Angiopoietins/TIE2 System and VEGF Are Involved in Ovarian Function in a DHEA Rat Model of Polycystic Ovary Syndrome

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Polycystic ovary syndrome (PCOS) is the most common endocrinological pathology among women of reproductive age. It is characterized by anovulation, oligo- or amenorrhea, hyperandrogenism, obesity, and insulin resistance. PCOS patients present with elevated levels of vascular endothelial growth factor (VEGF) in serum and follicular fluid. In this study, we examined the ovarian expression of angiopoietins (ANGPT) and their receptor tyrosine kinase receptor (TIE2), involved in the stabilization of blood vessels, in a rat model of dehydroepiandrosterone-induced PCOS. We also analyzed the effect of ovarian VEGF inhibition on ANGPT/TIE2, follicular development, and vascular stability. VEGF levels were increased in the PCOS ovaries, whereas the levels of its receptor fetal liver kinase-1 were decreased. In addition, the periendothelial cell area and the ANGPT1 to ANGPT2 ratio in the ovary were increased in the PCOS group. Percentage of primary follicles was increased and the percentage of preantral follicles and corpora lutea was decreased in the PCOS group. VEGF inhibition decreased the percentage of primary follicles close to control values. Interestingly, despite the presence of cysts in the ovaries from VEGF inhibitor-treated PCOS rats, its percentage was lower than the PCOS group without treatment. In summary, this study describes an alteration not only in the VEGF/fetal liver kinase-1 system but also in the ANGPT/TIE2 system in a dehydroepiandrosterone-induced PCOS rat model. This leads to an increase in periendothelial cell recruitment. We also demonstrated that ovarian VEGF inhibition can partially restore the accumulation of small follicles in PCOS rats and reduces cyst formation, improving ovulation and follicular development. Therefore, the inhibition of VEGF could be considered, in addition to other currently applied treatments, as a new strategy to be studied in PCOS patients to restore ovarian function. (Endocrinology 153: 3446–3456, 2012)
Nonetheless, the development and differentiation of a structurally and functionally mature vascular network requires the coordinated action of a variety of factors. These include the angiopoietins (ANGPT)-1 and ANGPT2, which act via the tyrosine kinase receptor (TIE2) (5). ANGPT1 is necessary for the recruitment of perivascular cells that lead to the maturation and stabilization of newly developed capillaries (5, 6). Although ANGPT1 induces the phosphorylation of TIE2, which subsequently transduces a biological effect, ANGPT2 binds to TIE2 with the same affinity as ANPGT1 but does not phosphorylate the receptor. For this reason, ANGPT2 is considered a natural antagonist of ANGPT1 (5). Proliferation of vascular endothelial cells by ANGPT1 is controversial (7). This factor has been reported to induce ERK activation, the hallmark of proliferation (8). Cell migration is also controlled by Rho family GTPases, Rac and Rho. The activation of Rac1 by ANGPT1 is suggested to be involved in endothelial cell migration (9).

The expression of Angpt1 and Angpt2 mRNAs in the ovary has been demonstrated in different species (5, 10, 11), suggesting a role for these factors in ovarian angiogenesis. In a previous report, we have demonstrated that the expression of ANGPT1 and ANGPT2 proteins and their receptor TIE2 increases during follicular development and is mainly localized in theca cells of follicles in both immature, gonadotropin-stimulated and adult rats. TIE2 receptor immunostaining in granulosa cells is absent in all follicular stages (12). The coexpression of ANGPTs and TIE2 receptor in theca cells implies that the ANGPT system may exert both autocrine and paracrine actions. We have also demonstrated that ANGPT1 alters steroidogenesis, reduces ovarian apoptosis, and stimulates cell proliferation in rat antral follicles. These ovarian actions of ANGPT1 may be exerted by regulating ovarian vascular stability and/or by a direct effect on follicular cells (13).

Alterations of ovarian angiogenesis contribute to the pathophysiology of different ovarian conditions such as polycystic ovary syndrome (PCOS), ovarian hyperstimulation syndrome, uterine bleeding, subfertility, and endometriosis (14). PCOS is the most common endocrinological pathology among women of reproductive age, affecting more than 5% of the female population (15). It is characterized by chronic anovulation, oligo- or amenorrhea, and hyperandrogenism (16), and it is often associated with obesity and insulin resistance (17). Accordingly, PCOS has implications for both reproductive function and long-term health. Within the ovary, the stroma and inner theca hyperplasia leads to hypersecretion of androgens and the formation of multiple cysts (18). In addition, PCOS is characterized by abnormalities in angiogenesis.

