Metformin regulates ovarian angiogenesis and follicular development in a female Polycystic Ovary Syndrome rat model.

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Polycystic Ovary Syndrome (PCOS) is a frequent pathology that affects more than 5% of women of reproductive age. Among other heterogeneous symptoms, PCOS is characterized by abnormalities in angiogenesis. Metformin has been introduced in the treatment of PCOS to manage insulin resistance and hyperglycemia. Besides its metabolic effects, metformin has been shown to improve ovulation, pregnancy and live birth rates in PCOS patients. In the present study, we used a dehydroepiandrosterone (DHEA)-induced PCOS rat model to analyze the effect of metformin administration on ovarian angiogenesis. We found that metformin was able to restore the increased levels of VEGF, ANGPT1 and ANGPT1/ANGPT2 ratio and the decreased levels of PDGFB and PDGFD observed in the DHEA-treated rats. These effects could take place, at least in part, through a decrease in the levels of serum insulin. We also found an improvement in follicular development, with a lower percentage of small follicles and cysts and a higher percentage of antral follicles and corpora lutea after metformin administration. The improvement in ovarian angiogenesis is likely to restore the accumulation of small follicles observed in PCOS rats and to reduce cyst formation, thus improving follicular development and the percentage of corpora lutea. These results open new insights into the study of metformin action not only in glucose metabolism but also in ovarian dysfunction in PCOS women.

Polycystic Ovary Syndrome (PCOS) is a frequent pathology that affects more than 5% of women of reproductive age. Its symptoms are heterogeneous and range from chronic anovulation, oligo- or amenorrhea and hyperandrogenism to obesity and insulin resistance (1, 2). In addition, PCOS is characterized by abnormalities in angiogenesis. The angiogenic process needs to be tightly regulated to accomplish the formation of competent vasculature. Several angiogenic factors, acting in a coordinated manner, are responsible for the correct formation of new blood vessels (3). The main angiogenic factor is the vascular endothelial growth factor (VEGF), which mediates endothelial cell migration and proliferation (3). Another family of angiogenic factors is the Angiopoietin family (ANGPTs), which is mainly involved in the regulation of vessel stability and permeability. This family comprises the agonist ANGPT1, the antagonist ANGPT2 and the receptor TEK (3). The Platelet-Derived Growth Factor (PDGF) family plays a critical role in the recruitment of pericytes to new vessels (4, 5). This family consists of five ligands, PDGFA, PDGFB, PDGFAB, PDGFC and PDGFD, and two receptors, PDGFRα and PDGFRB (6). Only PDGFRB and its ligands, PDGFB and PDGFD, are involved in vascular permeability. Beyond its role in angiogenesis, we and other authors have shown that PDGF pathway is required for theca cell development, follicular development and steroid production in the ovary (7, 8). Recently, we have found a decrease in the levels of both PDGFB and PDGFD in follicular fluids (FF) from PCOS patients (9). In a rat ovarian hyperstimulation syndrome

Abbreviations:

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.
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Received September 17, 2014. Accepted January 9, 2015.

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Endocrinology EARLY RELEASE: ENDOCRINE SOCIETY

(OHSS) model, we have demonstrated that ovarian levels of PDGFB and PDGFD are decreased, suggesting a role or these proteins in the pathogenesis of OHSS (10).

Ovarian angiogenesis is altered in different gynecological conditions such as PCOS, OHSS, uterine bleeding, subfertility, and endometriosis (3). In this regard, FF from PCOS patients have high levels of VEGF and ANGPT1 and low levels of PDGFB and PDGFD (9, 11, 12). It is known that PCOS patients have increased risk of developing OHSS after stimulation with gonadotropins for assisted reproductive techniques (ART). VEGF has been proposed as a main candidate in the pathophysiology of OHSS (13). In PCOS patients, VEGF levels show a significant increase after human Chorionic Gonadotropin (hCG) administration during ovarian hyperstimulation, which may be related to this higher risk of developing OHSS (14, 15).

Metformin (1,1-dimethylbiguanide hydrochloride) is an oral antihyperglycemic drug widely used for the treatment of type 2 diabetes. Its primary mechanism of action is through the activation of the AMP activated kinase (AMPK), which acts as an energy sensor by monitoring the AMP/ATP status of the cell (16, 17). Activation of AMPK produces the pleiotropic beneficial effects of this drug, such as the suppression of endogenous glucose production and the improvement in lipid metabolism (17). Metformin has been introduced in the treatment for PCOS to manage insulin resistance and hyperglycemia. However, the effects of this drug on PCOS are wide and exceed those related to glucose metabolism. Several studies have shown that metformin enhances lipid profiles, decreases body weight and regularizes menstrual cycles in PCOS patients (18–20). In addition, metformin has been shown to improve ovulation, pregnancy rates (21–25) and live birth rates (26, 27) in PCOS patients. However, the effect of metformin on fertility is still controversial. The available meta-analyses that have evaluated the effect of metformin on the ovary have shown different results. For this reason, in 2007, the ESHRE/ASRM group recommended the use of metformin in PCOS only in women with glucose intolerance and not for ovulation induction (28). Nevertheless, in the last years, new evidence has been found about the possible benefits of metformin in fertility treatment of PCOS patients, since it leads to an increase in ovulation and pregnancy rates (21, 23–25). Moreover, while some studies have found no differences in live birth rates in PCOS patients treated with metformin (23, 29), other studies have reported an increase in this parameter compared to untreated patients (25–27). These controversies could be due to the different criteria used for patient selection. However, the mechanisms by which metformin acts in the ovary to enhance fertility are still unclear. Better understanding these mechanisms would help to clarify the use of this drug in PCOS patients.

Metformin has an effect on the regulation of angiogenesis in different in vivo and in vitro models, being able to either stimulate (30–32) or hinder (33) neovascularization, mainly by regulating VEGF expression. In ovarian cancer, metformin has been proposed as an adjunctive therapy due to its antiangiogenic action and its ability to decrease VEGF expression (34–37). In murine models of either diabetes or obesity, where neovascularization is altered, metformin is able to decrease angiogenesis, thus preventing vascular dysfunctions (38, 39). In PCOS, Tan et al showed that metformin treatment increases trombospondin and omentin-1 serum expression in women (40, 41). Interestingly, metformin has been shown to decrease the risk of developing OHSS both in animal models and in ART patients (42, 43) as well as in PCOS patients (24, 26, 44). Whether this prevention is related to a decrease in angiogenic factor expression remains to be demonstrated.

