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Involvement of the ANGPTs/Tie-2 system in ovarian hyperstimulation syndrome (OHSS)

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ABSTRACT

Ovarian hyperstimulation syndrome (OHSS) is a disorder associated with ovarian stimulation. OHSS features are ovarian enlargement with fluid shifting to the third space. Disturbances in the vasculature are considered the main changes that lead to OHSS. Our aim was to analyze the levels of angiopoietins 1 and 2 (ANGPT1 and 2) and their soluble and membrane receptors (s/mTie-2) in follicular fluid (FF) and in granulosa-lutein cells culture (GLCs) from women at risk of developing OHSS. We also evaluated the effect of ANGPT1 on endothelial cell migration. In ovaries from an OHSS rat model, we analyzed the protein concentration of ANGPTs, their mTie-2 receptor, and platelet-derived growth factor PDGF-B, -D and PDGFR-β. ANGPT1 levels were increased in both FF and GLCs from women at risk of OHSS. Incubation of these FF with an ANGPT1 neutralizing antibody decreased endothelial cell migration. In the ovaries of OHSS rat model, mTie-2 protein levels increased and PDGF-B and -D decreased. In summary, these results suggest that ANGPT1 could be another mediator in the development of OHSS.

1. Introduction

Angiogenesis is a rare event under physiological conditions in adults. In the female reproductive tract, it is restricted to follicular development, corpus luteum formation, and uterine endometrial proliferation during the menstrual cycle (Findlay, 1986; Reynolds and Redmer, 1992).

Numerous inducers of angiogenesis have been identified. These include the members of the FGF-2 (Basic Fibroblast Growth Factor) and Vascular Endothelial Growth Factor A (VEGFA) families, angiopoietins (ANGPTs), transforming growth factors (TGFs) and the platelet-derived growth factor (PDGF) (Carmeliet, 2000; Hanahan et al., 1996; Neufeld et al., 1999; Otrock et al., 2007). Although VEGFA is the main initiator of angiogenesis, the formation and differentiation of a structurally and functionally mature vascular network probably requires the coordinated action of various factors. These include ANGPT1 and ANGPT2, which act via the tyrosine kinase receptor Tie-2 (Maisonpierre et al., 1997). Unlike VEGFA, ANGPT1 is unable to stimulate endothelial cell proliferation (Davis et al., 1996), but is required for the stabilization of newly developed capillaries (Maisonpierre et al., 1997; Suri et al., 1996). ANGPT1 is able to induce the phosphorylation of Tie-2, which subsequently transduces a biological effect. ANGPT2 binds to Tie-2 with the same affinity as ANPGT1, but does not phosphorylate the receptor, thus acting as a natural antagonist of ANGPT1 (Maisonpierre et al., 1997). Previous studies have demonstrated expression of Vegfa, Angpt1 and Angpt2 mRNAs in the ovary of rats (Koos, 1995; Maisonpierre et al., 1997; Phillips et al., 1990), cows (Goede et al., 1998) and monkeys (Hazzard et al., 1999), suggesting a role of these factors in ovarian angiogenesis. In our laboratory, we have shown protein expression and cellular localization of ANGPT1, ANGPT2 and their receptor Tie-2, as well as of VEGFA and its receptor KDR, during folliculogenesis in the rat ovary (Abramovich et al., 2009).

Another potent angiogenic factor is PDGF, which recruits pericytes and muscle cells to stabilize the blood vessels (Heldin and Westermark, 1999). In particular, expression of PDGF family members has been identified in mouse, rat, and human ovaries (Sleer and Taylor, 2007; Yoon et al., 2006). These factors exert their effects through binding and subsequent activation of two structurally related tyrosine kinase receptors: PDGF receptor alpha (PDGFRα) and PDGF receptor beta (PDGFRβ) (Heldin et al., 1998). PDGFA, PDGFB, and PDGFC bind to PDGFRα, while PDGFB and PDGFD bind to PDGFRβ (Fredriksson et al., 2004).

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In our laboratory, we have demonstrated that inhibition of VEGFA or ANGPT1 by intrabursal administration of Trap (VEGF inhibitor) or ANGPT1 antibody causes an imbalance in the ratio of antiapoptotic:proapoptotic proteins, which leads a larger number of follicles to atresia in the rat ovary (Abramovich et al., 2006; Parborell et al., 2008). In addition, we have recently observed that a selective inhibitor of PDGF receptor (AG1295) increases the number of atretic follicles from eCG-treated prepubertal rats. In this same model, the treatment with AG1295 causes the presence of hemorrhagic follicles (unpublished results). This is of special interest because defects in ovarian angiogenesis contribute to a variety of disorders, including anovulation and infertility, pregnancy loss, polycystic ovary syndrome (PCOS), ovarian hyperstimulation syndrome (OHSS) and ovarian neoplasm (Geva and Jaffe, 2000; Neulen et al., 1995).

OHSS is an iatrogenic complication associated with ovarian stimulation for the treatment of infertility (Budev et al., 2005; Rizk et al., 1997). OHSS occurs in 5–10% of patients undergoing ovulation induction therapy, and the severe form takes place in 0.5–5.0% (Aboulghar and Mansour, 2003; Delvigne and Rozenberg, 2002). It is widely accepted that the main clinical components of this syndrome are marked enlargement of the ovaries, which contain luteal cysts and hemorrhagic cysts along with the shifting of fluid to the third space, including the peritoneal cavity (Golan et al., 1989).

