystic ovary syndrome.

In some cases, ovarian hypersensitivity to gonadotrophins is the consequence of mutations in the FSH receptor, which allow hCG to bind to it (Smits *et al*., 2003; Vasseur *et al*., 2003; Montanelli *et al*., 2004a, b; Delbaere *et al*., 2005). In such instances, a history of cases can be found in the family (Vasseur *et al*., 2003; Montanelli *et al*., 2004a).

On the other hand, OHSS as a consequence of abnormally high levels of endogenous gonadotrophins may be seen in molar pregnancies and diandric or digynic triploids (Cappa *et al*., 1976), in which the concentration of hCG is high. Molecules structurally similar to gonadotrophins, if present at high concentrations, such as in the case of thyroid-stimulating hormone in hypothyroidism, may occupy their receptors and lead to hyperstimulation during pregnancy (Guvenal *et al*., 2006; Borna and Nasery, 2007). Interestingly, FSH and LH secretion in gonadotroph adenoma may lead to enlarged ovaries, but, if no hCG is present, ascites occurs (Kihara *et al*., 2006).

The range of clinical manifestations of OHSS is the logical consequence of the processes that define the syndrome (Table II). Enlarged ovaries may themselves produce abdominal discomfort. Increased vascular permeability (VP) leads to two groups of clinical problems: (i) fluid accumulation in the abdomen and other body cavities: the shift of serum from the intravascular space to the free abdominal cavity causes a sensation of heaviness in the abdomen and breathing difficulties due to limited diaphragmatic mobility (Delvigne and Rozenberg, 2003). Abdominal pain already present due to ovarian enlargement increases with fluid accumulation. In severe forms of OHSS, respiratory function may worsen as a result of pleural effusion (Delvigne and Rozenberg, 2003); (ii) haemoconcentration and reduced blood perfusion: haemoconcentration causes reduced general organ perfusion. Oliguria and renal insufficiency may occur, and liver function may also be affected, with a consequential elevated concentration of blood transaminases. In addition, haemoconcentration increases the risk of thromboembolic events. In very severe forms, renal failure and reduced perfusion in other vital organs, such as the brain and heart, may lead to coma and death (Rizk, 2006).

The objective of this review is to present a thorough evaluation of the information available in the literature regarding the importance of vascular endothelial growth factor (VEGF) in the pathophysiology of OHSS and possible approaches for preventing its development. A search of the literature was performed using PubMed and the authors' files.

Table II. Clinical and laboratorial features in the different stages of OHSS[a]

OHSS stage	Clinical features	Blood tests features
Mild	Abdominal distention/discomfort Mild nausea/vomiting Diarrhoea Enlarged ovaries	No important alterations
Moderate	Mild features + ultrasonographic evidence of ascites	Elevated haematocrit (htc) (>41%) Elevated WBC (>15 000) Hypoproteinaemia
Severe illness	Mild + moderate features +:	Haemoconcentration (Htc >55%)
	Clinical evidence of ascites	WBC >25 000
	Hydrothorax	Creatinin clearance <50 ml/min
	Severe dyspnea	Creatinine >1.6
	Oliguria/anuria	Hyponatremia (Na^+ <135 mEq/l)
	Intractable nausea/vomiting	Hypokalaemia (K^+ >5 mEq/l)
	Tense ascites	Elevated liver enzymes
	Low blood/central venous pressure	
	Rapid weight gain (>1 kg in 24 h)	
	Syncope	
	Severe abdominal pain	
	Venous thrombosis	
Critical	Anuria/acute renal failure thromboembolism Arrhythmia Pericardial effusion Massive hydrothorax Arterial thrombosis ARDS Sepsis	Worsening of severe findings

[a]Adapted from Navot *et al.* (1992). ARDS, adult respiratory distress syndrome; WBC, white blood cells.

The role of VEGF in the pathophysiology of OHSS

Although other mechanisms, such as increased peripheral arteriolar dilatation (Balasch *et al*., 1998), have been proposed as causes of the haemodynamic alterations seen in OHSS, there is now a general consensus that women with ovaries primed with FSH/LH and subsequently exposed to hCG develop a clinical picture in which the ultimate pathophysiological step is increased VP (Vlahos and Gregoriou, 2006). Since hCG has no direct vasoactive properties (Gómez *et al*., 2002), investigations have aimed to detect the vasoactive substance responsible for this condition.

Initially, the association between high levels of E_2 and the occurrence of OHSS justified the belief that this sex steroid was the determinant of the syndrome (Haning *et al*., 1983; Asch *et al*., 1991). However, clinical observations have demonstrated that a high E_2 level is neither necessary for, nor a sufficient cause of OHSS: women with very low E_2 blood concentrations due to desmolase gene mutation can develop OHSS (Pellicer *et al*., 1991), and no matter how high E_2 blood levels may be,

OHSS does not occur if hCG is not administered (Schenker, 1993; Aboulghar and Mansour, 2003). Moreover, studies on the E_2 molecule thrust aside relevant direct vasoactive effect (Delvigne and Rozenberg, 2002; Villasante *et al*., 2007). It is now known that the association of a high E_2 level with OHSS is a mere marker of granulosa (eventually lutein) cell activity.

Subsequent studies have focused on the substances present in the follicular and ascitic fluid of hyperstimulated women. Cytokines and growth factors (interleukins IL-2, IL-6, IL-8, IL-10, IL-18, VEGF) are known to be implicated in the inflammatory processes associated with late follicular maturation, ovulation, corpus luteum function and embryo implantation, as these molecules are present in the aforementioned fluids in women with OHSS. Other substances, such as histamine, prolactin, prostaglandins and renin-angiotensin, have been proposed as participants in OHSS pathophysiology (Rizk *et al*., 1997) (Table III).

Any molecule with an important role in OHSS pathophysiology should fulfil a number of prerequisites. Its expression should be increased by the hCG molecule and should be higher in cases of OHSS. Its effect on VP should be clear and strong. The inhibition of such an effect in hyperstimulated women should inhibit the clinical manifestations of OHSS. Information gathered by studies carried out in the last decade have pointed to VEGF as crucial for the development of the syndrome, as this protein has been shown to fulfil the aforementioned prerequisites. A number of other molecules (including angiogenin, IL-6, IL-10 and IL-18) take part in cascades of events that determine conditions which are also relevant in the development of OHSS, such as ovarian neovascularization, inflammatory response and inhibition of hepatic albumin production (Rizk, 2006). Even increased VP is an outcome that depends on other substances, such as soluble cell adhesion molecules (see section 'Downstream mechanisms of action'). As already mentioned, the scope of this review is the VEGF system.