Several animal models, including rodents, sheep and nonhuman primates, have been established to study PCOS. However, a whole animal model that mimics all features associated with human PCOS has not been yet established (45). The dehydroepiandrosterone (DHEA) rat model has been widely applied to mimic human PCOS in rodents (46–49). We have recently described that, besides the main features of human PCOS such as anovulation, absence of cyclicity, and alterations in steroidogenesis, this model presents increased ovarian VEGF and ANGPT1 levels (50). We hypothesized that metformin regulates ovarian angiogenesis and follicular development in the DHEA rat model of PCOS. Therefore, we aimed to study the effect of metformin treatment on follicular and vascular development, the levels of ovarian angiogenic factors and serum insulin concentration in a DHEA-induced PCOS rat model.

Materials and Methods

Animal treatment

Immature (21 days old) female Sprague Dawley rats were subcutaneously injected with DHEA (6 mg per 100 g body weight per 0.2 ml sesame oil), daily for 15 consecutive days to induce the hyperandrogenic PCOS condition. Control animals were injected with 0.2 ml of sesame oil (control group).

Then, DHEA-treated rats were divided into two experimental groups. One group received metformin (Sigma-Aldrich, D150959–5G) (300 mg/kg) orally dissolved in the drinking water daily for the 15 days of the DHEA treatment (51). A different group of control rats was treated with metformin for 15 days. The dose of metformin administered was equivalent to the dose administered to human patients (52). Throughout the treatment, animals were weighed, the water intake was measured and the

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amount of metformin was adjusted in fresh water daily to keep the metformin dose constant. Six rats per group were used in each experiment and each experiment was performed at least three times.

Rats were killed by CO_2 asphyxiation or decapitation on day 16, as previously (50). Serum was collected after blood centrifugation and stored until use. In addition, the ovaries were removed and cleaned of adhering tissue. One ovary was then frozen and the other was fixed in Bouin solution (Biopur Diagnostics) for subsequent assays. All protocols and experiments were approved by the Animal Experimentation Committee of the Instituto de Biología y Medicina Experimental (IByME-CONICET, Buenos Aires, Argentina) and conducted according to the guide for the care and use of laboratory animals of the National Institute of Health (USA).

Ovarian morphology

The ovaries were removed and immediately fixed in Bouin solution (Biopur, Argentina) for 12 hours 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 (53). 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 primordial (PrimordF) (presence of one squamous granulosa cell layer), primary (PrimF) (presence of one cubic granulosa cell layer), preantral (PAF) or early antral (EAF), according to the presence or absence of an antrum, preovulatory (POF) and corpora lutea (CL). Morphological characteristics of atretic follicles (AtF) 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 (54, 55). The cystic follicle was considered as a large follicle containing four or five plicated layers of granulosa cells surrounding a very large antrum (56, 57) or a large fluid-filled structure with an attenuated granulosa cell layer and thickened theca interna cell layer (57). The percentage of different structures was determined in four ovarian sections from each ovary; six ovaries per group.

Histochemistry and immunohistochemistry

Tissue sections were deparaffinized in xylene and rehydrated by graduated ethanol washes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS, and nonspecific binding was blocked with 2% BSA for 20 minutes. Sections were incubated with rabbit polyclonal antismooth muscle cell α-actin (Ab 18 147, 1:100; Abcam Inc., Cambridge, MA, USA) or biotinylated lectin BS-1 (from *Bandeiraea simplicifolia*, 20 μg/ml, 1:100) overnight at 4°C. After washing, the slides were incubated with biotinylated antirabbit IgG (except for lectin BS-1) and after 30 minutes with avidin-biotinylated horseradish peroxidase complex (Vectastain ABC system; Vector Laboratories, Burlingame, CA, USA). Protein expression was visualized with diaminobenzidine staining (Roche, catalog 11718096001). The reaction was stopped with distilled water, stained with hematoxylin, and dehydrated before mounting with mounting medium (Canada Balsam Synthetic; Biopack, Buenos Aires, Argentina).

The images were digitized using a camera (Nikon, Melville, NY, USA) mounted on a conventional light microscope (Nikon). Finally, the images were converted to TIFF format (bilevel scale) for their analysis. The percentages of the endothelial and periendothelial areas were processed using Image Pro® Plus 3.0 (Media Cybernetics, Silver Spring, MA, USA). Areas were determined in three ovarian sections from each ovary; six ovaries per group. Only follicles and stroma were counted in the ovarian sections to avoid errors due to the differences in the percentage of CL between groups. Stained zonae pellucidae in the lectin BS-1 histochemistry were not considered in the analysis. The microphotographs were analyzed by an observer blinded to the treatment type.

Western blots

Ovaries were immediately frozen at −80 C until protein extraction. Ovaries were resuspended in 400 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-tosylamide-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 minutes at 10 000 xg, and the resulting pellets were discarded. Protein concentration in the supernatant was measured by the Bradford assay. After boiling for 5 minutes, 30 μg protein was applied to an 8%-12% sodium dodecyl sulfate-polyacrylamide gel, and electrophoresis was performed at 150 V for 1.30 hours. The resolved proteins were transferred onto nitrocellulose membranes for 2 hours. The blot was preincubated in blocking buffer [5% nonfat milk, 0.05% Tween 20 in 20 mM TBS (4 mM Tris-HCl; 100mMNaCl, pH 8)] for 1 hour at room temperature and incubated with appropriate primary antibodies (ANGPT1, Abcam 8451, 1:500; ANGPT2, Abcam 65 835, 1:500; TIE2, sc-9026, 1:200; FLK1, sc-6251, 1:200; PDGFB, Abcam 16 829, 1:200; PDGFD, sc-30 196, 1:1000; PDGFRβ, sc-432, 1:3000; Actin B, sc-1616, 1:3000) in blocking buffer overnight at 4 C. Then, it was incubated with antirabbit or antimouse secondary antibodies conjugated with horseradish peroxidase (Sigma-Aldrich 4914; 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, which was used as an internal control.