Several studies have shown that VEGFA is an important candidate as a mediator of OHSS (Agrawal et al., 1999; Lee et al., 1997; McClure et al., 1994; Neulen et al., 1995; Pellicer et al., 1999). VEGFA concentrations in serum, peritoneal fluid and follicular fluid of patients at risk of OHSS are positively related to the development of the syndrome (Agrawal et al., 1999). Nevertheless, no report has extensively studied the protein levels of ANGPT1, ANGPT2 and their receptor Tie-2 in patients at risk of development OHSS or in a rat OHSS model. There is only one report showing that the levels of ANGPT-2 increase significantly in the serum from infertile women (controlled ovarian stimulation, COS) by day 30–40 after hCG administration. Besides, the levels of soluble Tie-2 (sTie-2) do not change during follicular stimulation or luteal phase (Molskness et al., 2006). In another study from the same laboratory, Hurliman et al. (2010) noted that both ANGPT1 and ANGPT2 are present in macaque serum and human serum from women during COS protocols (Hurliman et al., 2010).

Therefore, the aim of the present study was to investigate the levels of ANGPT1, ANGPT2 and soluble Tie-2, in follicular fluid (FF) from women at risk of developing OHSS. In addition, we analyzed the expression of ANGPT1, 2 and membrane Tie-2 (mTie-2) in cultured granulosa lutein cells from these patients. We also evaluated the effect of ANGPT1 neutralizing antibody on endothelial cell migration in the presence of FF from these patients. Furthermore, we examined the levels of ANGPT1, ANGPT2 and mTie-2, as well as of PDGF-B, -D and its receptor PDGFRβ, in ovaries from an immature rat OHSS model stimulated by equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG).

2. Materials and methods

2.1. Subjects and collection of FF

This study was performed in 39 patients aged 25–41 years old undergoing Assisted Reproductive Technology ART at the Reproductive Medicine Center Pregna (Buenos Aires, Argentina). Written informed consent was given by all the patients before recruitment. The study was approved by the ethics committee of the Institute of Biology and Experimental Medicine (IByME, Buenos Aires, Argentina).

Patients with pelvic pathologies such as endometriosis, uterine fibroids or pelvic inflammatory disease were excluded from the study. The patients were classified into two groups: control group ($n = 20$) and OHSS group ($n = 19$). The criteria for considering a patient at risk of developing OHSS were a serum E_2 level > 3000 pg/ml on the day of hCG administration and the retrieval of >20 oocytes.

When follicles reached 17 mm, hCG (5000 UI/ml Pregnyl®, Organon SA) was injected. Oocyte retrieval (OR) was conducted under vaginal ultrasound guidance 34 h later. During OR, human follicular fluid (FF) was extracted from 16 to 20 mm follicles. Follicular fluid from all follicles of each patient was collected. Only macroscopically clear fluids, indicating lack of contamination, were considered in the study. Samples of FF visibly contaminated with blood were excluded from the study.

The FF was centrifuged immediately for 10 min at 2000*g* to remove cellular components and debris and then transferred to sterile polypropylene tubes. The supernatant was stored at −80° C until assayed.

2.2. Measurement of ANGPT1 and soluble Tie-2

The levels of ANGPT-1 and sTie-2 in FF were analyzed with a commercially available ELISA kit (Quantikine; R&D Systems Inc., Minneapolis, MN). Intra-assay and inter-assay coefficients of variation were 11.5% and 18% for ANGPT-1 and 9% and 8% for sTie-2, respectively.

2.3. Human granulosa-lutein cells (GLCs) culture

Human granulosa-lutein cells (GLCs) were obtained by follicular aspiration from patients undergoing ART as described above. Briefly, follicular aspirates of each patient collected during oocyte retrieval were centrifuged at 400*g* for 10 min. After removing the supernatant, the layers of GLCs with the red blood cell pellet were resuspended in 1 ml of DMEM:F12 (1:1 vol/vol) containing 10 mM HEPES supplemented with fungizone (250 μg/ml) and gentamicin (10 mg/ml) (Life Technologies, Inc., Gaithersburg, MD) in a sterile 50-ml centrifuge tube and centrifuged at 180*g* for 5 min. The resuspended pellet was layered carefully on a Percoll cushion (density = 1.085, 3 ml percoll/ml cell suspension) (Sigma–Aldrich, MO, USA) in 15-ml sterile tubes and centrifuged at 600*g* for 30 min. Granulosa cells were aspirated from the interface and resuspended in 5 ml of lysis buffer (NH_4Cl 0.15 M; $KHCO_3$ 10 mM; Na_2EDTA 0.1 mM) for 5 min at room temperature to remove red blood cells, and then centrifuged at 180*g* for 5 min. The cell number was evaluated and cells were plated at a density of 3×10^5/well in 24-well dishes in culture medium supplemented with 10% FBS. Then, cells were washed and cultured in serum free medium supplemented with hCG (Endocorion, Ellea, 1 IU/ml) (Gruemmer et al., 2005). After 48 h, lysis buffer was added and protein extracts were stored at −20 °C until use in further experiments.