In humans, five different VEGF mRNAs have been detected, encoding the isoforms $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (Neufeld *et al*., 1999). The isoforms $VEGF_{121}$ and $VEGF_{165}$, also named VEGF A, are normal products of the ovary (Olson *et al*., 1994; Gómez *et al*., 2002). The receptors for VEGF belong to the tyrosine kinase receptor family (De Vries *et al*., 1992). Two specific endothelial cell membrane receptors for VEGF have been identified: VEGFR-1 (Flt-1) and VEGFR-2 (Flk1/KDR) (Waltenberger *et al*., 1994; Shalaby *et al*., 1995). VEGFR-1 is also produced as a soluble receptor (sVEGFR-1) through the alternative splicing of the precursor mRNA (Kendall *et al*., 1996). These receptors are present mainly in the endothelium, but also in the ovarian follicles (Gómez *et al*., 2003a, b).

The binding of the isoforms $VEGF_{121}$ and $VEGF_{165}$ to VEGFR-2 determines the phosphorylation of the receptor intracellular domains (Guo *et al*., 1995), a critical phase in downstream signalling, and one which is implicated in endothelial reorganization, membrane ruffling and chemotactic contraction (Waltenberger *et al*., 1994) (Fig. 1).

The first indication of the role of VEGF as the main promoter of increased VP in OHSS was provided by an *in vitro* study in which the incubation of ascitic fluid from hyperstimulated women with rhVEGF antiserum significantly decreased the VP activity of said fluid (McClure *et al*., 1994).

Since then, attention has focused on the expression of VEGF and its receptors during the ovarian stimulation process, on how gonadotrophins (mainly hCG) affect its expression and on the association between such changes and the exacerbation of the clinical symptoms of OHSS. The identification of the cell types in which VEGF is produced or found is essential for a valid insight into the role that each tissue plays in this process. In this way, the first steps have been taken towards understanding the downstream pathways through which the activated VEGFR increases VP. Finally, the molecular and clinical consequences of blocking specific points of the process may confirm the cause–effect relationships involved in OHSS pathophysiology, and shed some light on specific treatment options for this syndrome.

Temporal relationship between hCG surge, VEGF expression and increased VP

Rodents

Studies employing a well established OHSS rat model have shown that ovarian VEGF mRNA levels and VP increase during stimulation with gonadotrophins (pregnant mare serum gonadotrophin—PMSG), which precedes hCG administration (Gómez *et al*., 2002). Gonadotrophins used for ovarian stimulation also increase the expression of ovarian VEGF receptor 2 (VEGFR-2) mRNA (Gómez *et al*., 2003a). The administration of hCG further augments all these parameters, pushing them to their maximum levels. A linear correlation is found between increased expression of VEGF/VEGFR-2 mRNAs and enhanced VP, with both peaking 48 h after hCG injection (Fig. 2).

Immunohistochemistry shows the presence of VEGF and VEGFR-2 proteins in the granulosa-lutein and endothelial cells of the entire corpus luteum (Gómez *et al*., 2002). Prior to hCG administration, the vessels are the main target for the receptor antibody, and only a dispersed and weak staining is observed in granulosa cells. Following hCG, and in parallel to the neoangiogenesis process, there is a strong staining in the whole corpus luteum (blood vessels and granulosa-lutein cells) (Gómez *et al*., 2002). VEGFR-2 was previously thought to be almost exclusively expressed by endothelial cells (Quinn *et al*., 1993). However, these findings corroborate the claims of authors that some granulosa-lutein cell populations act as endothelial cells in the ovarian tissue (Antczak and Van Blerkom, 2000).

In these animals, the administration of PMSG or hCG does not produce any changes in VEGF mRNA levels in the mesentery (Gómez *et al*., 2002). The stimulation of oophorectomized animals with gonadotrophins confirms the absence of any alteration in VEGF expression or VP, indicating that VEGF production takes place in the ovary (Gómez *et al*., 2002).

Humans

It is known that the presence of both LH-like activity and ovarian function (corpora lutea and/or antral follicles) are an absolute requirement for the onset of OHSS, as the syndrome disappears or fails to develop when an oophorectomy is performed (Amarin, 2003) or when hCG is not administered at the end of controlled ovarian hyperstimulation (COH) with gonadotrophins (Schenker, 1993; Aboulghar and Mansour, 2003).

Table III. Agents suspected of playing a major role in OHSS pathophysiology

Agent	Reference
Estradiol	Haning *et al*. (1983); Asch *et al*. (1991)
Progesterone	Ujioka *et al*. (1997)
IL-2	Orvieto *et al*. (1995)
IL-6	Friedlander *et al*. (1993); Revel *et al*. (1996); Aboulghar *et al*. (1999)
IL-8	Revel *et al*. (1996); Chen *et al*. (2000)
IL-10	Manolopoulos *et al*. (2001)
IL-18	Barak *et al*. (2004); Gutman *et al*. (2004)
VEGF	McClure *et al*. (1994);
Angiogenin	Aboulghar *et al*. (1998)
Endothelin	Balasch *et al*. (1995)
Prostaglandins	Katz *et al*. (1984); Simon *et al*. (1998)
Renin-angiotensin	Navot *el al*. (1987)
Kinins	Kobayashi *et al*. (1998); Ujioka *et al*. (1998)

IL, interleukin; VEGF, vascular endothelial growth factor.