Quantification by 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, USA). OD data are expressed as arbitrary units ± SEM.

Enzyme-linked immunosorbent assay (ELISA)

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; MN, USA) according to the manufacturer's instructions. This kit is designed to measure rat VEGF 164. The intra- and

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interassay coefficients of variation for VEGF were 3.7 and 7.9%, respectively.

Radioimmunoassay (RIA)

Serum insulin was measured by RIA using human insulin (Beta Laboratories, Argentina) for iodination and standard, and antibovine insulin antibody (Sigma I8510, St. Louis, MO, USA) as previously described (58). Rats were killed at noon, after 5 hours of light, a time at which food intake is negligible (59). The minimum detectable concentration was 0.02 ng/ml, and the intra-assay coefficient of variation was 6.8% (five animals per group).

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 post-test. Values of $P < .05$ were considered significant. Data were statistically analyzed using Prism v5.0.

Results

Follicular development

In a previous work, we characterized the alterations in follicular development in rats injected with DHEA for 15 days (50). In the present work, we analyzed the effect of metformin on the alterations in follicular development produced by DHEA. For this purpose, we determined the percentage of each structure in H&E-stained histological sections from the different experimental groups. The percentage of primordial follicles was not different between groups (Figure 1A). In contrast, the percentage of primary follicles was higher in the PCOS group than in the control group (control: 4.48 ± 1.15; PCOS: 9.55 ± 0.90; $P < .05$). When rats were treated with metformin, the percentage of primary follicles was reduced to values similar to those of the control group (PCOS+MET: 5.42 ± 2.14) (Figure 1B). In agreement with that previously described (50), preantral follicles were decreased in the PCOS group (control: 5.37 ± 0.66; PCOS: 3.18 ± 0.66; $P < .05$) and metformin did not change this decrease (PCOS+MET: 0.74 ± 0.33) (Figure 1C). Regarding early antral follicles, their percentage was not different between the control and PCOS groups. Interestingly, metformin significantly increased this percentage (control: 44.36 ± 2.12; PCOS: 46.71 ± 2.71; PCOS+MET: 62.52 ± 5.04; $P < .01$ vs control and PCOS groups) (Figure 1D). In contrast, there were no differences between the percentage of atretic follicles in the

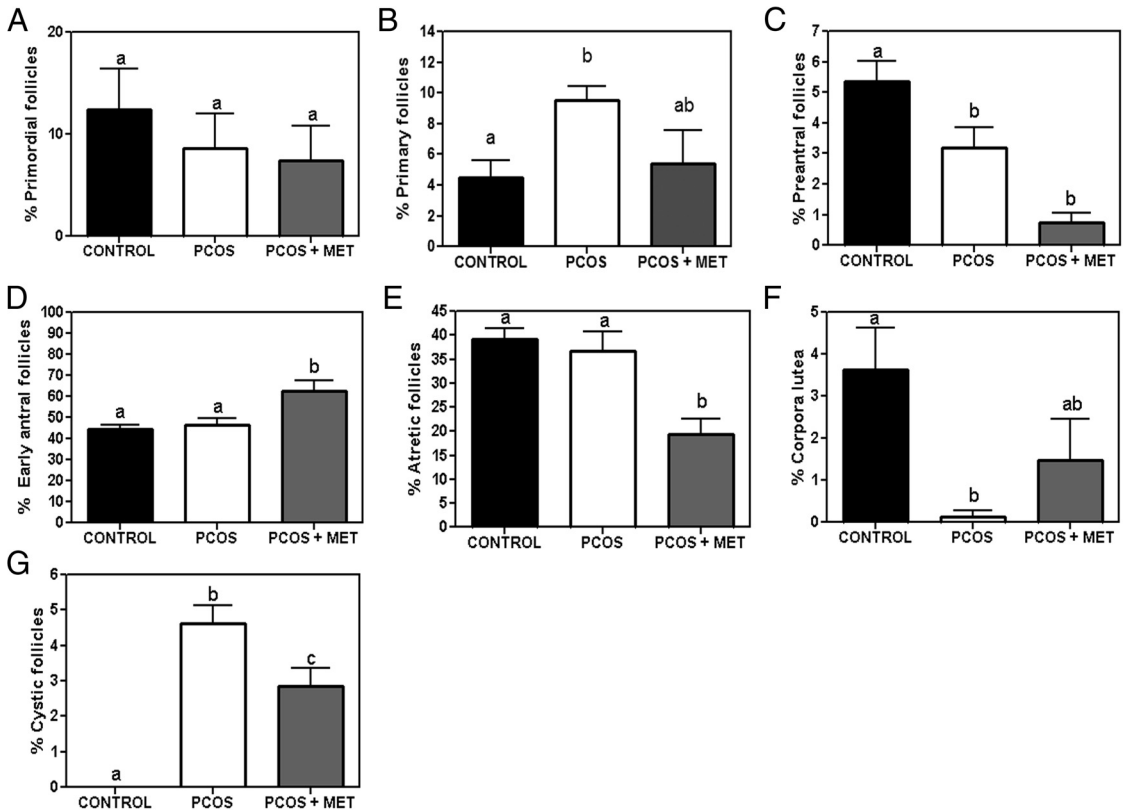

Figure 1. Follicular development in control, PCOS and metformin-treated PCOS rats. The graphs show the quantification of the percentage of each structure in ovarian sections stained with hematoxylin-eosin (H&E). A) % primordial follicles ($P > .05$). B) % primary follicles (a vs b $P < .05$). C) % preantral follicles (CONTROL vs PCOS $P < .05$; CONTROL vs PCOS+MET $P < .01$). D) % early antral follicles (a vs b $P < .01$). E) % atretic follicles (CONTROL vs PCOS+MET $P < .01$; PCOS vs PCOS+MET $P < .05$). F) % corpora lutea (a vs b $P < .01$). E) % cysts (a vs b $P < .001$; a vs c $P < .001$ b vs c $P < .05$). Different letters represent significant differences. Results were obtained from 3 experiments. Six rats per group were used in each experiment.