2.4. Migration assay

To assess the effect of ANGPT1 neutralizing antibody on endothelial cell migration in the presence of FF from patients at risk of developing OHSS, a wound healing assay using the EA.hy926 endothelial cell line was performed. This cell line was donated by Dr. Cora-Jean C. Edgell (University of North Carolina, USA). EA.hy926 cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, NY, USA/Sigma Aldrich) with 10% fetal bovine serum (FBS) in the presence of 100 U/ml penicillin G and 100 mg/ml streptomycin sulfate at 37 °C with 5% CO_2. For migration analysis, EA.hy926 cells were detached by trypsinization, resuspended in the same medium and plated at 3×10^5 cells per well of a 24-well plate and grown to confluence. The cell monolayers were wounded

by a 1000 μl micropipette tip in one direction. After the injury, the cell culture was washed with PBS to remove cellular debris. The wounded cells were incubated with FF (25%) (from both control patients and patients at risk of developing OHSS) preincubated 1 h with or without antibody against ANGPT-1. For immunoneutralization, anti-human ANGPT-1 antibody was added to FF at an antibody/angiogenic factor molar ratio of 1000:1 and incubated for 1 h at 37 °C (von Otte et al., 2006). As a negative control, serum-free DMEM/F12 was used. Cells were then incubated with FF for 15 h at 37 °C. Cell migration was monitored at initial wounding (t 0 h) and at 15 h (t 15 h) under a phase-contrast microscope. Pictures were acquired at the same magnification and location at the bottom of the dish. The result was calculated as percentage of cell migration (cell free area at t 0 h – cell free area at t 15 h) respect to negative control. Endothelial cell migration in negative control is arbitrary presented as 100%. Quantification was carried out using ImageJ software (National Institutes of Health, Bethesda, MD).

2.5. Animals, stimulation protocol and experimental design

Committee of the Experimental Medicine and Biology Institute (IByME-CONICET) approved all procedures in this study in accordance with the standards of the National Institutes of Health, as described in the Guide for Care and Use of Laboratory Animals.

We used an animal model that develops OHSS in immature Sprague-Dawley rats (21–23 days, 60–80 g), as described by Kitajima et al. (2004, 2006).

The rats were randomly divided into two groups. The control group (n = 10) was injected with eCG (10 UI, 23rd day of life at 09:00 h), and with hCG 48 h later (10 UI, 25th day of life at 09:00 h). The OHSS group (n = 10) received excessive doses of eCG (50 UI/day) injected for 4 consecutive days (from the 21st to the 24th day of life, at 09:00 h), followed by hCG (25 UI, 25th day of life at 09:00 h).

Rats were killed by CO_2 asphyxiation 48 h after the hCG injection. The ovaries were removed, cleaned of adhering tissue in culture medium and used for western immunoblot assays.

2.6. Western blot

Ovaries from both the control and OHSS groups were removed and placed on ice. Briefly, the ovaries were resuspended in five volumes of lysis buffer (20 mM Tris–HCl pH 8, 137 mM NaCl, 1% Nonidet P-40 and 10% glycerol) supplemented with protease inhibitors (0.5 mM PMSF, 0.025 mMN-CBZ-l-phenylalanine chloromethyl ketone, 0.025 mMN-p-tosyl-lysine chloromethyl ketone and 0.025 mM l-1-tosylamide-2-phenyl–ethylchloromethyl ketone) and homogenized with an Ultra-Turrax (IKA Werk, Breisgau) homogenizer. Rat ovarian samples were centrifuged at 4 °C for 10 min at 10,000g and the resulting pellets were discarded. In addition, the FFs were centrifuged for 20 min at 14,000 rpm and the supernatant resuspended in lysis buffer. On the other hand, cultured granulosa cells obtained by follicular aspiration from patients at risk of developing OHSS and controls were lysed in the lysis buffer. The cell lysates were centrifuged at 10,000g for 10 min at 4 °C. We also performed a protein homogenate from an endothelial cell line (EA.hy926). Protein concentration was measured by the Bradford assay. After boiling for 5 min, 40 μg of protein was applied to a 12% SDS–polyacrylamide gel and electrophoresis was performed at 25 mA for 1.5 h. The resolved proteins were transferred for 2 h onto nitrocellulose membranes. The blot was preincubated in blocking buffer (5% nonfat milk, 0.05% Tween-20 in 20 mM TBS pH 8.0) for 1 h at room temperature and incubated overnight with appropriate primary antibodies (ANGPT-1 1/1000, ANGPT-2 1/1000, Tie-2 1/200, PDGF-B 1/100, PDGF-D 1/1000, PDGFR-β 1/1000, actin B 1/3000) (Santa Cruz Biotechnology, Inc.) in blocking buffer at 4 °C. The proper loading was evaluated by staining the membranes with Ponceau-S in the case of detection of secreted proteins in FF, and normalized to actin B in the case of detection of ovarian proteins of rats. The protein levels were analyzed by densitometry using Scion Image for Windows (Scion Corporation, Worman's Mill, CT). Optical density data are expressed as arbitrary units ± SEM.

2.7. Data analysis

Data are expressed as the mean ± SEM. In the case of animal studies, the results were obtained from at least four experiments, using 10 animals per group. Differences between groups were tested for significance using the independent samples t test for parametric variables. Statistical significance was defined as $p < 0.05$. For statistical analysis of data, we used the statistical program Prism v5.0.

3. Results

3.1. Levels of ANGPT1 and sTie-2 in follicular fluid from patients at risk of OHSS and non-OHSS patients

The mean of ANGPT1 levels in follicular fluid (FF) increased significantly in women who developed OHSS as compared to those who did not ($p < 0.05$) (Fig. 1A). In contrast, there were no significant differences between both groups with respect to sTie-2 levels (Fig. 1B).

