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VEGF, VEGFR-1 and VEGFR-2 immunoreactivity in the porcine arteries of vascular subovarian plexus (VSP) during the estrous cycle

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Abstract: Vascular endothelial growth factor (VEGF) is an important angiogenic factor in the female reproductive tract. It binds to cell surface through ligand-stimulatable tyrosine kinase receptors, the most important being VEGFR-1 (flt-1) and VEGFR-2 (flk-1). The broad ligament of the uterus is a dynamic organ consisting of specialized complexes of blood vessels connected functionally to the uterus, oviduct and ovary. Endothelial cells form an inner coating of the vessel walls and thus they stay under the influence of various modulators circulating in blood including ovarian steriods involved in developmental changes in the female reproductive system. The aim of the present study was to immunolocalize VEGF and its two receptors: VEGFR-1 and VEGFR-2 in the broad ligament of the uterus in the area of vascular subovarian plexus during different phases of the estrous cycle in pig and to determine the correlation between immunoreactivity of the investigated factors and phases of the estrous cycle. The study was performed on cryostat sections of vascular subovarian plexus stained immunohistochemically by ABC method. Specific polyclonal antibodies: anti-VEGF, anti-VEGFR-1 and anti-VEGFR-2 were used. Data were subjected to one-way analysis of variance. Our study revealed the presence of VEGF and its receptors in endothelial and smooth muscle cells of VSP arteries. All agents displayed phase-related differences in immunoreactivity suggesting the modulatory effect of VEGF, VEGFR-1 and VEGFR-2 on the arteries of the VSP in the porcine broad ligament of the uterus. (www.cm-uj.krakow.pl/FHC)

Key words: VEGF - VEGFR-1 - VEGFR-2 - VSP - Estrous cycle - Pig

Introduction

Since VEGF was first discovered in 1983 by Harold F. Dvorak and coworkers, a great majority of investigations have been conducted and many studies have described the role of this factor, its localization, modulatory effects and influence of other angiogenic and non-angiogenic factors as well as hormones that may regulate its expression [9, 15, 24, 25, 32, 34, 46, 52, 54, 56, 57, 58].

VEGF in the reproductive tract was first described in the uterus of the mouse, then human, rat, ewe, rabbit and monkey [6, 10 , 14, 41]. Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) is an endothelial-cell-specific mitogen and thus, a prime regulator of vascular formation and function promoting migration of ECs (endothelial cells) and the formation of new vessels, regulating their permeability, inducing vascular leakage and fenestrations in ECs of small venules and capillaries [6, 44]. It is a basic, heparin-binding, homodimeric glycoprotein of 45 kD secreted by various tissues and cells such as: macrophages, glandular, epithelial, stromal, glial, tumor, smooth muscle cells, keratinocytes and ovarian cysts [47]. VEGF also regulates vascular tone, production of vasoactive molecules: von Willebrand factor, nitric oxide, cytokines [5, 24, 33, 49] and recruits progenitor endothelial cells from the bone marrow [8]. It has been found to be a key regulator of angiogenesis and vasculogenesis during early embryonic development [4, 20] and in early postnatal life [13].

Production of VEGF is regulated by several mechanisms: hypoxia [15, 50, 52], PDGF, EGF, TGF-β, steroid hormones, deregulated glucose values [53], cell differentiation, oncogenes [46] but all of these need further detailed investigations.

The VEGF family consists of several members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, PLGF -

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placental growth factors and VEGF-E [22, 26, 29, 31, 38]. VEGF-A is best known and described as a major regulator of normal and abnormal angiogenesis [11, 12]. Several isoforms of VEGF were discovered. Initially, there were four variants. They consisted of 121, 165, 189 and 206 amino acids of almost indentical biological activities [16]. Nowadays, it is proven that more isoforms are generated including VEGF 183 [21] and VEGF 145 [40].

VEGF is a multifunctional factor. Its biological effects are mediated by two main tyrosine kinase receptors: VEGFR-1 and VEGRF-2 expressing different roles *in vivo* and thus responsible for functional variety of VEGF activities [8, 43, 45, 47, 57, 58]. Both receptors after ligand-induced dimerization activate intracellular signaling casades leading to their autophosphorylation [55, 56]. Activated receptor kinase answers in a biochemical and physiological fashion to stimulate DNA synthesis and determine the cellular response. However, VEGFR-2 reveals strong ligand-dependent tyrosine phosphorylation, whereas the response of VEGFR-1 is weak [56, 59].