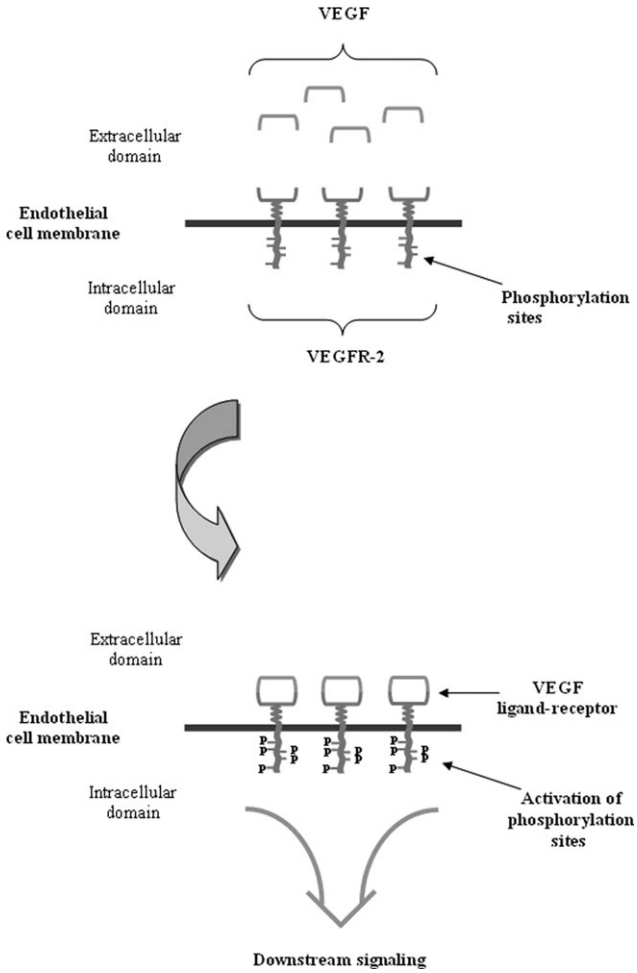

Figure 1: Activation of VEGFR-2 downstream signaling. VEGF binds to its receptor in the endothelial cell membrane and receptor intracellular domains are phosphorylated.

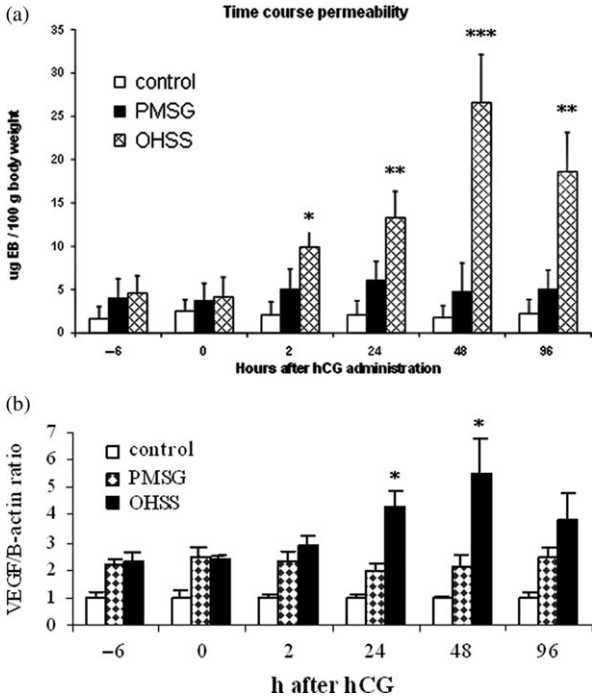

Figure 2: (**a**) Time-course permeability values.
The symbols represent (by Kruskal–Wallis test) significant differences among OHSS, control and PMSG groups at each time point. The PMSG and OHSS groups were different from controls at all time points, but OHSS was also different from PMSG at 2, 24, 48, and 96 h after hCG. $*P < 0.05$; $**P < 0.005$; $***P < 0.001$. EB, evan blue (adapted from Gómez *et al*., 2002). (**b**) VEGF gene ovarian expression quantified by RT–PCR. Whole VEGF expression in the OHSS and PMSG groups was significantly higher than that in the control group at any time point. Whole VEGF expression started to increase in the OHSS ovaries 2 h after hCG, reaching significance compared with PMSG after 24 and 48 h. $*P < 0.05$ (from Gómez *et al*., *Endocrinology* 2002;**143**:4339–4348; *Copyright 2002, The Endocrine Society*). EB, evan blue.

VEGF has a very strong angiogenic effect that has been documented to take place in the ovary (Keck *et al*., 1989; Leung *et al*., 1989; Phillips *et al*., 1990; Yamamoto *et al*., 1997) and to induce vascular hyperpermeability (Bates and Harper 2002; Bates *et al*., 2002) by interacting with VEGFR (Waltenberger *et al*., 1994; Gille *et al*., 2001).

In situ hybridization studies have shown that VEGF expression in human granulosa cells begins before hCG administration, in the same way it does in rats (Kamat *et al*., 1995). Human granulosa cells cultured *in vitro* also express VEGF (Koos and Olson, 1991). Granulosa-lutein cells collected at ovum retrieval in patients undergoing IVF express VEGF mRNA (Yan *et al*., 1993; Neulen *et al*., 1995), which is stimulated by hCG *in vitro*, particularly in cells from women who later develop OHSS (Wang *et al*., 2002). *In vivo*, it has been demonstrated that hCG administration increases VEGF expression in granulosa-lutein cells, particularly in patients at risk of OHSS due to a strong ovarian response (Fig. 3) (Yamamoto *et al*., 1997; Pellicer *et al*., 1999; Wang *et al*., 2002). VEGF serum levels are associated with the probability of developing OHSS and with its clinical picture (Abramov *et al*., 1997; Artini *et al*., 1998; Agrawal *et al*., 1999; Chen *et al*., 1999). Some reports have failed to detect such a correlation (Ludwig *et al*., 1999; Pellicer *et al*., 1999), but most