A range of animal models, including rodents, sheep, and nonhuman primates, has been established to study the PCOS condition. However, at present, a whole animal model that mimics all features associated with human PCOS has not been established (19). In this study, dehydroyeandrosterone (DHEA) model was used because it exhibits the main features of human PCOS such as anovulation, absence of cyclicity, and alterations in steroidogenesis (19). Hence, this model has been widely applied to mimic human PCOS in rodents (20–25).

Follicular fluid (FF) levels and ovarian expression of VEGF are increased in PCOS patients (26, 27). This increase in ovarian VEGF production can be reflected in an increase in serum VEGF concentration in these patients (28–30). Furthermore, it has been demonstrated that ovarian stromal blood flow is also increased in women with polycystic ovaries, and it correlates with VEGF serum concentrations (28, 31). VEGF levels show a significant rise after human chorionic gonadotropin administration during ovarian hyperstimulation in PCOS patients, resulting in a higher risk of suffering ovarian hyperstimulation syndrome (28, 32). However, the reasons for the high ovarian VEGF levels are not known yet. Furthermore, no studies have been performed analyzing other members involved in the angiogenic process in PCOS. In the present study, we examined the ovarian expression of ANGPT and their receptor TIE2 in a rat model of DHEA-induced PCOS. In addition, we analyzed in this model the effect of VEGF inhibition on ANGPT system, follicular development, and vascular stability.

Materials and Methods

Animal treatment

Immature (21 d old) female Sprague Dawley rats were injected sc with DHEA (6 mg per 100 g body weight per 0.2 ml sesame oil), daily for 15 d to induce the hyperandrogenic PCOS condition. Control animals were injected with 0.2 ml of sesame oil (control group). For VEGF inhibition, on d 14 of DHEA treatment, PCOS animals were anesthetized with ketamine HCl (80 mg/kg; Holliday-Scott S.A., Buenos Aires, Argentina) and xylazine (4 mg/kg; König Laboratories, Buenos Aires, Argentina), and the ovaries were exteriorized through an incision made in the dorsal lumbar region. Then DHEA-treated rats were divided into two groups. One of them was injected under the bursa of both ovaries with 1 lg of VEGF inhibitor Trap (recombinant mouse soluble VEGF receptor 1/Fc Chimera; R&D Systems, Inc., Minneapolis, MN) in 5 ml of PBS with 0.1% BSA (PCOS + Trap group). The other group received vehicle under the bursa (PCOS group). Six rats per group were used in each experiment and each experiment was performed at least three times. After injection, the ovaries were replaced and the incisions closed with skin adhesive. The rats were killed by decapitation on d 16, according to our previous work (33). The ovaries were removed and
cleaned of adhering tissue in culture medium. One ovary was then frozen, and the other one was fixed in neutral-buffered formalin for subsequent assays. The experimental protocols were approved by the Animal Experimentation Committee of the Instituto de Biología y Medicina Experimental.

Ovarian morphology

To evaluate changes in general structure, the ovaries were removed and immediately fixed in 4% neutral-buffered formalin for 12 h and then embedded in paraffin. Five-micrometer step sections were mounted at 50-μm intervals onto microscope slides to prevent counting the same structure twice, according to the method described by Woodruff et al. (34). One set of slides was stained with hematoxylin-eosin (H/E) to count the number of different structures per ovary section, and the others were used for immunohistochemistry assays. Follicles were classified as primary (PrF) (presence of one cubic granulosa cell layer), preantral (PF) or early antral, according to the presence or absence of an antrum and corpora lutea (CL). Morphological characteristics of atretic follicles include the degeneration and detachment of the granulosa cell layer from the basement membrane, the presence of pyknotic nuclei in this cell type, and oocyte degeneration (35, 36). The cystic follicle was considered as a large follicle containing four or five plicated layers of granulosa cells surrounding a very large antrum (37, 38) or a large fluid-filled structure with an ing three ovarian sections from each ovary (three sections per ovary; five ovaries).