Vascular endothelial growth factor is expressed at high levels in various normal male and female tissues [2, 36] as a regulatory factor participating in mammalian reproduction. Both, VEGF and its receptors are suggested to be influenced by ovarian steroids involved in developmental changes and functional activity in female reproductive tract [7, 14, 18, 19, 30, 32, 37, 51].

To gain a better understanding of the VEGF and its receptors expression in the broad ligament of the uterus - dynamic tissue, responding to steroid hormones and locally produced growth factors and cytokines throughout the cycle - our study was aimed at the detailed cellular localization of these agents.

This study is based on the hypothesis that during a normal reproductive cycle, levels of VEGF and its receptors in the endothelium and smooth muscle cells of VSP arteries might fluctuate and might be dependent on different stages of the cycle. Current studies characterized VEGF in terms of its role in vascular biology but many conclusions remain unclear with regard to hormonal regulation. Thus, it seemed to be important to examine VEGF and its receptors localization and expression in the area of dynamic hormonal influence - VSP of the broad ligament of the uterus.

Materials and methods

Animals and tissue preparation. The study was performed on 16 cyclic pigs in the following phases of the estrous cycle: follicular (17-21) n=4, early luteal (1-5) n=4, mid luteal (6-12) n=4 and late luteal (13-16) n=4. Tissues of vascular subovarian plexus (VSP) were excised below the ovary and then fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB, pH=7.4). After several washes in 0.1M PB, all tissue samples were stored in 18% sucrose for cryoprotection. Ten μm cryostat sections were mounted on gelatin/chrome-alum-coated slides. Next, sections were stained immunohistochemically for VEGF, VEGFR-1 and VEGFR-2 activity.

Immunostaining for VEGF, VEGFR-1 and VEGFR-2. Immunostaining was done on consecutive sections (3 per uterus). In order to block endogenous peroxidase, sections were treated with hydrogen peroxide in methanol, washed in 0.1M PBS (phosphate buffer saline, pH=7.4), treated with glycine to block the activity of aldehyde groups and then blocked with 10% normal goat serum (NGS) for 1 h at room temperature. Next, sections were incubated overnight at room temperature with primary rabbit polyclonal antibodies: anti-VEGF (A:20, sc-152) in dilution 1:200, anti-VEGFR-1 (c-17, sc-316) in dilution 1:150, anti-VEGFR-2 (N-931, sc-505) in dilution 1:100 (Santa Cruz Biotechnology, Inc.). After overnight incubation with these antibodies, sections were washed in PBS and incubated again with anti-rabbit biotynylated IgG (Vectastatin ABC Kit) diluted 1:400 for 1 h. The following incubation with avidin-biotin-peroxidase complex (ABC Reagent, Vectastatin Kit) lasted 45 min at room temperature. The reaction was visualized by incubating sections in 0.3 mg/ml 3,3‘-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide in PBS. Finally, sections were dehydrated and cover-slipped with DePeX. Negative controls were incubated with 10% NGS instead of the primary, secondary or both antibodies.

The intensity of immunostaining was estimated by measuring the optical density using Olympus DP Soft. Optical density is the method of measuring the intensity of visible products of tissue staining. The measured values range from 0 to 250 where 0 means black and 250 white colour. When the of immunostaining is strong the values are close to 0. However, all data in this study are presented as absolute values, so the stronger is tissue reaction the higher is the value of optical density. 20 pixel values were measured in the endothelium and muscular layer of each from 21 chosen arteries (7 from each section). That gave 1680 measurements per phase (420 measurements/uterus) for endothelium and the same for muscular layer. The data was subjected to one-way analysis of variance (Statistica 5,0). Differences among phases were tested by Tukey‘s test. All values were expressed as means (±SEM).

Results

Light microscopic analysis of the sections revealed estrous phase-related differences in expression of VEGF, VEGFR-1 and VEGFR-2 through the examined stages of the estrous cycle. The positive immunostaining was found in endothelial and smooth muscle cells of the arteries in the area of VSP.

Endothelial cells

Quantitative evaluation of the optical density of the VEGF, VEGFR-1 and VEGFR-2 immunoreactivity revealed their significant variability depending on the estrous cycle. The highest immunoreactivity of VEGF was displayed during the follicular phase of the estrous cycle (Figs. 1A, 2A) ($P \leq 0.001$). The intensity of staining decreased significantly during early luteal phase (Figs. 1B, 2A) to increase again during mid luteal phase (Figs. 1C, 2A). The weakest staining for VEGF was detected during early luteal phase and it did not significantly differ from the VEGF expression during late luteal phase (Figs. 1D, 2A) of the estrous cycle.