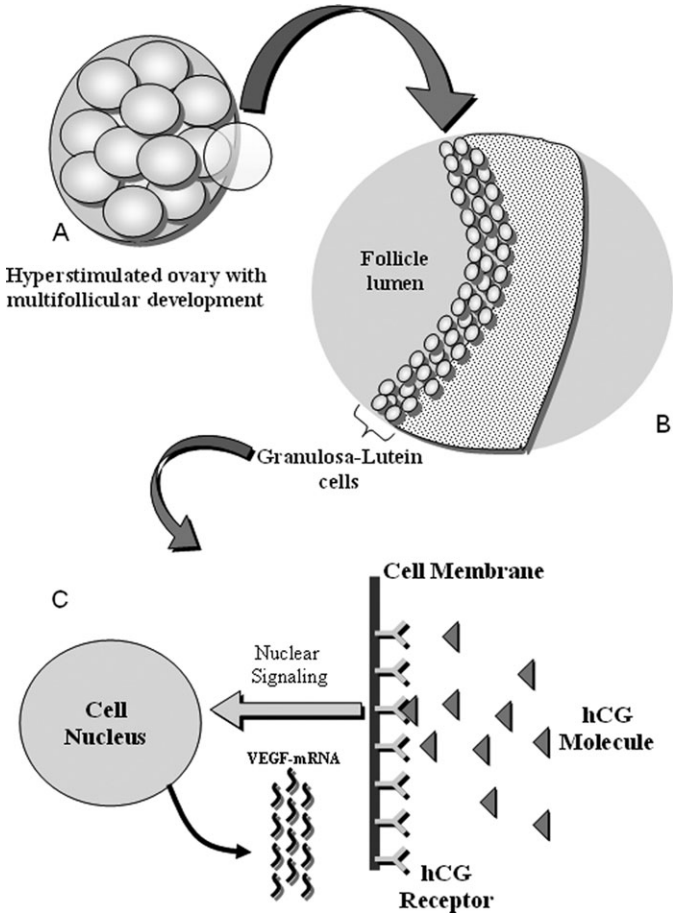

Figure 3: First steps in the pathophysiology of ovarian hyperstimulation syndrome.
A high number of granulosa-lutein cells are stimulated by hCG, which determines the increased production of VEGF-mRNA. VEGFR-2 mRNA production in granulosa-lutein and endothelial cells (see Fig. 4) is also increased by exposure to hCG.

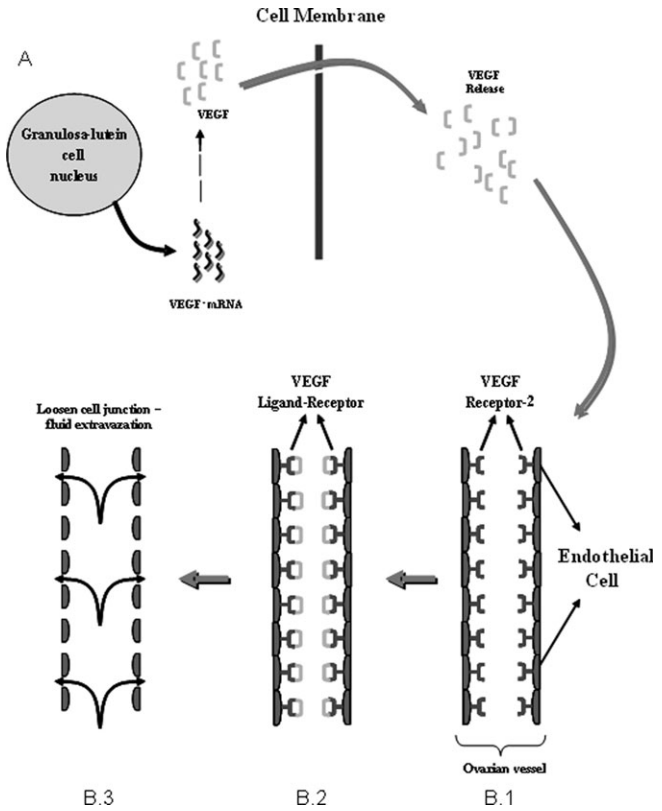

Figure 4: Ovarian hyperstimulation syndrome pathophysiology.
High amounts of VEGF are produced and released by granulosa-lutein cells (**A**) and bind to their receptors in the endothelial cells membrane (B.1, B.2). This determines downstream signaling that augments vascular permeability (B.3). This final effect seems to be related with changes in the actin fibres and in cellular shape, which in turn may be due to factors such as the rearrangement of endothelial junctional proteins like vascular endothelial cadherin (Bates *et al*., 1999; Villasante *et al*., 2007).

of these publications measured serum VEGF rather than plasma VEGF, thereby introducing the contribution of other non-reproductive cells, such as granulocytes or platelets.

Similarly to that observed in rats, the presence of VEGFR-2 in granulosa-lutein cells after hCG administration has been confirmed in humans (Wang *et al*., 2002).

In vitro studies with human lung microvascular endothelial cells have shown that the endothelium contains hCG receptors and responds to this gonadotrophin by releasing VEGF and increasing the amount of VEGFR-2 in the cell surface, suggesting that endothelial cells are involved in the pathogenesis of OHSS as VEGF producers (Albert *et al*., 2002). Figs 3 and 4 summarize the phases of VEGF expression and increased VP.

In the ovaries of women treated with gonadotrophins, the presence of VEGF in endothelial cells might be explained by the production of VEGF by these cells, or as the result of a rapid release of VEGF from granulosa-lutein cells into the vessels. Another question that requires clarification is whether only the vessels of the ovary or the entire vascular tree participate in the mechanisms leading to OHSS. Other endothelial cells in the body might also be a target for VEGF, and this could explain why accumulation of protein-rich fluid is observed not only in the abdominal cavity but also as a general circulatory disturbance in some cases (Manau *et al*., 1998, 2002a).

In spite of occasional systemic disturbances, a strong body of evidence attributes the principal pathophysiological events of typical OHSS to the gonads. It has been demonstrated that the ovary is the main source of VEGF and other cytokines produced during hyperstimulation (Rizk *et al*., 1997; Schenker, 1999), and that increased capillary permeability and ascites are phenomena predominantly related to the ovaries (Blumenfeld *et al*., 1997). Furthermore, parameters of ovarian activity during stimulation (E_2 levels and number of oocytes retrieved) correlate closely with VEGF gene expression (Doldi *et al*., 1997). Finally, patients who become pregnant after oocyte donation do not develop OHSS, despite showing high levels of free VEGF (Pau *et al*., 2006).