The release of ANGPTs to FF was also measured by western blot. The results for ANGPT1 confirmed those obtained by ELISA ($p < 0.05$) and the levels of ANGPT2 were similar in both groups (Fig. 2A and B). Besides, there was a marked increase in the ANGPT1:ANGPT2 ratio in FF from patients at risk of OHSS compared to patients who did not develop OHSS (Fig. 2C).

3.2. ANGPT1 and 2 in granulosa-lutein cells from control patients and patients at risk of OHSS

To evaluate if granulosa-lutein cells (GLCs) from patients at risk of OHSS and non-OHSS patients were able to produce ANGPT1 and 2 in the presence of hCG, we performed GLC culture in presence of said hormone (1 IU/ml). Using western blot analysis, we observed that GLCs expressed higher levels of ANGPT1 in patients at high risk of OHSS compared to control patients (Fig. 3A). No significant change was observed in ANGPT2 levels in both groups of patients (Fig. 3B). Therefore, there was a marked increase in the ANGPT1:ANGPT2 ratio in GLCs from patients at risk of OHSS compared to patients who did not develop OHSS (Fig. 3C). In addition, we showed that GLCs do not possess the membrane receptor for ANGPTs, mTIE-2 (data not shown). As an internal control, we demonstrated that the antibodies recognize the phosphorylated and unphosphorylated Tie-2 receptor in endothelial cells.

3.3. Effect of ANGPT1 neutralizing antibody on endothelial cell migration in the presence of follicular fluid from patients at risk of OHSS

To analyze the specific effect of ANGPT1 on ovarian angiogenesis in patients at risk of OHSS, we decided to evaluate the effect of FF on endothelial cell migration in the presence of a neutralizing antibody against ANGPT1. For this purpose, a wound healing in vitro assay was applied on endothelial cell culture. Recolonization of wounded areas was evaluated after 15 h by measuring the width of the gap in the same areas. At 15 h, the size of the gap was measured, normalized to each native wound surface (t

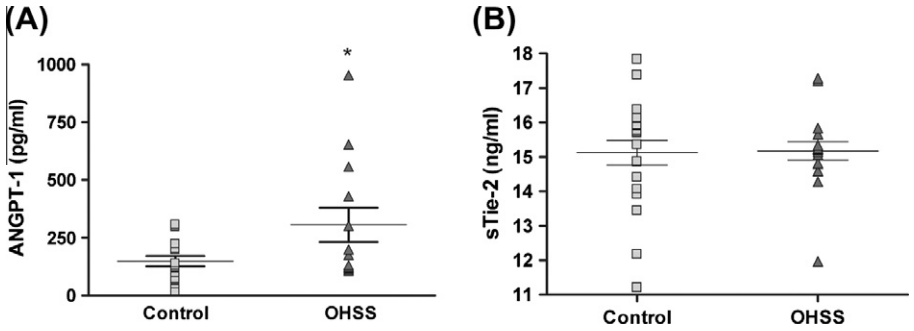

Fig. 1. Levels of ANGPT1 (A) and sTie-2 (B) in follicular fluid from patients at risk of OHSS and non-OHSS patients measured by ELISA. (A) Follicular fluid levels of ANGPT1 in control patients and patients at risk of developing OHSS. (B) Follicular fluid levels of soluble Tie-2. Data are expressed as mean ± SEM. (OHSS: $n = 19$, control: $n = 20$; $^{*}p < 0.05$.)

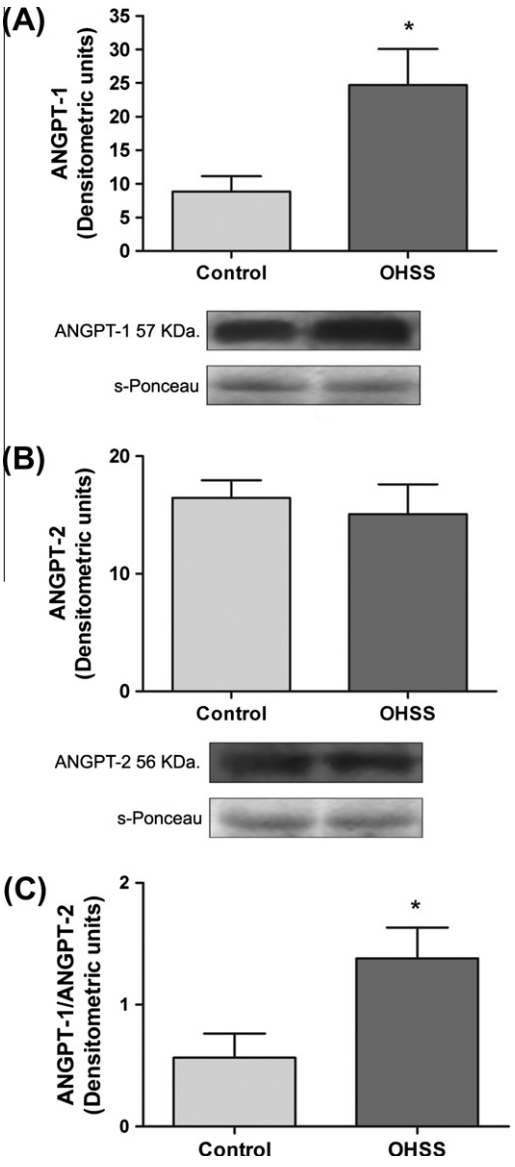

Fig. 2. Expression of ANGPTs in follicular fluid from OHSS and non-OHSS patients measured by western blot. (A) Densitometric quantification of ANGPT1 and (B) ANGPT2. (C) ANGPT1:ANGPT2 ratio. Data are expressed as arbitrary units ± SEM normalized to the invariable band in the membrane stain with Ponceau-S. Representative immunoblots of ANGPT1 and ANGPT2 content are shown in the lower panel. (OHSS: $n = 19$, control: $n = 20$; $^{*}p < 0.05$.)