Immunohistochemistry

Tissue sections were deparaffinized in xylene and rehydrated by graduated ethanol washes. Endogenous peroxidase activity was blocked with hydrogen peroxide in PBS solution, and non-specific binding was blocked with 2% BSA overnight at 4 C. Sections were incubated with rabbit polyclonal anti-smooth muscle cell α-actin (Abcam 18147, 1:100; Abcam Inc., Cambridge, MA) or anti von Willebrand factor (Dako A0082, 1:300; Dako, Carpinteria, CA) overnight at 4 C. After washing, the slides were incubated with biotinylated antirabbit IgG and after 30 min with avidin-biotinylated horseradish peroxidase complex (Vectastain ABC system; Vector Laboratories, Burlingame, CA). Protein expression was visualized with diaminobenzidine staining. The reaction was stopped with distilled water, stained with hematoxylin, and dehydrated before mounting with mounting medium (Canada Balsam Synthetic; Biopack, Buenos Aires, Argentina). To perform this study, six randomly selected fields were analyzed from each ovarian section (six sections per ovary; six to eight ovaries per group).

Western blots

Ovaries were immediately frozen at −70 C until protein extraction. Ovaries were resuspended in 500 μl 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 phenylmethylsulfonyl fluoride, 0.025 mM N-CBZ-L-phenylalanine chloromethyl ketone, 0.025 mM N-p-tosyl-lysine chloromethyl ketone, and 0.025 mM l-1-lysylamide-2-phenyl-ethylchloromethyl ketone) and phosphatase inhibitors (25 mM sodium fluoride, 0.2 mM sodium orthovanadate, and 10 mM glycerophosphate) and homogenized with an Ultra-Turrax (IKA-Werke GmbH & Co., Staufen, Germany) homogenizer. Samples were centrifuged at 4 C for 10 min at 10,000 × g, and the resulting pellets were discarded. Protein concentration in the supernatant was measured by the Bradford assay. After boiling for 5 min, 40 μg protein was applied to a 12% sodium dodecyl sulfate-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 Tris-HCl; 100 mM NaCl, pH 8) for 1 h at room temperature and incubated with appropriate primary antibodies (ANGPT1, Abcam 8451, 1:500; ANGPT2, Abcam 65835, 1:500; TIE2, sc-9026, 1:200; FLK1, sc-6251, 1:500) in blocking buffer overnight at 4 C. Then it was incubated with antirabbit or antigoto secondary antibodies conjugated with horseradish peroxidase (1:1000) and finally detected by chemiluminescence and autoradiography using x-ray film. The density in each band was normalized to the density of the β-actin band that was used as an internal control.

Quantification for Western blot assay

For quantification, a screening was performed on blots with x-ray film using different times of exposure to optimize the signal. The levels of protein were compared and analyzed by densitometric studies using Scion Image for Windows (Scion, Frederick, MD). OD data are expressed as arbitrary units ± SEM (n = 8).

Enzyme-linked immunosorbent assay

Ovarian protein extracts were used to measure the levels of ovarian VEGF. For this purpose we used a commercially available ELISA kit (Quantikine rat VEGF kit; RRV00; R&D Systems) according to the manufacturer’s instructions. This kit is designed to measure rat VEGF 164. The intra- and interassay coefficients of variation for VEGF were 3.7 and 7.9%, respectively.

Radioimmunoassay

Progesterone ovarian content were examined by RIA after acetone extraction, as previously described (40). Briefly, each ovary was homogenized in acetone. Labeled steroids were added as internal standards, and the percentage of recovery was between 60 and 80%. Following centrifugation, the samples were evaporated to dryness. Distilled water was added, and samples were extracted twice with diethyl ether. The phase was transferred and evaporated to dryness and the remaining residue dissolved in methanol. After adding distilled water, the samples were submitted to a solvent partition. The upper layer was discarded and the lower phase evaporated. Finally, samples were
stored in distilled water for later analysis by RIA. Progesterone was measured using a specific antibody supplied by Dr. G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO). Under these conditions, the intra- and interassay coefficients of variation were 8.0 and 14.2%, respectively (n = 6).

Serum LH and FSH were determined by RIA using kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases. Results were expressed in terms of rat LH and FSH standards. Assay sensitivities were 0.015 ng/ml for LH and 0.1175 ng/ml for FSH. The intra- and interassay coefficients of variation for LH were 7.2 and 11.4%, respectively, and 8.0 and 13.2%, respectively, for FSH.