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control and the PCOS group. However, metformin was able to significantly decrease the percentage of these follicles (control: 39.14 ± 2.35; PCOS: 36.80 ± 3.97; PCOS+MET: 19.48 ± 3.06; $P < .05$ vs control and PCOS groups) (Figure 1E).

In order to analyze the effect of metformin on follicular development of control rats, we determined the percentage of each structure in a group of rats treated only with metformin. No differences were found between the control and the metformin-treated control in any of the structures analyzed (control PrimordF: 12.4 ± 4.0, control+MET PrimordF: 3.1 ± 3.1; control PrimF: 4.5 ± 1.1, control+MET PrimF: 4.5 ± 1.3; control PAF: 5.4 ± 0.7, control+MET PAF: 5.5 ± 0.8; control EAF: 44.4 ± 2.1, control+MET EAF: 47.7 ± 3.2; control AtF 39.1 ± 2.4, control+MET AtF: 40.3 ± 3.0; control CL: 3.6 ± 1.0, control+MET Pr: 4.0 ± 1.4).

Corpora lutea and cystic structures

To analyze the effect of metformin on ovulation and the formation of ovarian cysts after DHEA administration, we calculated the percentage of CL and cysts in ovarian sections from the different groups. As expected, the percentage of CL was lower in the PCOS group than in the control group. In the rats that received metformin during DHEA treatment, the percentage of CL in the ovaries increased to values similar to those of the control group (control: 3.64 ± 0.99; PCOS: 0.13 ± 0.13; PCOS+MET: 1.48 ± 0.97; $P < .01$ control vs PCOS) (Figure 1F).

As expected, control ovaries did not present follicular cysts. Interestingly, metformin treatment significantly decreased the percentage of cystic structures in PCOS rat ovaries (PCOS: 4.61 ± 0.53; PCOS+MET: 2.86 ± 0.50; $P < .05$) (Figure 1G).

Vascular density and stability

We next analyzed the effect of metformin on endothelial cell density in the rat ovaries. To this end, we stained endothelial cells with the lectin *Bandeiraea simplicifolia* and quantified the positively stained area. PCOS ovarian sections showed an increase in the percentage of endothelial cell area that did not reach statistical significance. However, metformin treatment significantly decreased the percentage of endothelial cell area in ovarian sections of DHEA-treated rats (control: 0.98 ± 0.08; PCOS: 1.38 ± 0.19; PCOS+MET: 0.75 ± 0.05; $P < .05$ PCOS vs PCOS+MET) (Figure 2A).

To analyze the effect of metformin on ovarian vascular stability, ovarian sections from rats of the different experimental groups were immunostained with smooth muscle cell α-actin antibody, and the percentage of positively stained periendothelial area was quantified. The percentage of periendothelial cell area was larger in the PCOS group than in the control group (control: 5.2 ± 0.3; PCOS: 7.7 ± 0.3; $P < .01$). Metformin administration significantly reduced the area of periendothelial cells in ovarian sections (PCOS+MET: 5.5 ± 0.4; $P < .01$ vs. PCOS group) (Figure 2B).

Angiogenic factor expression

To further analyze whether metformin has an effect on the expression of ovarian angiogenic factors, we measured the levels of VEGF, ANGPT1, ANGPT2, PDGFB, PDGFD and the receptors KDR, TEK and PDGFRB in ovaries from the different experimental groups. Metformin administration reversed the increased levels of VEGF observed in PCOS rats to control values (control: 45.82 ± 6.70; PCOS: 81.81 ± 12.23; PCOS+MET: 28.64 ± 5.77; $P < .01$ PCOS vs PCOS+MET). No changes were found in the decreased levels of KDR after metformin treatment (Figure 3).

Regarding the ANGPT/TEK system, the ovarian levels of ANGPT1 were higher in PCOS rats than in control rats (control: 0.28 ± 0.04; PCOS 0.47 ± 0.04; $P < .05$). Metformin decreased ANGPT1 levels to values similar to those of the control group (PCOS+MET: 0.33 ± 0.02).

ANGPT2, the antagonist of the ANGPT family, was lower in PCOS ovaries and metformin did not change its levels (control: 1.07 ± 0.08; PCOS: 0.81 ± 0.03; PCOS+MET: 0.74 ± 0.05). However, the higher ANGPT1/ANGPT2 ratio observed in PCOS rats was partially reversed after metformin treatment, reaching values similar to those of the control group (control: 0.18 ± 0.04; PCOS: 0.46 ± 0.08; PCOS+MET: 0.29 ± 0.05) (Figure 4). The ANGPT1/ANGPT2 ratio reflects a tendency to a stable and less permeable vasculature. The receptor TEK was higher in PCOS ovaries with no effect of metformin on its levels (control: 0.07 ± 0.01; PCOS: 0.15 ± 0.02; PCOS+MET: 0.14 ± 0.01).

We also studied the expression of the members of the PDGF family involved in angiogenesis regulation and found a decrease in PDGFB (control: 1.13 ± 0.17; PCOS: 0.51 ± 0.13; $P < .05$), PDGFD (control: 1.77 ± 0.19; PCOS: 0.95 ± 0.09; $P < .05$), and their receptor PDGFRB (control: 1.30 ± 0.14; PCOS: 0.81 ± 0.09; $P < .05$), in PCOS rats compared to control rats. Interestingly, metformin increased the levels of PDGFB (PCOS+MET: 0.81 ± 0.06) and PDGFD (PCOS+MET: 1.80 ± 0.21) to levels comparable to those of the control group. No effect was observed on the levels of PDGFRB (Figure 5).

Serum insulin concentration

To analyze the action of metformin on insulin concentration in the DHEA-treated rat model, we measured the

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concentration of insulin in sera from the control, PCOS and PCOS+MET groups. We found a significant increase in the levels of insulin in PCOS rat serum compared to control rats (control: 0.08 ± 0.01 ng/ml; PCOS: 0.24 ± 0.05 ng/ml; $P < .01$). Interestingly, metformin treatment decreased the levels of insulin in PCOS rats (PCOS+MET: 0.06 ± 0.01 ng/ml; $P < .001$ vs PCOS group) (Figure 6).