It is surprising that, among women who display high parameters of ovarian response and who should, therefore, run the same risk of OHSS, only some develop the syndrome. This discrepancy may be related to soluble proteins that bind to VEGF. The VEGF soluble receptor sVEGFR-1 is reported to act as a modulator of VEGF bioactivity (Horning *et al*., 1999). The soluble molecule competes with the full-length VEGFR to bind with VEGF and inhibit VP (Kendall and Thomas, 1993; Roeckle *et al*., 1998). Another molecule, α_2-macroglobulin α2M, a major serum-binding protein associated with tissue remodelling during ovulation and corpus

luteum maintenance (Gaddy-Kurten *et al*., 1989), is also thought to determine the availability of free VEGF to bind to VEGFR-2 (McElhinney *et al*., 2002). High levels of these proteins may decrease free VEGF and protect against OHSS. High follicular fluid concentration of sVEGFR has been reported to be associated with poor ovarian response (Neulen *et al*., 2001), and high serum concentration of α2M (McElhinney *et al*., 2002) is thought to be related with a lower risk of developing OHSS.

This issue was recently reappraised. In order to evaluate the possible association between VEGF ligand-receptor interactions and the development of early and late OHSS, levels of VEGF and sVEGFR-1 receptors were measured in women who developed OHSS after ovarian stimulation for IVF and others who did not (Pau *et al*., 2006). During the luteal phase, hyperstimulated patients presented total and free VEGF levels significantly higher than those observed in women who had not undergone hyperstimulation, including those with a strong ovarian response (more than 20 oocytes retrieved). Women who did not develop OHSS, among whom both normal and strong responses to stimulation were observed, presented significantly higher plasma levels of the natural antagonist sVEGFR-1. In late-onset OHSS, a similar pattern was seen: hyperstimulated women had significantly higher amounts of free and bound VEGF and lower sVEGFR-1 during the first trimester of pregnancy (Pau *et al*., 2006).

Serum levels of α_2M have also been analysed in early and late-onset OHSS (Pau *et al*., 2006). No difference was observed between the α_2M levels of patients with and without early-onset OHSS. Serum levels of α_2M were even higher in women with late-onset OHSS during week 9 of pregnancy.

The ability of α_2M to bind and inactivate VEGF is well known (Soker *et al*., 1993; Bhattacharjee *et al*., 2000), but its relevance in OHSS is not confirmed. As already stressed, the investigation of angiogenic factors in women undergoing ovarian stimulation is complex (Molskness *et al*., 2004). The abundance of molecules that may be involved in the control of angiogenesis during the luteal phase and early pregnancy has been addressed only partially.

Downstream mechanisms of action

Very little is known about the steps through which the complex VEGF ligand-receptor increases VP. Endothelial cell-to-cell junctions and vascular endothelial (VE)-cadherin, an interendothelial adhesion molecule, have been tested as downstream targets of VEGF during OHSS pathogenesis, namely capillary hyperpermeability (Villasante *et al*., 2007). Cultures of human endothelial cells from umbilical veins (HUVEC) were treated with varying doses of E_2, hCG, VEGF and anti-human VEGF antibodies. Culturing HUVEC with high doses of E_2 produced no significant changes in VE-cadherin concentration, but hCG and VEGF individually produced a significant increase in VE-cadherin release. Anti-human VEGF antibodies prevented this increase in the release of VE-cadherin. Permeability assays demonstrated that, while E_2 did not alter the arrangement of HUVEC *in vitro*, hCG and VEGF caused changes in the actin fibres and in cellular shape (Fig. 5). These findings suggest an association with capillary permeability. Addition of an anti-human VEGF antibody to the culture medium inhibited the morphological changes induced by VEGF in endothelial cells. Finally, endothelial monolayers

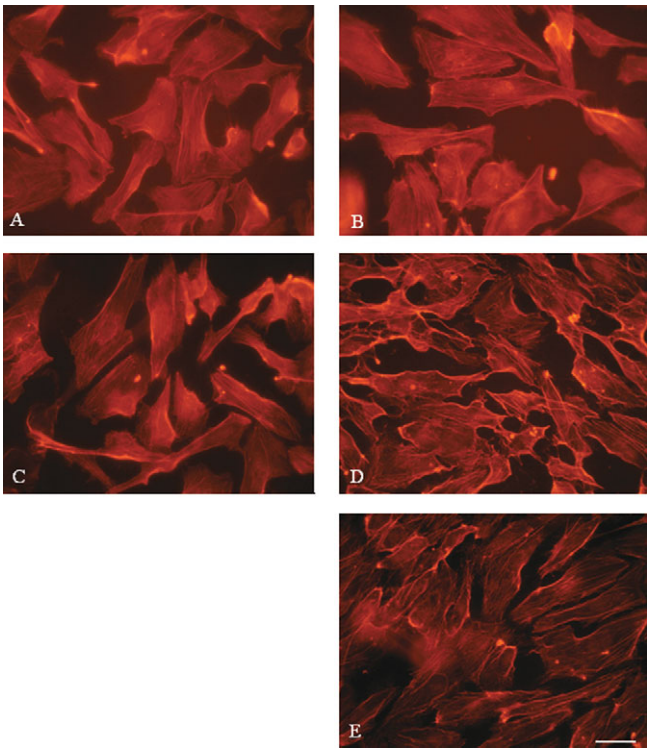

Figure 5: Morphological changes in the organization of actin cytoskeleton in human umbilical vein endothelial cells (HUVEC) after adding E2, hCG, VEGF and VEGF plus hCG for 24 h.
Cells are stained with TRITC-phalloidin and fluorescent microscopy is used for the analysis of actin filament organization. Control monolayer HUVEC in basal conditions is seen in (**A**). (**B**) shows the lack of effect of E2 added to the culture medium. In contrast, the addition of hCG induces contraction of the endothelial membrane (**C**). Addition of VEGF and VEGF + hCG (**D** and **E**) induces a considerable change in cellular shape due to rearrangement of actin filaments being irregularly aligned within the cells. Bar. 20 μm (adapted from Villasante *et al*., 2007). Copyright 2007, The Endocrine Society.

cultured in the presence of VEGF displayed significantly increased permeability, which may be due to rearrangement of endothelial junctional proteins such as VE-cadherin, as described elsewhere (Bates *et al*., 1999). Addition of anti-human VEGF antibody to the culture medium inhibited this effect.

Adhesion molecules like VE-cadherin seem to play a role in the development and progression of increased capillary permeability in severe OHSS. This may be clinically relevant, as preliminary observations in women who have developed OHSS while undergoing COH showed a 4-fold increase in VE-cadherin levels after hCG administration that remained elevated until OHSS resolved (Villasante *et al*., 2003). The fact that E_2 alone is unable to modify the release of VE-cadherin suggests that it is irrelevant to OHSS pathogenesis.