**Data analysis**

Data are expressed as the mean ± SEM. Representative gels and tissue sections are shown in the figures. Statistical analysis was performed using an unpaired Student t test or one-way ANOVA following Tukey or Newman-Keuls posttests. To analyze the contingency tables, a χ² test was performed. Values of P < 0.05 were considered significant.

**Results**

**Levels of VEGF/FLK1 system proteins**

To validate this model to study angiogenesis in PCOS, we measured the levels of VEGF in the protein extracts of ovaries of control rats and rats injected with DHEA for 15 d. Levels of VEGF were significantly higher in the PCOS ovaries compared with control ovaries (control: 40.0 ± 6.1 pg/mg protein; PCOS: 72.7 ± 13.3 pg/mg protein; P < 0.05, n = 5) (Fig. 1A). This result is in accordance with the increase in ovarian VEGF levels in PCOS patients, validating the DHEA model used in this study to determine changes in angiogenesis in PCOS.

Regarding the receptor FLK1, we analyzed the levels of this protein in the three experimental groups by Western blot technique. FLK1 was decreased in the ovaries of PCOS group compared with control ovaries. Trap treatment did not change the levels of this receptor (control: 0.49 ± 0.09; PCOS: 0.27 ± 0.06; PCOS + Trap: 0.16 ± 0.03; P < 0.05 between control and PCOS) (Fig. 1B).

**Ovarian blood vessel stability**

To evaluate changes in ovarian vascular stability, ovarian sections from rats of the different experimental groups were immunostained with smooth muscle cell alpha actin antibody (periendothelial cell marker), and the percentage of vascular area positively stained was quantified. The results showed that the percentage of periendothelial cells area was larger in the PCOS group than in the control group (control: 6.65 ± 0.28; PCOS: 9.17 ± 0.50; P < 0.05). Furthermore, the treatment with Trap significantly reduced the area of periendothelial cells in the ovary, even below the values observed in the control group (PCOS + Trap: 3.67 ± 1.01; P < 0.05 vs. control group) (Fig. 2A).

To rule out the possibility that the observed changes in periendothelial cell area are due to variations in the extent or size of the vascular blood vessels, we used an endothelial cell marker Von Willebrand. We evaluated the total endothelial cell area and the transversal area of each vessel. No differences were found in any of the parameters analyzed (Fig. 2B).

**Levels of ANGPT/TIE2 system proteins**

ANGPT1 recruits perivascular cells leading to the maturation and stabilization of newly developed capillaries. To take into account the differences observed in the area of these perivascular cells among the groups analyzed, we evaluated changes in the angiogenic system ANGPT/TIE2. Specifically, we measured ANGPT1, ANGPT2, and their receptor TIE2 by Western blot. Although ANGPT1 was increased in the ovary of the PCOS group (control: 0.63 ± 0.07; PCOS: 0.89 ± 0.06; P < 0.05), ANGPT2 was de-
increased in this group (control: 0.80 ± 0.07; PCOS: 0.47 ± 0.06; P < 0.05), leading to an increased ANGPT1 to ANGPT2 ratio, resulting in an increase in vessel stability. Trap treatment had no effect on this ANGPT1/ANGPT2 increase (Fig. 3, A–C). Regarding the TIE2 receptor, the levels in the PCOS ovaries were higher than those found in control group (control: 0.60 ± 0.09; PCOS: 1.17 ± 0.13; P < 0.001). Surprisingly, Trap treatment increased the levels of the TIE2 receptor to values even higher than in the PCOS group (PCOS: 1.17 ± 0.13; PCOS + Trap: 1.93 ± 0.24; P < 0.01) (Fig. 3D).

Follicular development
To characterize alterations in follicular development in rats injected for 15 d with DHEA and the effect of Trap treatment on these changes, we determined the percentage of each structure in H/E-stained histological sections from the different experimental groups (Fig. 4A). The percentage of PrF was increased in the PCOS group (control: 4.69 ± 1.17; PCOS: 8.84 ± 0.80; P < 0.05). Trap treat-

