Direct injection of vascular endothelial growth factor into the ovary of mice promotes follicular development

Ramiro Quintana, M.D.,[a] *Laura Kopcow, M.D.,*[a] *Carlos Sueldo, M.D.,*[a]
Guillermo Marconi, M.D.[a] *Nidia Gomez Rueda, M.D.,*[a] *and Rosa Inés Barañao, Ph.D.*[b]

Instituto de Ginecología y Fertilidad (IFER), Buenos Aires, Argentina

Objective: To investigate the effects of an ovarian injection of vascular endothelial growth factor (VEGF) on antral follicle development, neoangiogenesis, and apoptosis.

Design: Controlled laboratory study.

Setting: University-affiliated fertility center.

Animal(s): Balb/c female mice (n = 32) were studied.

Intervention(s): Mice were divided into four groups: control group (C) n = 6, no treatment; hyperstimulated group (HS), n = 8, ovaries were stimulated with 7.5 IU pregnant mare serum gonadotropin (PMSG) and 10 IU of hCG; VEGF group (V), n = 8, injected with 0.1 mL of VEGF (0.2 μg) in each ovary; V+HS, n = 8 injected with VEGF and 2 weeks later hyperstimulated.

Main Outcome Measure(s): Number of antral and luteinized follicles, number of vessels, and percentage of Bcl-2-positive cells.

Result(s): The number of antral follicles with VEGF was higher than in the C and HS groups (16.0 ± 2.5 vs. 6.0 ± 0.9 and 11.3 ± 0.6, respectively, p<0.005). All treatments significantly increased the number of vessels (C: 5.0 ± 0.5 vs. V: 20.0 ± 4.8, p<0.005 and V+HS: 22.2 ± 1.2, p<0.01), as well as increased Bcl-2-positive cells compared to controls (C: 0; V: 11.8 ± 3.5, p<0.005; V+HS: 12.5 ± 3.7, p<0.005).

Conclusion(s): Our findings demonstrated that a direct injection of VEGF into the mouse ovary results in the development of an enhanced vascular network promoting follicular development and diminishing apoptosis. (Fertil Steril® 2004;82(Suppl 3):1101–1105. ©2004 by American Society for Reproductive Medicine.)

Key Words: VEGF, ovary, folliculogenesis, vascularization

The regulation of folliculogenesis in the mammalian ovary is a complex process. Gonadotropins induce ovarian follicle growth that is coincident with increased follicular vasculature, suggesting a role of angiogenesis in follicular development.

As suggested from expression analysis (1, 2), one of the prime candidates regulating ovarian blood vessel formation is vascular endothelial growth factor (VEGF) (3). The VEGF was originally isolated because of its specific ability to stimulate microvascular endothelial cells to proliferate and migrate, as well as to enhance vascular permeability (4, 5). Vascular endothelial growth factor is a 34-kD heterodymeric glycoprotein that plays an important role in the process of fetal and neonatal development (6–8). During adult life, neoangiogenesis enters a period of quiescence, except in pathological situations (such as hypoxia, healing, rheumatoid arthritis, tumoral growth); it also remains active in cyclic and physiologic processes of the female reproductive system and it is recognized as essential for follicular development, ovulation, luteinization, menstruation, implantation, and pregnancy (9). Vascular endothelial growth factor is produced by the majority of cell types and it participates in the development of blood vessels in the thecal layer of follicles (10–12), acting through two tyrosine kinase family receptors, namely Flt-1 and Flt-1/KDR (4, 13).

An inverse relationship between VEGF and apoptosis in lung and pancreatic cancer as well as in granulosa cells has been described (14, 15); therefore, VEGF has been proposed as an important antiapoptotic factor.

To investigate whether additional induction of perifollicular angiogenesis with VEGF would support subsequent follicular development and decrease apoptosis, we studied the effect of a direct injection of VEGF into the ovaries of mice, followed (in some groups of mice) by gonadotropin treatment to stimulate follicular growth. We also evaluated the degree of vascularization by anti-mouse CD34 monoclonal antibodies and the apoptosis of follicular cells by B-cell lymphoma/leukemia 2 (Bcl-2) determination.