Prevention of OHSS—targeting VEGF

Prevention of VEGF overexpression

VEGF overexpression observed after hCG administration is related with this molecule's high biological activity, which is 6–7 times that observed when LH binds to the same receptor (Yen *et al*., 1968). This is the consequence of the longer half-life of

hCG and affinity for the common receptor (Yen *et al*., 1968). Although the LH dose necessary to trigger ovulation is not considered financially acceptable or clinically feasible, use of this hormone might avoid increasing OHSS-inducing VEGF expression.

In rats, the use of hCG, FSH and LH (the latter at a dose with either the same number of units as the hCG dose or six times the number of hCG IU) were equally effective in triggering the final process of oocyte maturation and ovulation after ovarian stimulation (Gómez *et al*., 2004). FSH and hCG, as well as a high dose of LH, exerted similar biological actions, including increased VP due to excessive VEGF expression. A low dose of LH resulted in significantly lower VEGF expression and VP than that seen in the other three groups. In this way, similar rates of ovulation were achieved, while undesired vascular changes were impeded.

In humans, contradictory results have been obtained with respect to the LH dose high enough to trigger ovulation but low enough to prevent VEGF overexpression. Initial multicentre studies showed that a single dose of 15 000 IU rLH (recombinant LH) was more efficient than one of 5000 IU rLH in achieving optimal oocyte maturation in IVF (The European Recombinant LH Study Group, 2001). Such findings suggest that the incidence of OHSS is lower when doses of 15 000–30 000 IU rLH are employed rather than hCG. A similar reduction in the incidence of OHSS was observed when a GnRH analogue was employed to induce an endogenous LH/FSH surge and oocyte maturation (Diedrich *et al*., 2001). It was also reported that the circulatory dysfunction frequently seen in women undergoing controlled ovarian stimulation for assisted reproduction was less intense when rLH was employed instead of hCG (Manau *et al*., 2002b). The publication in question also reported the same number of mature oocytes and similar implantation rates in embryos derived from women treated with 5000 IU hCG or rLH, suggesting that the dose of rLH necessary to trigger oocyte maturation and avoid OHSS is lower than initially expected. Native GnRH has also been used to trigger endogenous LH + FSH surge in ovulation induction cycles in which the risk of OHSS was considered to be high (Blumenfeld *et al*., 1994).

In animals, FSH clearly drives many events at mid-cycle, such as oocyte maturation (Pellicer *et al*., 1989), luteinization, corpus luteum formation and follicular rupture (Galway *et al*., 1990; Montgomery-Rice *et al*., 1993; Zelinski-Wooten *et al*., 1998). However, in humans, the concept that FSH might function in the same way as LH or hCG in mid-cycle events is far from orthodox. In fact, there are reports of a hereditary mutation in the FSH receptor, which allows the binding of hCG and the subsequent development of spontaneous OHSS in pregnant women (Vasseur *et al*., 2003; Montanelli *et al*., 2004a). This raises interesting questions about the limits of the specificity of functions of each gonadotrophin, and the mechanisms through which they are established.

Inhibition of VEGFR-2 phosphorylation

SU5416

SU5416(Z-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone) is a small synthetic tyrosine kinase molecule which inhibits angiogenesis in different cancers by preventing the initial VEGF-dependent VEGFR-2 phosphorylation and subsequent downstream signalling. SU5416 does not affect surface expression of the receptor or its affinity for VEGF. In rodents, injections of SU5416 every 48 h were shown to significantly inhibit the increase in VP induced by hCG after ovarian stimulation in the OHSS model (Gómez *et al*., 2002). Interestingly, if SU5416 injections are given daily during the ovarian stimulation protocol, but withdrawn when hCG is administered, their capacity to prevent OHSS is annulled (Gómez *et al*., 2002).

By blocking VP in hyperstimulated rats through inhibition of VEGFR-2 phosphorylation, the aforementioned study was the first to show a cause–effect relationship between increased VEGF expression and capillary permeability *in vivo*. However, due to its side effect profile (thromboembolism, vomiting) (Glade-Bender *et al*., 2003; Kuenen *et al*., 2003), and to the possibility of interference with early pregnancy development through its blocking of implantation-related ovarian (Wulff *et al*., 2001; Zimmermann *et al*., 2001a, b, 2003; Pauli *et al*., 2005) and uterine (Rockwell *et al*., 2002; Heryanto *et al*., 2003) angiogenesis, SU5416 cannot be used clinically to treat OHSS.

Dopamine agonists

Background information on the inhibition of VEGFR-2 signalling

Another approach to blocking downstream signalling of the VEGF ligand-receptor complex is the dopamine (Dp)/dopamine receptor 2 (Dp-r2) pathway, the activation of which is involved in the regulation of angiogenic events (Eljarmak *et al*., 1985; Basu and Dasgupta, 1997; Basu *et al*., 2004). Dopamine's binding to its receptor determines a dose-dependent inhibition of VEGFR-2 signalling (Gómez *et al*., 2006). This pathway has been explored in oncological treatments. Administration of high doses of dopamine agonists simultaneously blocks tumour-related angiogenesis and VP in a mouse cancer model by interfering with VEGF/VEGFR-2 signalling (Basu *et al*., 2001). *In vitro* studies have suggested that the molecular mechanism underlying this action involves the internalization of VEGFR-2, which is induced by the activation of the Dp-r2 (Basu *et al*., 2001).

A study of ovarian gene expression in OHSS produced interesting results regarding the relationship between VEGF and dopamine (Gómez *et al*., 2003b). Among 14 000 genes whose expression in hyperstimulated rats was studied using microarray technology, only eight were significantly down-regulated. One of these was the tyrosine hydroxylase (TH) gene (Table IV). TH is the enzyme responsible for dopamine synthesis. In this way, high VEGF expression and activity in OHSS seem to be associated with reduced dopamine production.