ment decreased this percentage close to control values (PCOS + Trap: 5.62 ± 0.61). The percentage of PF was lower in the PCOS rats compared with the control group. VEGF inhibition did not result in the restoration of control values in this follicular stage (control: 6.21 ± 0.80; PCOS: 4.04 ± 0.54; PCOS + Trap: 3.18 ± 0.27; P < 0.05). We did not detect differences in the percentage of early antral or atretic follicle in the groups analyzed. However, the percentage of CL were significantly decreased in both the PCOS and PCOS + Trap groups (control: 6.30 ± 0.91; PCOS: 0.33 ± 0.23; PCOS + Trap: 1.00 ± 0.48; P < 0.05) (Fig. 4B). Based on these results, we then compared the number of rats that presented CL vs. the number of rats that did not present these structures among the groups analyzed. The number of PCOS rats that presented CL was significantly lower than in the control group (control: 66.7%, six of nine; PCOS: 11.1%, one of nine; P < 0.05). Interestingly, the number of rats treated with Trap that presented CL was no different from control group (control: 66.7%, six of nine; PCOS + Trap: 44.4%, four of nine) (Fig. 5). Accordingly, we measured progesterone levels in each experimental group. As was expected, progesterone content was lower in the PCOS ovaries compared with the control group (control: 0.69 ± 0.07 μg/μg protein; PCOS: 0.27 ± 0.06 μg/μg protein; P < 0.01, n = 6). Although there were no significant differences in ovarian progesterone in the Trap-treated PCOS group compared with PCOS alone, a trend toward the recovery of control values was observed (PCOS: 0.27 ± 0.06 μg/μg protein; PCOS + Trap: 0.40 ± 0.07 μg/μg protein; n = 6).

Gonadotropin levels
LH and FSH were measured in rat serum obtained from animals belonging to the experimental groups. No differences were found in the levels of serum FSH among the groups analyzed (control: 1.8 ± 0.2 ng/ml; PCOS: 2.5 ± 0.2 ng/ml; PCOS + Trap: 2.2 ± 0.2 ng/ml; n = 15). LH was significantly decreased after DHEA administration (control: 4.5 ± 0.5 ng/ml; PCOS: 1.6 ± 0.1; n = 15; P < 0.01). Trap treatment did not reverse DHEA effect on LH levels (PCOS + Trap: 1.6 ± 0.2; n = 15).

Cyst formation
To determine the effect of VEGF inhibition on cyst development, we analyzed the percentage of cyst structures
in ovarian sections stained with H/E. As expected, ovaries from the PCOS group showed a high percentage of cysts, whereas these structures were absent in the control rats. Although there were cysts in the ovaries from VEGF inhibitor-treated PCOS rats, this percentage was significantly lower than PCOS group without treatment (PCOS: 4.81 ± 0.55; PCOS Trap: 3.16 ± 0.57; P < 0.05) (Fig. 4, B, d).

**Discussion**

Alterations in ovarian angiogenesis have been reported in PCOS patients. Specifically, an increase in ovarian VEGF synthesis, vascular blood flow, and VEGF serum levels has been extensively described in these women (26, 28, 30, 31). However, to our knowledge, there are no further studies describing other possible alterations in this complex process. The aim of the present study was to evaluate changes in other central angiogenic protein family, the ANGPT/TIE2 system in a DHEA rat model of PCOS. In addition, we evaluated the effect of local VEGF inhibition on ovarian follicular development, cyst formation, expression of ANGPT/TIE2 system, and vascular stability. We described for the first time an increase in ANGPT/TIE2 system in this PCOS rat model. Moreover, VEGF inhibition can partially restore follicular development and decrease cyst formation.

In this work, we showed an increase in the periendothelial cell area in a PCOS group compared with the control group. We also analyzed the total endothelial cell area and vessel size. However, no differences were found in these parameters. This is consistent with our previous study in which we demonstrated that the endothelial cell area in ovaries injected intrabursa with Trap was no different from controls (33). Taken together, these results suggest that differences observed in the periendothelial cell area among the groups analyzed are due to differences in periendothelial cell number attached to the vessels and not to differences in the amount or the size of the vessels present in the ovaries. It has been reported that, in some circumstances, VEGF is able to recruit pericytes and other periendothelial cells to the newly formed blood vessels (41, 42). However, there are other factors, such as the ANGPT/TIE2 system that specifically acts stabilizing the vasculature by allowing periendothelial cells to cover vessels.