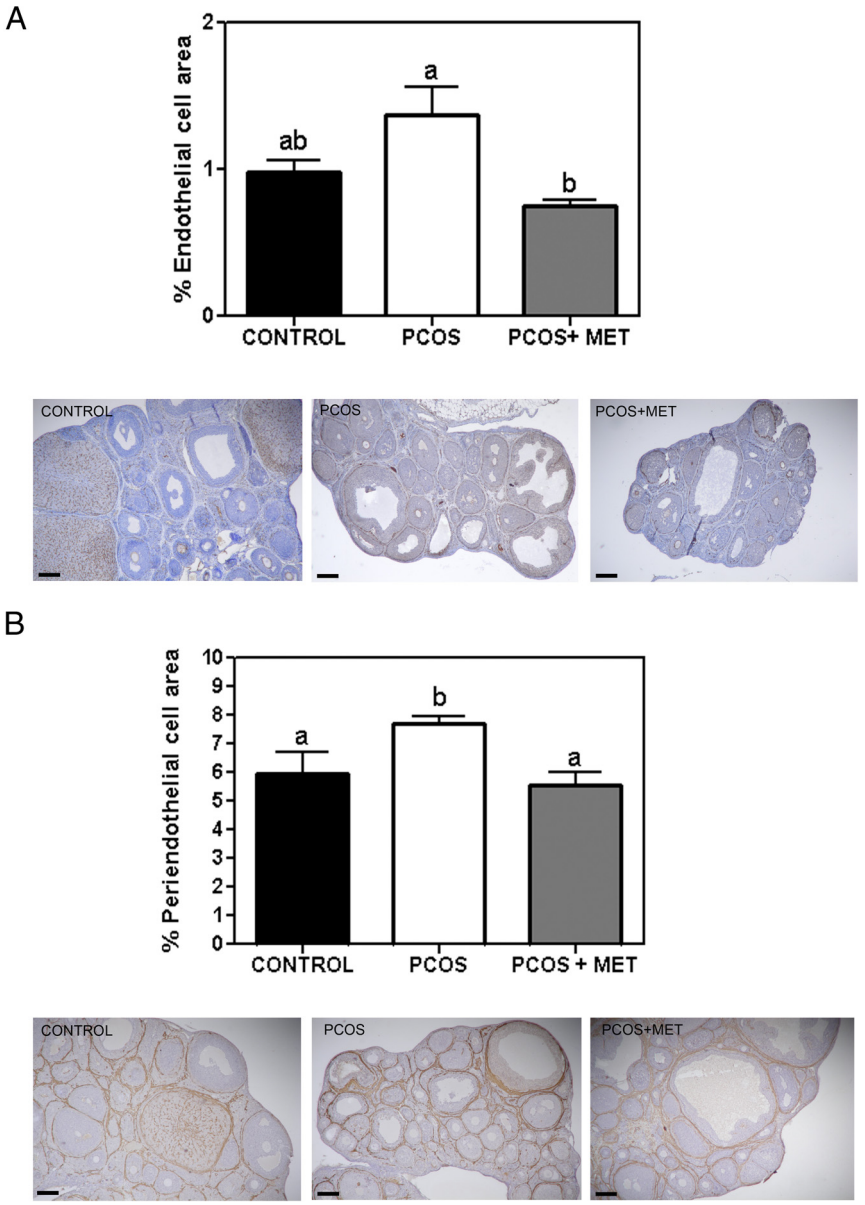

Figure 2. Effect of metformin treatment on ovarian vessels. A. Immunostaining of endothelial cells in control, PCOS, and metformin-treated PCOS rats. Different letters indicate significant differences. (a vs b $P < .05$). Upper panel: Quantification of endothelial cell area in ovarian sections stained with lectin BS-1 in the three groups analyzed. Lower panel: Representative histological sections of control, PCOS and PCOS+MET ovaries stained with lectin BS-1. The photographs show vascular networks and transversal sections of blood vessels. Scale bars represent 200 μm. Areas were determined in three ovarian sections from each ovary; six ovaries per group. B. Immunostaining of periendothelial cells in control, PCOS, and metformin-treated PCOS rats. Different letters indicate significant differences (a vs b $P < .05$). Upper panel: Quantification of periendothelial cell area in ovarian sections stained with antismooth muscle cell α-actin antibody in the three groups analyzed. Lower panel: Representative histological sections of control, PCOS and PCOS+MET ovaries stained with antismooth muscle cell α -actin antibody. Scale bars represent 100 μm. Areas were determined in three ovarian sections from each ovary; six ovaries per group.

Discussion

The biguanide metformin has been extensively used for PCOS treatment, especially in women with hyperglycemia and insulin resistance. Despite the described beneficial effects of metformin on ovarian physiology, the mechanisms of action of this biguanide in the ovary are still unclear. In the present study, we demonstrated for the first time a regulation of ovarian angiogenesis by metformin in a PCOS rat model. Metformin restored the decrease in the percentage of endothelial and periendothelial cell area and the levels of ovarian VEGF, ANGPT1, the ANGPT1/ANGPT2 ratio and PDGFs. Metformin also normalized serum insulin concentration in PCOS rats. Moreover, we found an improvement in follicular development with an increase in the percentage of CL and a decrease in the percentage of cystic structures.

It is widely accepted that metformin exerts beneficial effects in PCOS women besides its action on glucose metabolism. Numerous studies have shown an increase in ovulation and pregnancy rates after metformin treatment in PCOS patients undergoing ART (21–25). Other studies have also found an increase in live birth rates in PCOS patients treated with metformin (26, 27). This may be due, at least in part, to an increase in ovulation rates and to a decrease in spontaneous abortion in the first trimester (60, 61). PCOS patients treated with metformin during gonadotropin-induced ovarian stimulation have less risk of developing OHSS than untreated patients (24, 26, 44, 62).