All this knowledge suggests that dopamine administration interferes with the VEGF effect observed in OHSS. Doses of dopamine agonists much lower than those used in the tumour model (Mueller *et al*., 1976) are sufficient to activate the Dp-r2 pathway, since they decrease prolactin secretion by the pituitary gland (Shelesnyak, 1955). Thus, low dose dopamine agonists are employed to treat hyperprolactinemia in humans (Mornex *et al*., 1978; Bigazzi *et al*., 1979; Robert *et al*., 1996; Ciccarelli *et al*., 1997; Liu and Tyrrell, 2001). Interestingly, these low doses do not produce any anti-angiogenic activity: states of high level VEGR-2-dependent vascular activity, such as corpus luteum physiology (Zimmermann *et al*., 2001a) or pregnancy development (Pauli *et al*., 2005), are

Table IV. Significantly[a] down-regulated genes in the ovaries of the OHSS rat model

Gene	Fold down-regulated
Gelatinase	13.24
Membrane bound C2 domain containing protein	11.37
ADP-ribosylation factor	10.39
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase	8.73
Carbonic anhydrase 3	7.71
Homer, neuronal immediate early gene	6.82
Tyrosine hydroxylase (TH)[b]	5.66
Inhibin alpha	4.32

[a]At least 3-fold down-regulated compared with baseline; [b]key enzyme in dopamine synthesis. A total of 83 up-regulated genes were identified in this model (Gómez *et al*., 2003b).

not affected (Mornex *et al*., 1978; Bigazzi *et al*., 1979; Robert *et al*., 1996; Ciccarelli *et al*., 1997; Liu and Tyrrell, 2001). The hypothesis that low doses of Dp-r2-activating drugs are capable of decreasing VP without affecting angiogenesis was tested in the context of OHSS in rodents and humans.

In the same established OHSS rat model previously described (Gómez *et al*., 2002), low dose dopamine agonist cabergoline (Cb2) reversed VEGFR-2 dependent VP without affecting luteal angiogenesis (Gómez *et al*., 2006) (Fig. 6), and no luteolytic effects were observed, as serum progesterone levels and luteal apoptosis were not altered. Cb2 administration did not affect VEGF/VEGFR-2 ovarian mRNA levels either. Densitometric analyses revealed that Cb2 administration decreased the general phosphorylation of VEGFR-2 by 42% with respect to controls. It is of note that a similar percentage of decreased total phosphorylation of VEGFR-2 was reported in a previous study of the effects of high doses of dopamine agonists in cultured endothelial cells (Basu *et al*., 2001). As previously mentioned, these authors suggested that high doses of dopamine agonists reduced the density of VEGFR-2 on the membrane of endothelial cells through a process of induced internalization. Their findings suggested that the receptor became inaccessible for VEGF, and that this led to a general inhibition of the VEGF/VEGFR-2 pathway, which resulted in decreased VP but also in angiogenesis. On the other hand, in the study with the rat model, it is unlikely that VEGFR-2 was internalized by low doses of Cb2. If this were the case, not only VP but also angiogenesis would have been blocked (Gómez *et al*., 2006). These data suggest that the effects of Cb2 on the reduced phosphorylation of one or several tyrosine sites other than that critical for the activation of VEGFR-2 are involved in the segregation of the VP and angiogenic components. The phosphorylation of the tyrosine sites in the transmembrane and C-terminal regions of the receptor are known to stimulate subsequent VEGFR-2 downstream signalling (Parast *et al*., 1998). Studies in Dp-r2 knockout models show that VEGFR-2 phosphorylation is increased in the absence of Dp-r2 inhibition, and is not reversed by the administration of dopamine agonists (Sarkar *et al*., 2004).

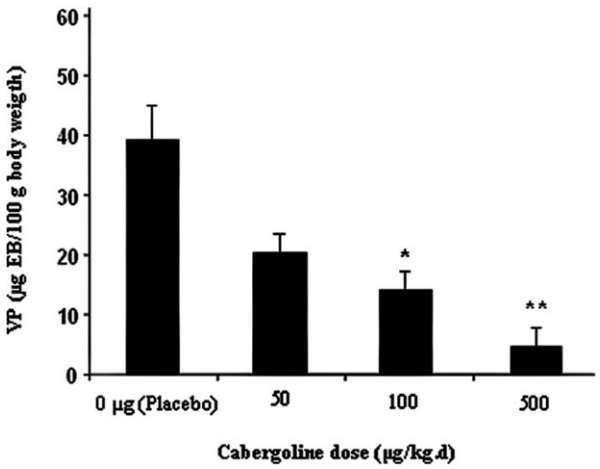

Figure 6: Cabergoline prevents vascular permeability (VP) in the hyperstimulated rat model
Vascular permeability (as micrograms of extravasated Evan Blue dye per 100 g animal weight) documented 48 h after hCG injection in OHSS rats supplemented with prolactin 5 mg and treated with cabergoline (Cb2) at 0 (control), 50, 100 and 500 µg/kg/day doses. A single Cb2 dose was given the day of hCG administration, whereas controls received glucosaline. The dose of 100 µg/kg/day was able do significantly reduce vascular permeability (VP) without affecting luteal angiogenesis. $*P < 0.05$, $**P < 0.005$, comparison against the control group. EB, evan blue (adapted from Gómez *et al*., 2006). Copyright 2006, The Endocrine Society.

Clinical studies for the prevention of OHSS

Results obtained with animal models, and the safe clinical profile of dopamine agonists, have led to studies with humans. Cb2 was administered to oocyte donors at high risk of developing OHSS (>25 pre-ovulatory follicles, E_2 >3000 pg/ml in serum) (Álvarez *et al*., 2007a). The dose used (5–10 µg/kg/day, 5–10 times lower than the 50 µg/kg/day used in rodents) is sufficient to block prolactin secretion, but does not interfere with ovarian function in humans (Vanrell and Balasch, 1983). Higher doses should be avoided, since they are thought to produce a risk of corpus luteum disruption (Bohnet *et al*., 1977), possibly by affecting luteal angiogenesis. The presence of Dp-r2 in human granulosa-luteal cells was confirmed by two different molecular methods. To our knowledge, this was the first report that described Dp-r2 in human ovarian cells. Results showed that prophylactic administration of Cb2 was associated with a significant reduction

Table V. Comparison of the incidence signs and symptoms of moderate and severe OHSS in patients treated with cabergoline and placebo (Álvarez *et al*., 2007)