To establish a relationship between the ANGPT/TIE2 system and the higher periendothelial cell recruitment of PCOS ovaries, we analyzed the expression of ANGPT1, ANGPT2, and TIE2 in this PCOS animal model. As expected, PCOS rats expressed higher ANGPT1 and its receptor TIE2 and lower ANGPT2 than control rats. These results suggest that this alteration leads to higher periendothelial cell recruitment and a trend toward vessel stabilization. To our knowledge this is the first study that shows an alteration in the ANGPT system in a rat model of PCOS. Brannian et al. (43) have demonstrated that prolonged in vivo exposure to the hypoglycemic agents thiazolidinediones decreases intraovarian expression of several genes that participate in vascular remodeling in an aging obese mouse model. Among these genes are angiopepin, angiopoietin-like 4, and resistin-like molecule-α. Our study is consistent with these findings, suggesting that the thiazolidinediones, a treatment commonly used to im-

**FIG. 3.** Levels of the ANGPT/TIE2 system proteins in control, PCOS, and Trap-treated PCOS rat ovaries. Histograms show the densitometric analysis for each protein. The density in each band was normalized to the density of the β-actin band. Lower panels show a representative blot for each protein analyzed. A, Densitometric quantification of ANGPT1 in the rat ovaries of the three groups analyzed. B, Densitometric quantification of ANGPT2 in the rat ovaries of the three groups analyzed. C, ANGPT1 to ANGPT2 ratio in the rat ovaries. D, Densitometric quantification of the receptor TIE2 in the rat ovaries of the three groups analyzed. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
prove PCOS symptoms in women, could be acting in the ovary, in part restoring the alterations in angiogenesis found in this study.

To further analyze alterations in angiogenesis in this PCOS rat model, we evaluated the levels of VEGF and its receptor FLK1 in the rat ovaries. The rat model used in this work mimics the increase in the levels of VEGF observed in patients, strengthening the use of these animals to study angiogenesis in PCOS ovaries. Surprisingly, PCOS rat ovaries expressed lower FLK1 levels than the control group. This can be due to a down-regulation of the membrane receptor of VEGF in the presence of excessive amounts of the ligand. There is controversy regarding serum levels of the soluble receptor fms-like tyrosine kinase 1 (also known as soluble VEGFR1) in PCOS patients. Because soluble VEGF receptor acts as a decoy receptor for this molecule, measurement of its levels can reflect VEGF bioactivity. The soluble receptor fms-like tyrosine kinase 1 has been measured in serum and FF from PCOS patients. Whereas some authors have found a decrease in this soluble receptor in both FF and serum (44), others have not observed any changes in the levels of this molecule in FF (45, 46). However, ovarian levels of the membrane VEGFR, FLK1 has been poorly studied in PCOS patients. Gomez et al. (46) showed that the levels of FLK1 in granulosa cells in culture from PCOS patients did not change compared with healthy women. In this study, we found a
It is known that DHEA-treated prepubertal rats present alterations in hormonal levels (21, 47–50). Unlike PCOS patients, we found in this study a decrease in LH levels in PCOS rats, whereas serum FSH levels remained unchanged. This decrease in LH levels could be due to a negative feedback of DHEA treatment on the rat hypothalamus-hypophysis axis. Whether these alterations lead to differences in ovarian structure and/or to the angiogenic factor changes observed in this work remains to be elucidated. Moreover, ovaries from control and PCOS rats are different in their cellular composition, making difficult the interpretation of protein concentration data obtained from whole ovary protein extracts. It is known that changes in ovarian morphology can lead to differences in the levels of angiogenic factors because the levels of these proteins secreted by each follicle depend on its follicular stage. However, it is worth noting that in a previous study, we showed that ANGPT1, ANGPT2, TIE2, VEGF, and FLK1 rise as the follicle develops (12). In this sense, the finding that ANGPT1, TIE2, and VEGF are higher, whereas ANGPT2 and FLK are lower in PCOS rats compared with control rats strengthens the fact that the alteration in angiogenesis observed in this work is not due to variability in follicular development.

Alterations in hormone levels and angiogenesis lead to defects in follicular development. To further characterize these changes, the ovarian percentage of each follicular stage, corpora lutea, and cysts were evaluated. In ovaries from PCOS rats, we found more primary follicles and less preantral follicles than in ovaries from control rats, without changes in early antral or atretic follicles. Moreover, PCOS ovaries show an expected increase in the percentage of cyst and a decrease in the percentage of periovulatory follicles. These results suggest that folliculogenesis is impaired in PCOS women, especially, the gonadotropin-independent transition from primary to preantral stages. Preantral follicles are increased in PCOS women, whereas in DHEA-treated rat ovaries, they are decreased. It is likely that preantral follicles in rats start to grow, although this development is abnormal. Follicles that start to develop tend to form cysts instead of growing normally to ovulate and form corpus luteum. It has been described that women with PCOS have more primordial, primary, and preantral follicles than normal women. Furthermore, the total follicle number is increased in the ovaries of these patients. Whether this increase is due to an alteration in circulating levels of FSH or a difference in sensitivity of the follicles within the ovary to gonadotropins remains unclear (51, 52).