Several studies have described a decrease in angiogenesis after met-

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formin treatment in different systems such as human umbilical vein endothelial cell line (HUVECs) (33, 63), diabetic rats (38), colon cancer (64) and ovarian cancer (34–37). In PCOS patients treated with metformin, Tan et al demonstrated an increase in serum and adipose tissue thrombospondin-1, with a consistent inhibition of PCOS serum-induced angiogenesis after 6 months of treatment (40). Due to the increased VEGF ovarian levels of PCOS patients and the reported beneficial effects of metformin on patient's fertility, we aimed to analyze the effect of metformin on the levels of ovarian angiogenic factors and vasculature development. We found an increase in endothelial and periendothelial cell area in PCOS rat ovaries compared to control ovaries. Interestingly, in ovaries from metformin-treated PCOS rats, these parameters were normalized. These results suggest that metformin regulates vascular formation and stability in developing ovarian follicles from DHEA-treated rats.

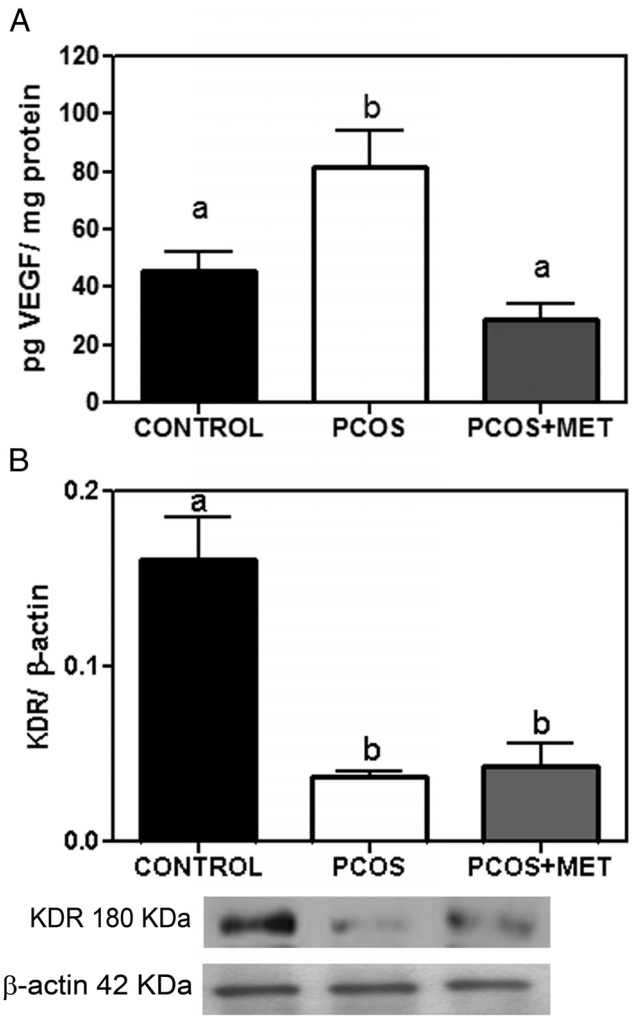

Figure 3. Levels of ovarian VEGF and its receptor KDR in control, PCOS and metformin-treated PCOS rats. A. VEGF concentration in ovarian protein extracts were measured by ELISA. (CONTROL vs PCOS $P < .05$; PCOS vs PCOS+MET $P < .01$). B. KDR levels in ovarian protein extracts were measured by Western blot. The density in each band was normalized to the density of the β-actin band. Different letters indicate significant differences. (a vs b $P < .001$). Data are expressed as the mean ± SEM. Results were obtained from 3 experiments. Six rats per group were used in each experiment.

While the follicle grows, different angiogenic factors participate in the formation of a correct thecal vasculature to assure a proper nutrition and hormonal supply to the developing follicle. VEGF, ANGPTs and PDGFs are some of the main angiogenic factors responsible for proliferation, vessel formation and subsequent stabilization of the new vessel. Members of these angiogenic families are altered in the ovaries of PCOS patients (9, 11, 12). Thus, we analyzed the levels of these angiogenic factors in the ovaries of PCOS rats treated with metformin. Regarding VEGF concentration, we found that metfomin was able to reduce the higher VEGF ovarian concentration present in PCOS rats. It has been described that metformin decreases VEGF expression in other systems, such as obese type 2 diabetic patients (65), endometriotic implants in rats (66), HUVECs and granulation tissue in rats (33), dimethylhydrazine-induced colon cancer in mice (64) and a rat model of OHSS (42). In this study, we demonstrated that metformin treatment is able to prevent the higher concentration of ovarian VEGF found in the DHEA-induced PCOS rat model. It is likely that in PCOS rat model, the excess of VEGF is overriding the decrease in the levels of the receptor KDR. Since VEGF is the main candidate involved in the pathogenesis of OHSS, the decrease in VEGF after metformin treatment can be related to the decrease in the risk of developing OHSS observed in PCOS patients stimulated with gonadotropins when treated with metformin.

Insulin is known to stimulate VEGF expression in different systems (67–70). Since a high percentage of PCOS patients have hyperinsulinemia and insulin resistance together with the ovarian pathology, we aimed to analyze the effect of DHEA on serum insulin levels and the involvement of metformin in the regulation of this effect. Interestingly, we found a significant increase in serum insulin concentration due to DHEA treatment. Metformin decreased insulin concentration to the control levels. These results are in agreement with a previous study that showed an increase in serum insulin levels in mice treated with DHEA, which was reversed after metformin treatment (51). Since metformin prevents the increase in the levels of serum insulin in DHEA-treated rats and insulin stimulates VEGF expression, one of the mechanisms by which metformin normalizes ovarian VEGF levels could be through a decrease in the levels of insulin. Further studies are needed to elucidate other possible mechanisms by which metformin decreases ovarian VEGF levels in the DHEA-induced PCOS rat model. It is worth to note that no studies have analyzed the levels of ovarian angiogenic

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factors in other hyperinsulinemic disorders. While risk factors of preeclampsia include insulin resistance and altered angiogenic factor levels, no studies have shown a relationship between these two conditions. Ovarian angiogenesis is in part regulated by circulating insulin so alterations in the levels of insulin could lead to altered ovarian angiogenesis.