	Cabergoline ($n = 35$)	Placebo ($n = 32$)	*P*-value
Haemoconcentration[a]	2	5	NS
Renal dysfunction[b]	0	0	–
Liver dysfunction[c]	2	2	NS
Thromboembolism	0	0	–
Ascites >9 cm^3 (%)	9 (25.7)	19 (59.4)	0.005
Moderate OHSS (%)	7 (20.0)	14 (43.8)	0.04
Severe OHSS (%)	4 (11.4)	6 (18.8)	NS

[a]haematocrit >45%; [b]creatinine >1.2 mg/dl; [c]aspartate transaminase or alanine transaminase >40 U/ml.

in the incidence of symptoms and signs of moderate/severe OHSS: more than 75% of women in the treatment group ($n = 63$) showed no symptoms, compared with 15% in the placebo group ($n = 57$) (Table V). Therefore, a specific treatment for OHSS is now available. Data about its efficacy and safety require corroboration, but the short-term use of dopamine agonists seems to represent no significant risk for patients. Very importantly, ovarian perfusion was studied by means of quantitative dynamic contrast enhanced magnetic resonance imaging. The result revealed increased ovarian VP in the placebo group after hCG administration, a finding that goes a long way towards explaining why withholding hCG prevents OHSS in at-risk women following gonadotrophin priming. In addition, the leading role of the ovarian vasculature in the genesis of ascites was confirmed by this report.

Prior to these studies (Gómez *et al*., 2006; Álvarez *et al*., 2007a), a number of reports suggested that dopamine/dopamine agonists had a positive effect in the prevention or treatment of OHSS. Improvements in urinary output and overall symptoms were reported in seven critically ill patients after they received an intravenous dopamine infusion (Ferraretti *et al*., 1992). Moreover, the administration of docarpamine (an oral dopamine prodrug) in 27 hospitalized patients gradually improved urinary output and ascites (Tsunoda *et al*., 2003). Cb2 was administered to 20 patients at risk of hyperstimulation on the evening after oocyte retrieval, and to 10 severely hyperstimulated hospitalized pregnant women (Manno *et al*., 2005). The authors reported the absence of OHSS in the group of at-risk patients and a prompt improvement in the hospitalized patients. However, a lack of appropriate control groups characterized all the above mentioned studies. Interestingly, the administration of Cb2 during ovarian stimulation to a group of women with polycystic ovarian syndrome and hyperprolactinemia was reported to reduce the incidence of OHSS (Papaleo *et al*., 2001). Furthermore, and somewhat intriguingly, ovarian enlargement and high E_2 levels associated with FSH-producing pituitary adenomas were reported to be successfully treated with Cb2 (Knoepfelmacher *et al*., 2006).

Concerning the safety of Cb2 use during infertility treatment, women at risk of OHSS that have received this drug have been reported to present fertilization, implantation and pregnancy rates similar to those of age-, embryo number- and quality-matched controls (Álvarez *et al*., 2007b). Ongoing and full-term pregnancies were also similar in each group, and no major perinatal problems were detected. Cb2 administration in early pregnancy does not seem to be harmful either: published studies have reported employing up to 7 mg per week, and the frequency of spontaneous and induced abortions and major congenital malformations is comparable with rates in the general population (Ricci *et al*., 2002).

Recently, the use of Cb2 and pergolide (another dopamine agonist) for the treatment of chronic conditions such as Parkinson's disease, hyperprolactinaemia and the restless leg syndrome has been consistently associated with an increased incidence of cardiac valve regurgitation (Schade *et al*., 2007; Zanettini *et al*., 2007). The real nature of this association needs to be fully comprehended. In any case, further research on other dopamine agonists that do not represent such risks is fundamental, and is already being carried out by our group.

Summary

There is now consensus that women whose ovaries have been primed with FSH/LH and subsequently exposed to hCG develop a clinical picture in which the key pathophysiological step is increased VP. Information gathered over the last decade has pointed to VEGF as being crucial to the development of OHSS syndrome. Studies in rodents and humans have shown that levels of ovarian VEGF and VEGFR-2 mRNA levels and VP are already increased by stimulation with gonadotrophins, which precedes hCG administration. The administration of hCG pushes all of these parameters to their maximum. A linear correlation is found between increased expression of VEGF/VEGFR-2 mRNAs and enhanced VP, with both peaking 48 h after injection of hCG.

Immunohistochemistry shows the presence of VEGF and VEGFR-2 proteins in the granulosa-lutein and endothelial cells of the entire corpus luteum. Prior to hCG administration, the vessels are the main target of the receptor antibody, but afterwards a strong staining is observed in the whole corpus luteum (blood vessels and granulosa-lutein cells.).

An extensive body of evidence confines the essential pathophysiological events of typical OHSS to the gonads. It has been demonstrated that the ovary is the main source of VEGF and other cytokines produced in hyperstimulation, and that increased capillary permeability and ascites are phenomena predominantly related to the ovaries.

Soluble proteins that bind to VEGF might exert a protective effect against OHSS by reducing the availability of free VEGF. High sVEGFR levels seem to reduce the risk of ovarian hyperresponse, whereas the role of α2M is less clear. The nature of the downstream mechanisms through which VEGF ligand-receptors alter VP is gradually becoming clearer. Adhesion molecules like VE-cadherin seem to play a role in the development and progression of increased capillary permeability in severe OHSS. *In vitro* studies show that hCG and VEGF alter VE-cadherin concentration in endothelial cell cultures and also determine changes in the position of actin fibres, cellular shape and capillary permeability. All these changes are prevented by anti-human VEGF antibodies. Furthermore, women undergoing COH who develop OHSS show a 4-fold increase in VE-cadherin levels after hCG administration, which continue to be elevated until OHSS resolves. The fact that E_2 alone is unable to modify the release of VE-cadherin suggests that it is irrelevant to the pathogenesis of OHSS.

Some approaches to preventing OHSS, which are based on its pathophysiology, are now applied. Studies show a reduced incidence of OHSS when rLH or a GnRH analogue is used to trigger the final steps of oocyte maturation. Prophylactic administration of Cb2, a dopamine agonist, is associated with a significant reduction in the incidence of symptoms and signs of moderate/severe OHSS. This drug inhibits VEGFR-2 phosphorylation and signalling. Its use is not associated with an inferior IVF outcome or obstetric/neonatal complications. A specific treatment is, therefore, available. Larger trials are necessary for confirming its efficacy and safety.

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