MATERIALS AND METHODS

Mice

For the experiments we have used a total of 32 Balb/c female mice, 8 weeks old, from the Laboratory Animals Unit of the Instituto de Biología y Medicina Experimental-CONICET: (Consejo Nacional de Investigaciones Científicas y Técnicas [National Research Council of Argentina], Buenos Aires, Argentina). The study was performed in accordance with the National Institutes of Health guidelines. Because the subjects of this study were not human beings, no institutional review board approval was required.

Mice were divided into four groups:

Control group (C): six female mice not given treatment.

Hyperstimulated group (HS): eight female mice stimulated during estrus with 7.5 IU of pregnant mare's serum gonadotropin (PMSG). Estrus was determined by vaginal cytology examinations. After 48 hours they were injected with 10 IU of hCG (intraperitoneally). Two hours later they were sacrificed.

VEGF group (V): eight female mice injected with 0.1 mL of VEGF (human recombinant vascular endothelial factor, Sigma, St. Louis, MO) (2 μg/mL) in each ovary. The VEGF injection was performed directly from the distal pole of the ovary into the medulla tissue, using a Hamilton syringe, model 1710 RN 81030 (capacity 100 μl) (Americas, Far East, & Pacific Rim; Hamilton Company, Reno, NY 89520-0012 U.S.A.) and introducing the needle (needle gauge 22s, model 91038 metal hub), from the distal pole of the ovary. The mice were sacrificed 15 days after the VEGF injection.

V+HS group: eight female mice were injected with VEGF in each ovary and 13 days after they were treated as with the HS group.

In each group, ovaries were removed and fixed in 4% paraformaldehyde solution, embedded in paraffin wax, and then sectioned serially at 2 μm in thickness for their later evaluation.

Number of Follicles

Ovarian samples were stained with hematoxylin and eosin. Different stages of follicles were classified as described by Salha et al. (16). All antral and luteinized follicles were counted in individual sections. To avoid counting the same follicle more than once, individual follicles having an oocyte with a nucleus were only evaluated, and the size of the follicle in which the oocyte was present was measured using an ocular micrometer. The number of follicles was counted per high power field during 10 observations at ×250 magnification.

Vascular Elements

The count of vascular elements was done by immunocytochemistry, using a rat anti-mouse CD34 monoclonal antibody (Research Diagnostics Inc., Flanders, NJ), which is a specific antigen for vascular endothelial cells and hemopoietic progenitor cells. As a second antibody we used a rabbit anti-rat immunoglobulin, biotynilated (DAKO Corporation, Carpinteria, CA) and the Histostain Plus Kit (Zymed Laboratories Inc., South San Francisco, CA) detection system revealed with 3,3′-Diaminobenzidine (DAB). Results were expressed as the average number of vessels staining red, counting four fields at ×250 magnification.

Expression of Bcl-2 Antigen

Bcl-2 expression was studied from the same paraffin-embedded samples on serial sections with an immunohistochemical method. Sections were deparaffinized in xylene and rehydrated through graded alcohols, followed by microwaving in 0.01 M of sodium citrate buffer for antigen retrieval. Endogenous peroxidase was blocked by treatment with 0.3% hydrogen peroxide for 30 minutes at room temperature, after which nonspecific binding was blocked by incubation with normal rabbit serum. Tissue sections were incubated for 60 minutes with anti-Bcl-2 monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 37°C after incubation for 60 minutes with anti-mouse peroxidase conjugate or anti-rabbit peroxidase conjugate (DAKO Corporation) Binding was visualized by incubating sections with DAB and lightly counterstaining with hematoxylin before permanent mounting.