To analyze the regulation of other angiogenic factors by metformin, we analyzed the effect of this drug on ANGPTs and their receptor TEK. As we have previously shown, ANGPT1 and TEK are increased while ANGPT2 is decreased in PCOS rats compared to control rats. Metformin treatment restored the levels of ANGPT1. In contrast, metformin did not change ANGPT2 and TEK levels. However, the ANGPT1/ANGPT2 ratio was decreased in the metformin-treated group, reaching values similar to those of the control group. This is consistent with the lower percentage of periendothelial cell area observed in metformin-treated PCOS rats compared to the untreated group. Regarding PDGFs, metformin restored the decreased levels of the two angiogenic ligands PDGFB and PDGFD in the ovaries of PCOS rats to control values. Metformin treatment normalizes the levels of angiogenic factors in the PCOS rat model induced by DHEA administration. Our study is the first to describe an effect of metformin administration on the regulation of the ANGPT/TEK and PDGF system. Interestingly, metformin did not have any effects on the expression of the angiogenic factor receptors analyzed. We cannot discard that other treatment durations with DHEA can also affect the levels of these receptors. Further studies are needed to elucidate this issue.

Improvement in vascular growth could eventually lead to an improvement in follicular development. To assess this fact, we analyzed the effect of metformin on the follicular development of PCOS rats. In agreement with that previously described (50), when we compared the control vs the PCOS group, we found an increase in primary follicles, a decrease in preantral follicles, and no changes in antral or atretic follicles. Besides, in PCOS ovaries, we observed the presence of cystic structures and a decrease in the percentage of CL (50). Metformin treatment decreased the percentage of primary and preantral follicles and increased the percentage of antral follicles. Moreover, the percentages of atretic follicles and cystic structures were decreased after metformin treatment. Interestingly, the percentage of CL was increased and reached values similar to those of the control group. These results suggest that metformin enhances follicular growth and favors the correct development of follicles and ovulation in the PCOS rat model. Interestingly, metformin does not modify follicle development in control conditions, suggesting that its effects are due to restoration of the alterations observed in the PCOS rat model. Several studies have described a higher ovulation rate in PCOS patients treated with metformin (18, 71, 72). However, the mechanisms by which metformin exerts this regulatory action on follicular development are still unknown. Restoration of the levels of angiogenic factors, which in turn enhances vascular development, is likely to promote proper fol-

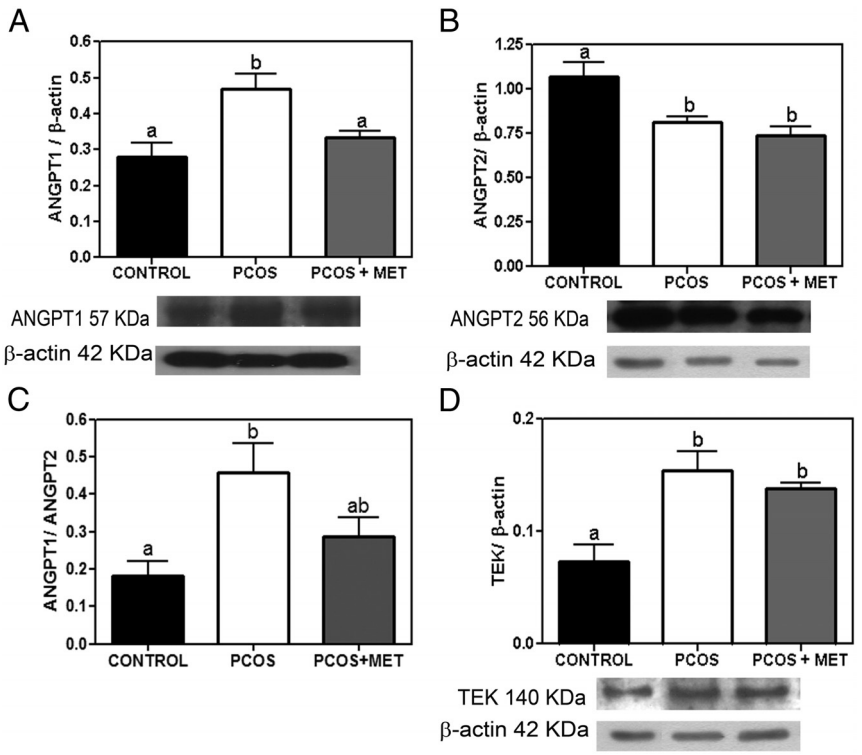

Figure 4. Levels of the ANGPT/TEK system proteins in control, PCOS and metformin-treated PCOS rat ovaries. The graphs show the densitometric analysis for each protein. The density in each band was normalized to the density of the β-actin band. *Lower panels* show a representative blot for each protein analyzed. Different letters indicate significant differences. Results were obtained from 3 experiments. Six rats per group were used in each experiment. A. Densitometric quantification of ANGPT1 in the rat ovaries of the three groups analyzed. (a vs b $P < .05$). B. Densitometric quantification of ANGPT2 in the rat ovaries of the three groups analyzed. (CONTROL vs PCOS $P < .05$; CONTROL vs PCOS+MET $P < .01$). C. ANGPT1/ANGPT2 ratio in the rat ovaries. (a vs b $P < .05$). D. Densitometric quantification of the receptor TEK in the rat ovaries of the three groups analyzed. (CONTROL vs PCOS $P < .01$; CONTROL vs PCOS+MET $P < .05$).