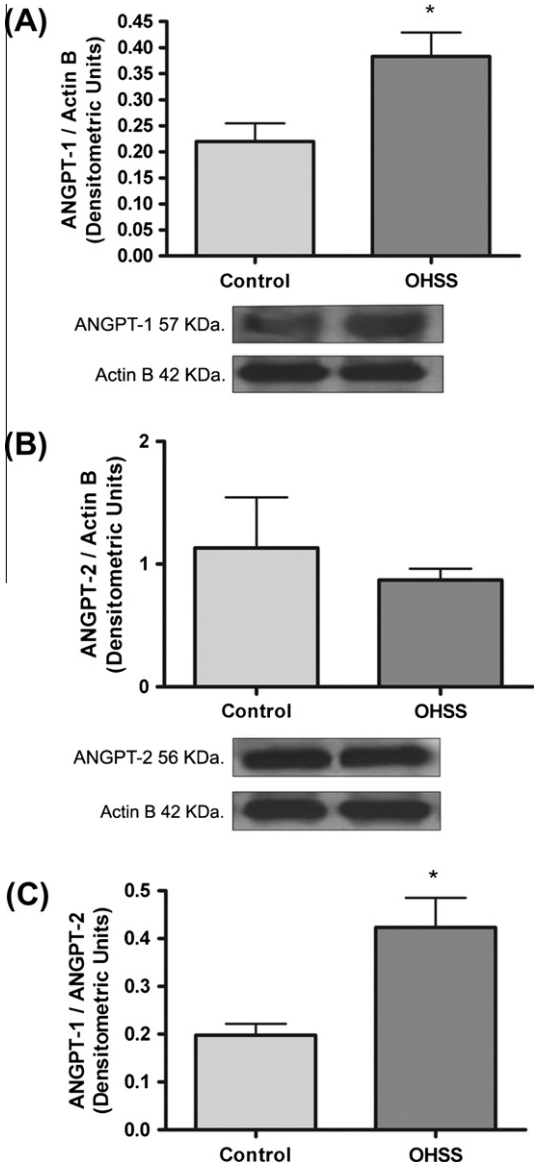

Fig. 3. Protein levels of ANGPT1 and 2 in cultured granulosa-lutein cells (GLCs) from OHSS and non-OHSS patients. (A) Densitometric quantification of ANGPT1 and (B) ANGPT2 in cultured GLCs. (C) ANGPT1:ANGPT2 ratio. Human granulosa-lutein cells (GLCs) were obtained by follicular aspiration from patients undergoing ART as described in Section 2. GLCs were incubated with hCG (1 UI/ml) for 48 h. The relative expressions of ANGPT1 and 2 were measured by western blot. Representative immunoblots of ANGPT1 and ANGPT2 content are shown in the lower panel. Data are expressed as arbitrary units ± SEM normalized to actin B. (OHSS: $n = 12$, control: $n = 15$; $^{*}p < 0.05$.)

0 h) and data were averaged. We performed a FF concentration curve from control patients to determine the optimal concentration at which maximum cell migration was observed. The cell migration improved as the concentration of FF increased, showing a significant increase in cell migration at a concentration of 25% (data not shown). This dose was used for subsequent assays. After 15 h, FF from patients at risk of OHSS induced cell migration at a higher extent than in control patients (Fig 4). Incubation with FF from patients at risk of OHSS in the presence of an antibody against ANGPT1 resulted in a significant decrease in cell migration compared to FF from patients at risk of OHSS without the addition of the ANGPT1 antibody ($p < 0.05$). Besides, it is important to note that in the absence of the neutralizing antibody against ANGPT1, endothelial migration was significantly greater in the presence of FF from patients at high risk of OHSS than in the presence of FF from the control group ($p < 0.001$).

3.4. Expression of ANGPTs/Tie-2 system in ovaries from an OHSS model developed in rat

In order to study the ovarian protein content of the members of the ANGPTs family from the OHSS rat model, we decided to evaluate the protein levels of ANGPT1, ANGPT2 and their membrane receptor Tie-2, in the ovaries of this model. As illustrated in Fig. 5A, the results showed a tendency toward a higher concentration of ANGPT1 in ovaries from OHSS rats. In addition, the ANGPT2 mean concentration was unchanged in both experimental groups (Fig. 5B). However, ovaries from the OHSS rat model showed that the levels of mTie-2 were significantly higher than in the control group ($p < 0.05$) (Fig. 5C).