A lymph node tissue was included as a positive control of Bcl-2. As a negative control, immunoglobulin of the same immunoglobulin class and concentration as the primary antibody were used. The negative control showed an absence of specific staining. Bcl-2 cells were identified by the presence of brown nuclear reactivity. The intensity of Bcl-2 staining was assessed in a blinded fashion at ×400 magnification by two independent observers. Results were expressed as percentages of positive Bcl-2 cells.

Statistical Analysis

Statistical comparisons were performed by Student's *t*-test and Kruskal–Wallis nonparametric ANOVA test, followed by Dunn's multiple comparison test. Regardless of the statistical test, only $P \leq .05$ was considered significant.

RESULTS

Number of Follicles

Figure 1 summarizes the number of antral and luteinized follicles in each group. The number of antral follicles ob-

FIGURE 1

Number of antral and luteinized follicles in four groups. The data represent the mean ±SD. Red column = control group; blue column = hyperstimulated group; green column = VEGF-treated group; yellow column = VEGF-treated and hyperstimulated group. *P<.005 vs. C; **P<.05 vs. C; ***P<.05 vs. HS; ****P<.01 vs. C; *****P=.028 vs. C.

Quintana. Effect of VEGF ovarian injection on folliculogenesis. Fertil Steril 2004.

FIGURE 2

Number of vessels in the four groups. The data represent the mean ±SD. Red column = control group; blue column = hyperstimulated group; green column = VEGF-treated group; yellow column = VEGF-treated and hyperstimulated group. *P<.05 vs. C; **P<.01 vs. C.
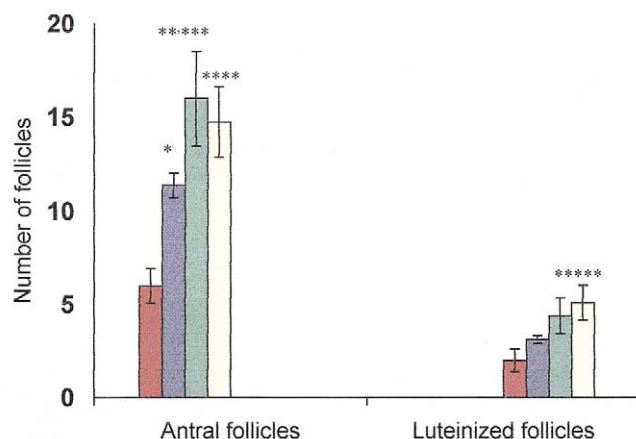
Quintana. Effect of VEGF ovarian injection on folliculogenesis. Fertil Steril 2004.

served in the different groups were: C = 6.00 ± 0.93; HS = 11.37 ± 0.65; V = 16.00 ± 2.54; and V+HS = 14.75 ± 1.88; and the number of luteinized follicles observed were: C = 2.00 ± 0.61; HS = 3.13 ± 0.21; V = 4.40 ± 0.96; and V+HS = 5.11 ± 0.94.

The number of antral follicles observed in mice treated with VEGF was significantly higher than in the control group (P<.05), and also higher than in the HS group (P<.05).

When mice were treated with VEGF and then HS, the number of antral follicles observed did not differ significantly from the one observed in the group treated only with VEGF (P = not significant); however, the number of luteinized follicles was significantly higher than in the control group (P<.028) (Fig. 1).

Number of Vessels

Figure 2 shows the number of vessels counted in each group. All treatments increased significantly the number of vessels; however, the highest angiogenesis were observed in the V+HS group of mice.
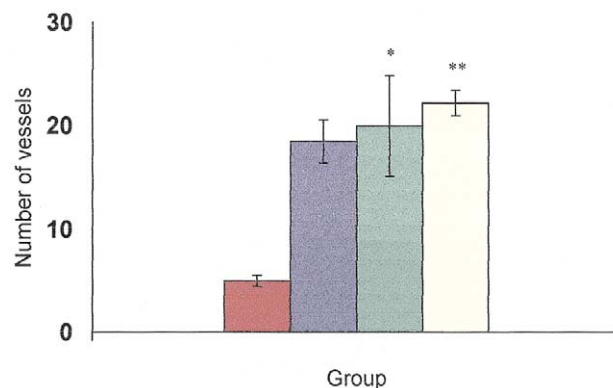
The number of vessels counted in each group was: C = 5.00 ± 0.55; HS = 18.50 ± 2.11; V = 20.00 ± 4.89 (P<.05 vs. C); and V+HS = 22.22 ± 1.23 (P<.01 vs. C).