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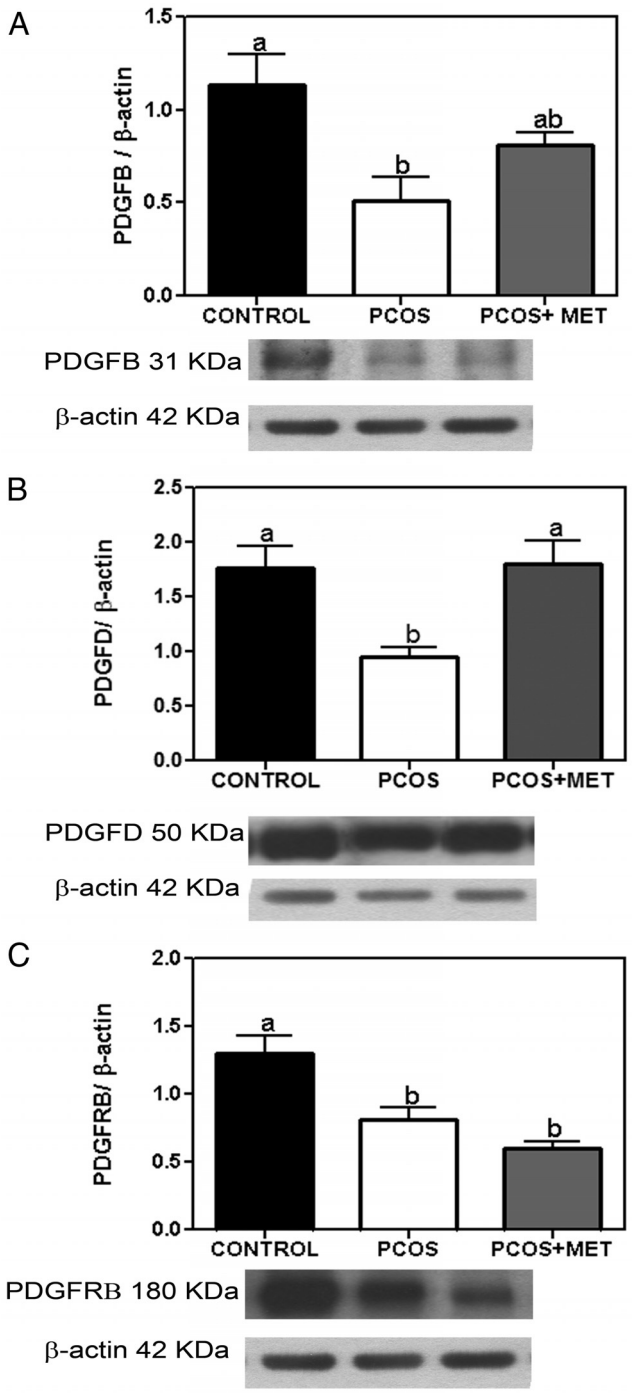

Figure 5. Levels of the PDGF/PDGFRB system proteins in control, PCOS, and metformin-treated PCOS rat ovaries. The graphs show the densitometric analysis for each protein. The density in each band was normalized to the density of the β-actin band. Lower panels show a representative blot for each protein analyzed. Different letters indicate significant differences. Results were obtained from 3 experiments. Six rats per group were used in each experiment. A. Densitometric quantification of PDGFB in the rat ovaries of the three groups analyzed. (a vs b $P < .05$). B. Densitometric quantification of PDGFD in the rat ovaries of the three groups analyzed. (a vs b $P < .05$). C. Densitometric quantification of the receptor PDGFRB in the rat ovaries of the three groups analyzed. (CONTROL vs PCOS $P < .05$; CONTROL vs PCOS+MET $P < .001$).

licular development, leading to an increase in ovulation in our rat model. The mechanisms by which metformin exerts these effects can be through a direct effect in the ovary or through a decrease in the levels of circulating insulin, which may improve follicular angiogenesis as well. Further studies are needed to elucidate the involvement of this mechanism in PCOS women.

It is worth mentioning that the DHEA rat model implies hyperandrogenemia and hyperinsulinemia. For that reason, the results of the present work are likely to be translated to PCOS women with hyperandrogenic and hyperinsulinemic phenotype. Other rodent models have different features that mimic different PCOS women phenotypes (prenatally androgenized mice, estradiol valerate, among others) (73). Considering that not all women diagnosed with PCOS have the same features, studies evaluating angiogenesis in other rodent models and in the ovaries of the different PCOS phenotypes will bring valuable information regarding this issue.

In summary, this is the first study that demonstrates a beneficial effect of metformin on follicular development and ovarian angiogenesis in a rat model of PCOS. Specifically, metformin is able to restore the increased levels of VEGF and ANGPT1 and the decreased levels of PDGFB and PDGFD observed in the DHEA-treated rats. The mechanisms of these metformin effects could be through a decrease in the levels of serum insulin. However, we cannot discard a direct effect of metformin on ovarian angiogenesis. The improvement in ovarian angiogenesis after metformin administration helps to restore the accumulation of small follicles observed in PCOS rats and reduce cyst formation, thus improving follicular development and ovulation. The results presented in this work

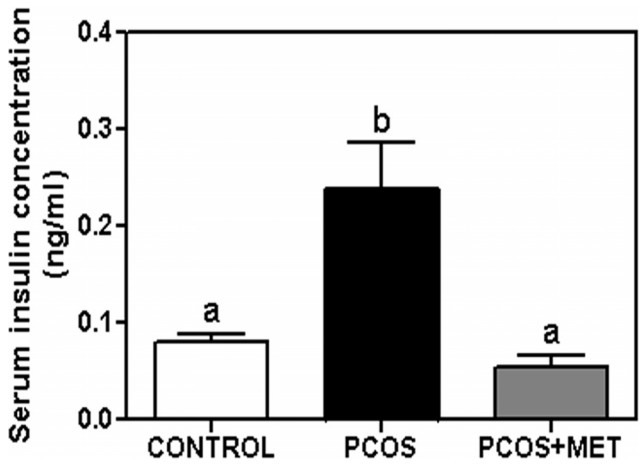

Figure 6. Effect of metformin treatment on serum insulin concentration. Insulin concentration was measured by RIA in serum obtained from control, PCOS and PCOS+MET groups of rats. Different letters indicate significant differences. (CONTROL vs PCOS $P < .01$; PCOS vs PCOS+MET $P < .001$). Results were obtained from 3 experiments. Six rats per group were used in each experiment.

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open new insights into the study of metformin action not only in glucose metabolism but also in ovarian dysfunction in PCOS women.

Acknowledgments

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Disclosure Summary: The authors have nothing to disclose.

This work was supported by .

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