3.5. Expression of PDGF-B, -D and PDGFR-β in ovaries from the OHSS model developed in rats

Since the PDGF angiogenic system is one of the factors responsible for the recruitment of pericytes and muscle cells to stabilize the blood vessels, we decided to evaluate the protein levels of PFGF-B, -D and PDGFRβ in the ovaries from different groups. The ovarian content of these angiogenic factors was measured by Western blot (Fig. 6). In the OHSS group, the protein levels of PDGF-B and -D significantly decreased compared with the control group ($p < 0.05$) (Fig. 6A and B). However, there were no significant changes in the protein levels of PDGF-B and -D receptor, PDGFR-β, in either experimental group (Fig. 6C).

4. Discussions

The data presented here demonstrate for the first time that ANGPT1 levels in FF from women at risk of developing OHSS are higher than in those from women who are not at such risk.

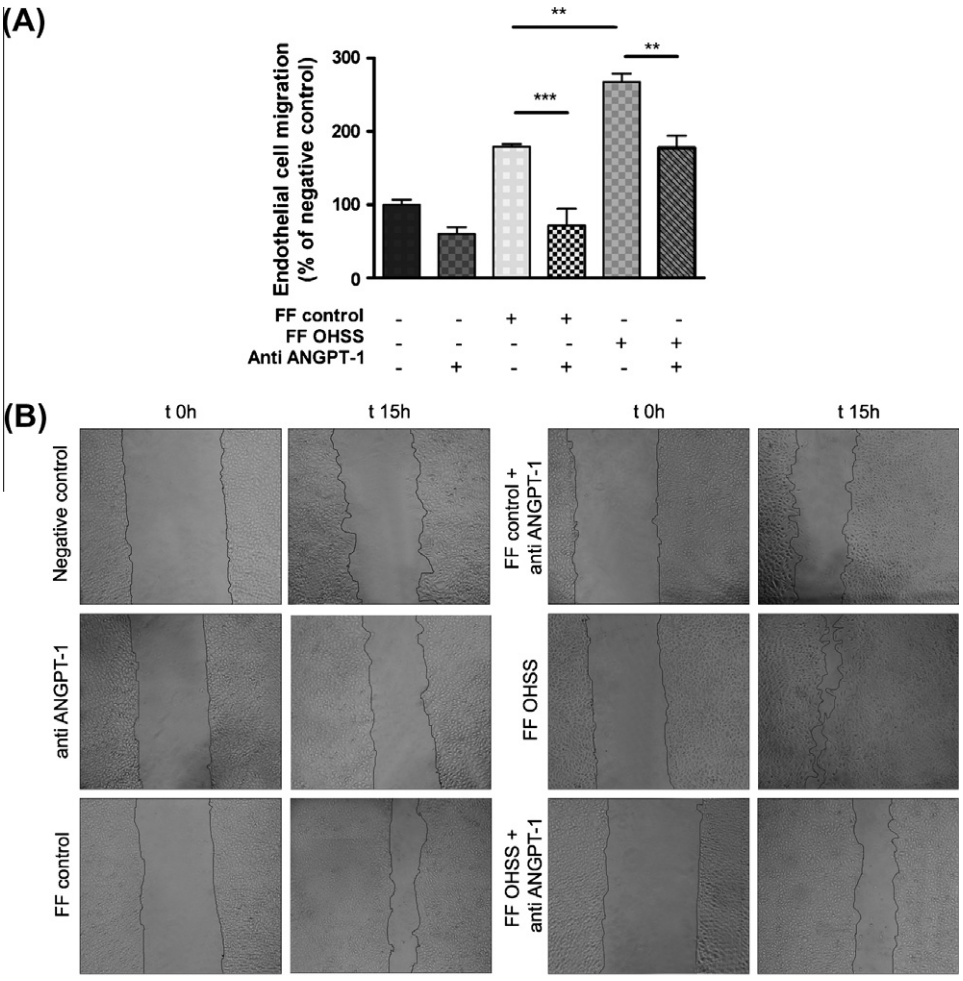

Fig. 4. Effect of follicular fluid on EA.hy926 endothelial cell migration in a wound-healing assay. (A) Quantification of the wound healing assay. The columns show the percentage of endothelial cell migration respect to negative control. Endothelial cell migration in the negative control is arbitrarily presented as 100%. Data are expressed as means ± SEM (OHSS: $n = 19$, control: $n = 20$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). Values represent the mean of three independent experiments. (B) Representative image of the induction of endothelial cell migration in a wound-healing assay by FF preincubated for 1 h at 37 °C with or without antibody against ANGPT-1. The molar ratio of antibody to ANGPT-1 was 1000:1. Images were taken immediately after scratching the cultures (t0) and 15 h later (t15). Original magnification 40×.