It is worth noting that human PCOS ovaries are enlarged and present 12 or more follicles measuring 2–9 mm in diameter with an increased density and area of stroma. They also exhibit multiple arrested midantral follicles that lead to antrum expansion, increased granulosa cell degeneration, and the development of cystic follicles with thin granulosa cell walls (53–55). Unlike these cysts, DHEA rat ovaries present large follicles containing four or five plicated layers of granulosa cells surrounding a very large antrum or large fluid-filled structures with an attenuated granulosa cell layer and thickened theca interna cell layer (37, 38). Because of the differences between PCOS animal models and human syndrome, it would be important to extend these results to PCOS patients.

Inhibition of VEGF has emerged as a potential therapy method for cancers, and there are some compounds that are currently used in clinics (56–59). Consequently, the use of these inhibitors has spread to other disorders involving angiogenic alterations, including inflammatory diseases, retinopathies, and age-related macular degeneration, and in this sense, some clinical trials are now in progress (60–62). Considering that PCOS is a pathology characterized by an increase in ovarian VEGF levels and an altered angiogenesis, we attempted to analyze the effect of local VEGF inhibition on follicular development, cyst formation, and ANGPT/TIE2 proteins in our rat model of PCOS.

VEGF inhibition restored the primary follicles percentage without altering other follicular stages. Interestingly, the percentage of cysts decreased in Trap-treated ovaries compared with PCOS ovaries. Taken together, these results could imply a partial restoration of follicular development in PCOS rats when VEGF levels decrease, avoiding the accumulation of small follicles and the formation of cysts. Supporting this, the number of rats that presented corpora lutea in the ovaries, which reflect the ovulation rate in these groups, was significantly increased after VEGF inhibition in PCOS rats.

Angiogenesis is a complex process in which several factors interact to form new and functional vasculature. Given the increase in the levels of VEGF seen in these patients, inhibition of this factor could influence vascular development of the growing follicles and alter the levels of other angiogenic factors. In this context, we analyzed the ANGPT/TIE2 system after Trap treatment. Our results indicate that, in these conditions, inhibition of VEGF does not restore the levels of ANGPT/TIE2 system to control values. Specifically, inhibition of VEGF did not change the levels of ANGPT1 or ANGPT2. Interestingly, it increased the levels of the receptor TIE2 to values even higher than control group. This reflects the relationship that exists among angiogenic factors and the influence that an im-
balance in one of them can produce in the levels of the others. Given the increase in the periendothelial cell area seen in PCOS rats, we measured this area in PCOS rats treated with Trap. Interestingly, the area of these cells was even lower than control rats. Considering that ANGPT1 and ANGPT2 levels did not change after VEGF inhibition, this substantial decrease suggests that VEGF could be involved in the recruitment of periendothelial cells per se or by regulating other factors other than ANGPT proteins. Ball et al. (63) have described that VEGF can stimulate platelet-derived growth factor (PDGF) receptors in bone marrow-derived human adult mesenchymal stem cells. Furthermore, pdgfb and pdgfrb knockouts show a compensatory up-regulation of VEGF levels (64). Given the fact that PDGFB and PDGF receptor-β play a critical role in the recruitment of pericytes to the newly formed vessels (65, 66), this decrease in periendothelial cell coverage after VEGF inhibition might be reflecting a decrease in PDGF signaling. However, further studies are needed to elucidate the mechanism by which the inhibition of VEGF decreases pericyte recruitment.

In summary, this is the first study to provide evidence of an alteration in ANGPT1/TIE2 system in a PCOS rat model developed by DHEA injection. This alteration leads to an increase in periendothelial cell recruitment by ovarian vessels and a resulting higher stability of follicular vasculature. In addition, we have demonstrated that local inhibition of VEGF in rat ovaries can partially restore the accumulation of small follicles observed in PCOS rats and reduces cyst formation, improving ovulation and follicular development. Therefore, inhibition of VEGF could be considered, in addition to other currently applied treatments, as a new strategy to be studied in PCOS patients to restore ovarian function.

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53. Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop

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