Expression of Bcl-2

Because Bcl-2 is an antiapoptotic or pro-survival protein, we have measured the percentage of ovarian cells that expressed this protein to quantify the inhibition of apoptosis in

each group of treated mice (Fig. 3). No positive cell was found in the control mice group.

We observed that HS, V, and V+HS mice presented increased percentages of Bcl-2-positive cells. The highest values were observed in the V and V+HS groups of mice.

Values obtained in each group were: C = 0; HS = 8.43 ± 1.85; V = 11.8 ± 3.57 (P<.05 vs. C); and V+HS = 12.58 ± 3.75 (P<.05 vs. C).

FIGURE 3

Percentage of cells with Bcl-2 expression. The data represent the mean ±SD. Red column = control group; blue column = hyperstimulated group; green column = VEGF-treated group; yellow column = VEGF-treated and hyperstimulated group. *P<.05 vs. C; **P<.05 vs. C.
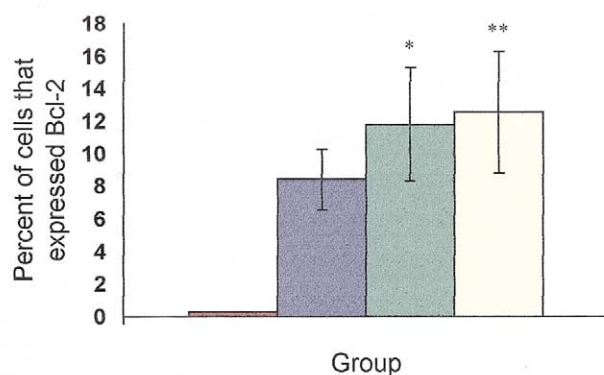
Quintana. Effect of VEGF ovarian injection on folliculogenesis. Fertil Steril 2004.

DISCUSSION

Perifollicular angiogenesis is significantly involved in follicular development. Follicles increase their granulosa cell number and acquire a peripheral theca layer in which new blood vessels form (neoangiogenesis) during the transition from a compact, avascular primary to a vascularized secondary follicle. On the basis of previous findings (12) that VEGF plays a major role in thecal angiogenesis, we performed an in vivo injection of VEGF into the ovary of mice to enhance the thecal angiogenesis associated with follicular development.

Our results have shown that by increasing vascularity, enhanced follicular development can be induced. Vascular density in histological studies of VEGF-treated ovaries significantly increased compared with the untreated ovaries. Also, the number of preovulatory follicles increased significantly in the ovaries injected with VEGF. Our data suggest that the regulation of perifollicular angiogenesis during follicular development is an important factor in the development of ovulatory follicles.

Vascular endothelial growth factor could increase the number of preantral follicles in the mouse ovary by a variety of mechanisms. Perhaps enhanced vascularity or vascular permeability near developing follicles could increase the delivery of endocrine or paracrine factors, such as growth factors, steroids, gonadotropins, or more generally, oxygen and nutrients to the developing follicles. Increased delivery of folliculotrophic substances could result in an increased rate of follicular recruitment from the primordial pool (increased follicle growth) or an inhibition of follicular atresia. Alternatively, recent evidence suggests that VEGF may have direct mitogenic effects on granulosa cells in vitro and could directly stimulate follicle growth in the rat ovary (17).