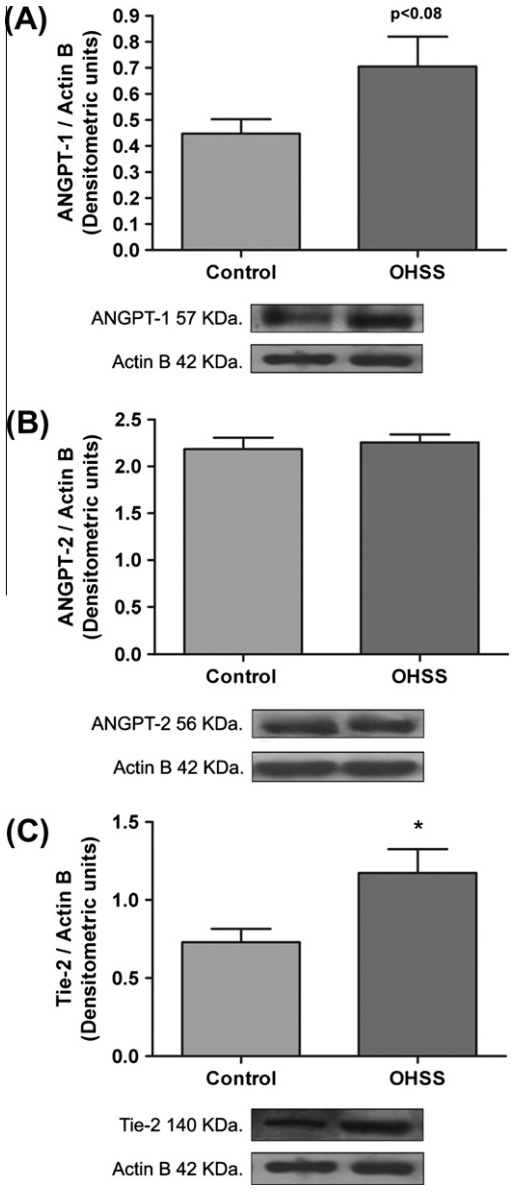

Fig. 5. Expression of the ANGPTs/Tie-2 system in the ovary in an OHSS model developed in rats. (A) Densitometric quantification of ANGPT1. (B) ANGPT2 and (C) mTie-2. Ovarian extract (40 μg of protein) was separated by commercial polyacrylamide gel and transferred to nitrocellulose membranes using an electroblotting apparatus. Optical density is expressed as arbitrary units ± SEM normalized to actin B ($n = 10$/group, $^{*}p < 0.05$). Representative immunoblots of ANGPT1, ANGPT2 and mTie-2 content are shown in the lower panel.

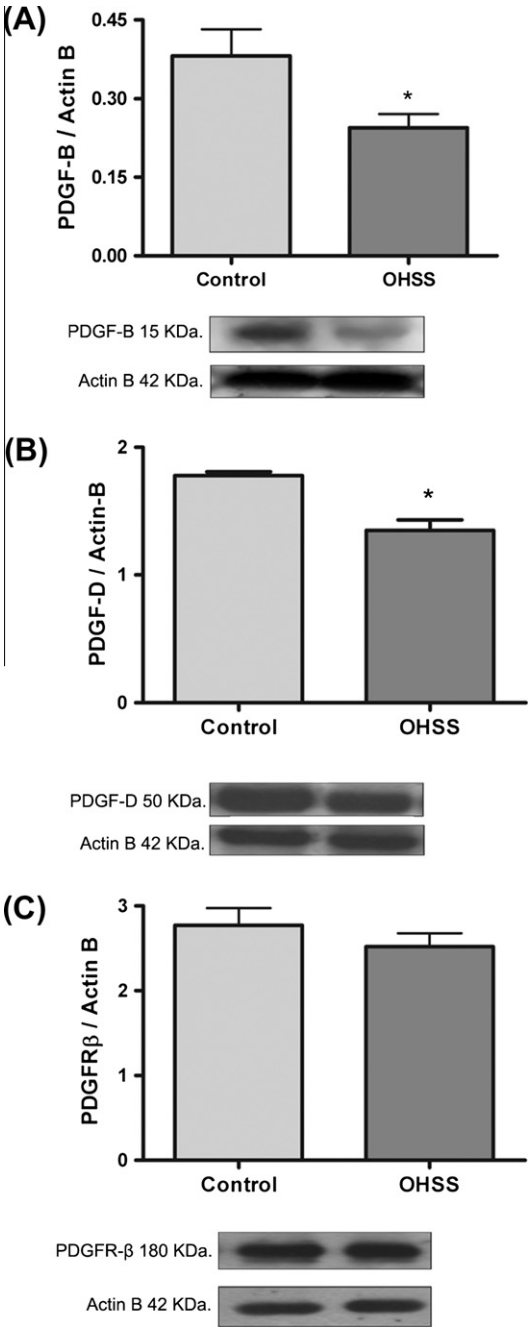

Fig. 6. Expression of PDGF-B, PDGF-D and PDGFR-β in the ovary in an OHSS model developed in rats. (A) Densitometric quantification of PDGF-B. (B) PDGF-D and (C) PDGFR-β. Ovarian extract (40 μg of protein) was separated by polyacrylamide gel and transferred to nitrocellulose membranes using an electroblotting apparatus. Optical density is expressed as arbitrary units ± SEM normalized to actin B ($n = 10$/group, $^{*}p < 0.05$; $^{**}p < 0.01$). Representative immunoblots of PDGF-B, PDGF-D and PDGFR-β content are shown in the lower panel.

However, the levels of soluble Tie-2 (sTie-2) did not change in FF from women at risk of developing OHSS. By western blot, we showed that ANGPT1 expression increased but ANGPT2 expression remained unchanged in cultured GLCs from patients at high risk of developing OHSS. Taking into account that we did not observe the presence of mTie-2 receptor in these cells, these results suggest that the ANGPT1 from granulosa lutein cells acts in a paracrine way on endothelial cells of the thecal compartment of the follicle and on the ovarian stroma. It is worth mentioning that we have previously shown, in rats, the presence of Tie-2 receptor in thecal and endothelial cells only. In contrast, this receptor was not detected in granulosa cells from different follicular stages (Abramovich et al., 2009). On the other hand, the soluble Tie-2 receptor is secreted by follicular and endothelial cells and acts as a receptor antagonist by sequestering free ANGPT1 (Molskness et al., 2006). These results suggest that there is a greater availability of ANGPT1 to membrane Tie-2 receptor, which would, in turn, increase patho-physiological angiogenesis in patients who are at risk of developing OHSS. In addition, Molskness et al. (2006) showed that serum ANGPT2 levels from patients at risk of OHSS did not change 36 h post-hCG administration but increased significantly by 30–40 days after hCG administration (Molskness et al., 2006). Serum levels of sTie-2 were unchanged during the process of follicular stimulation and the luteal phase. Our findings, together with the results described by others, suggest a strong role of ANGPT1 and ANGPT2 in ovarian physiological and pathological angiogenesis such as OHSS.