In agreement with our findings, Shimizu et al. (18) recently published a study on direct VEGF gene fragments injection in pigs' ovarian medulla. They concluded that the number of preovulatory follicles and the capillary density in the theca interna increased significantly in ovaries injected with VEGF gene fragment and treated with equine chorionic gonadotropin compared with those treated with equine chorionic gonadotropin alone. Despite similar results with our study, there was a difference in the period of time between VEGF (or gene fragment) injection and taking the ovarian biopsies for the study. We selected a 2-week interval between VEGF injection and ovarian biopsies based on previous studies showing vascular development in mice after this period of time (19) and to further enhance the effect of VEGF on vascularity instead of a more direct effect on permeability. Shimizu et al. performed the ovarian biopsy after only 7 days. Moreover, we are demonstrating the effectivity of the VEGF protein (they instead injected VEGF gene fragment) and its relationship with apoptosis.

Danforth et al. (20) recently published another study in which they investigated the influence of intrabursal administration of VEGF on the development of preantral follicles in the rat (primary and small secondary follicles) and showed an increment in a time- and dose-dependent manner (within 48–72 hours of administration). Also, they showed that estrogen (E) administration increased the expression of VEGF in the rat ovary.

Functional studies performed in nonhuman primates showed that administration of substances that inactivate VEGF block the development and function of preovulatory follicles as demonstrated by histological (1, 2) analysis or hormone measurements (21). This inhibition of follicular development is caused by arrests to both angiogenesis and antrum formation.

The availability of an adequate vascular supply to provide endocrine and paracrine signals may be important during the early stages of follicle growth as well as the later stages of follicle selection and dominance.

Using HX mice, Zimmermann et al. (22) demonstrated that exogenously administered gonadotropins are unable to drive follicular development to the preovulatory stage in the presence of an antiangiogenic agent in the form of VEGF receptor 2–neutralizing antibodies. This inhibition of follicular development is caused by the arrest of follicular angiogenesis and a reduction in antrum formation.

In our study VEGF has a similar effect as gonadotropins on angiogenesis and folliculogenesis. Gonadotropins drive follicular development, and a component of this function is derived from the effects of gonadotropins on the vasculature of the ovary. Gonadotropins may increase VEGF production and secretion of follicular epithelial cells, which in a paracrine manner acts on the VEGF receptor 2 located on the endothelial cells of the theca layer to induce angiogenesis (22). Therefore, it can be proposed that VEGF is one of the important mediators of gonadotropins in inducing folliculogenesis.

The results of our study show that a direct VEGF ovarian injection increases vascularization, the number of antral and luteinized follicles, and diminishes apoptosis compared to controls. When a comparison between HS and V is performed, a trend toward increased luteinized follicles, vascularization, and diminished apoptosis in the V group is observed (although the difference did not reach statistical significance), yet the treatment with VEGF significantly increases the ovarian response (antral follicles) compared to hyperstimulation alone.

On the other hand, when we compared V and V+HE, there was no difference in the number of antral follicles observed. Barboni et al. (11) have proposed that VEGF might play a local key role in gonadotropin-induced follicular development. Would the administration of VEGF substitute/replace the final effect of gonadotropins on folliculogenesis? Could this be the reason why VEGF and hyperstimulation did not have a synergistic effect?

Of significance, the increase in vascular density surrounding the antral follicles contributes to the inhibition of atresia. Early atretic follicles can regenerate when placed in culture, suggesting that the follicle remains in the atretic state due to a decrease in vascularity that limits access to nutrients, substrates, and trophic hormones (23). In addition to this enhancing effect on vascularization, we propose that there might be a direct beneficial effect of VEGF on apoptosis in the ovary.

Apoptotic cell death is a mechanism by which follicular atresia is induced (24). The VEGF reduces tumor cell apoptosis, whereas inhibition with anti-VEGF neutralizing antibodies induces apoptosis directly in tumor cells (25). Thus, in addition to its role in angiogenesis and vessel permeability, VEGF may act as a survival factor for the granulosa cells of follicles, and then may suppress atresia of the antral follicles, leading to an increased number of ovulated oocytes.

In summary, our findings demonstrated that a direct injection of VEGF into the ovary results in the development of an enhanced vascular network, promoting an increase in follicular development, and diminishing apoptosis in the mouse ovary.

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