In this study, we used an OHSS rat model to evaluate the possible involvement of the ANGPTs system. Previously, we demonstrated that in this OHSS model the ovarian weight, serum progesterone and estradiol levels, VEGF peritoneal concentration and protein expression of its main receptor, Flk-1, were higher than those in the control group (Scotti et al., 2011). It is important to note that the mature CL is highly vascularized, with 50–70% of the tissue comprised of periendothelial and endothelial cells (Redmer et al., 2001; Reynolds et al., 2000). ANGPTs act on vascular endothelial cells and contribute to blood vessel stabilization through the interaction with perivascular cells (Thurston et al., 2000). Based on these data, we decided to measure ANGPT1, ANGPT2 and mTie-2 protein levels in ovaries from an OHSS rat model. ANGPT2 levels were unchanged in this tissue from the OHSS group. Although it did not reach significant difference, we found a tendency toward higher levels of ANGPT1 in this group and a significant increase of mTie-2 protein concentration. The increase in the levels of the membrane receptor Tie-2 allows an increase in the ligand binding and a higher activation of the intracellular signal, which in turn, favors angiogenesis in this pathology. Taken together these results show a greater biological activity of ANGPTs/Tie-2 system in this rat model of OHSS which is consistent with the results obtained in FF of women at high risk of developing OHSS.

Increased levels of VEGFA (Artini et al., 1998; McClure et al., 1994; Molskness et al., 2004) are proposed to be one of the principal etiologic factors in the vascular dysfunction associated with OHSS. The balance between the ANGPT1:ANGPT2 ratio and VEGFA expression is important for angiogenesis and blood vessel regression (Goede et al., 1998; Hazzard et al., 1999; Wulff et al., 2000). Here, we observed an increase in ANGPT1 levels without changes in ANGPT2 levels in FF from patients who presented symptoms of OHSS. It is worth noting that angiogenesis requires vascular endothelial cell proliferation and migration. ANGPT1 mediated endothelial cell migration involves Dok-R, Rho, GTPases and Rac families (Abdel-Malak et al., 2008; Cascone et al., 2003; Jones et al., 2003; Master et al., 2001). Regarding this point, we analyzed the specific effect of ANGPT1 on ovarian angiogenesis in patients at risk of OHSS. For this purpose, we evaluated the effect of FF on endothelial cell migration in the presence of an ANGPT1 neutralizing antibody. The results showed that FF from patients at risk of OHSS induced cell migration to a larger extent than that from control patients. However, the incubation of FF from patients at risk of OHSS in the presence of ANGPT1 antibody resulted in a decrease in cell migration compared to that without the antibody. These results are consistent with those obtained by Agrawal et al. (1999) concerning the excessive ovarian angiogenesis described in OHSS patients. These authors showed that within the ovarian stromal blood vessels, Doppler blood flow velocities are higher in women who develop OHSS than in those who do not (Agrawal et al., 1999). All these results lead us to believe that the increased levels of ANGPT1 observed in OHSS cause a lack of regulation in the balance of the vascular system. However, ANGPT1 is a factor that mediates the stabilization of the microvascular endothelium. This angiogenic factor is involved in vascular maturation, but requires the coordinated action of several factors to achieve it. The PDGFs system is critically important for the expansion of the pericytes population and also for pericyte migration along the growing vessel (Hellstrom et al., 1999). In addition, Uutela et al. (2004) showed that PDGF-D and PDGF-B improve the smooth muscle cells (SMCs) coating of blood vessels and decrease their permeability (Uutela et al., 2004).

Considering these data, the decrease in ovarian PDGF-B and -D protein levels observed in the OHSS rat model could be associated with the increased permeability described in the pathology of OHSS.

Besides, our results suggest that in OHSS pericytes are initially stabilized by ANGPT signaling, but possibly fail to expand and spread along the microvessels or/and to establish a stable coverage of endothelial cells, producing an increased permeability. Studies are currently underway in our laboratory to elucidate the precise role of PDGFs in the pathology of OHSS.

In summary, the present study shows that the increase in ANGPT1 concentration in the FF observed on the day of oocyte collection may be an important nonsteroidal marker of OHSS. In addition, the results described regarding endothelial cell migration in the presence of FF from patients at risk of OHSS with ANGPT1 neutralizing antibody may provide new insights into the mechanisms by which ANGPT1 has an effect on ovarian disorders such as OHSS. We also demonstrated that mTie-2 protein levels increase and PDGF-B and -D protein levels decrease while ANGPT2 and PDGFR-β levels remain constant in ovaries from an OHSS rat model.

A better understanding of the mechanisms of the factors involved in pathological angiogenesis in the ovary may lead to new strategies in the treatment of OHSS.

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