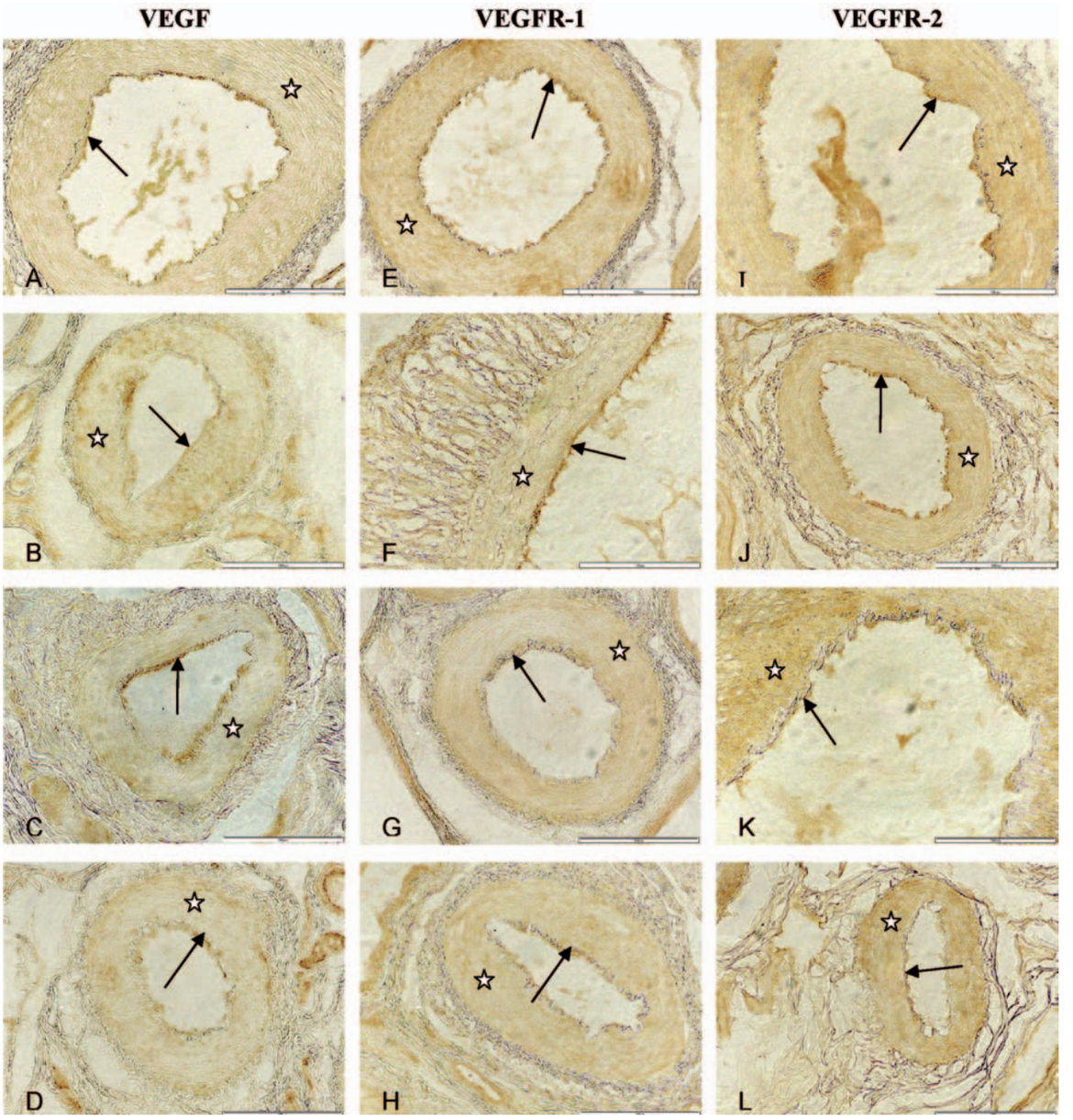

Fig. 1. Representative micrographs presenting the VEGF, VEGFR-1 and VEGFR-2 immunolocalization in the endothelial cells (arrowheads) and in the muscular layer (asterisks) of arterial vessels in the area of vascular subovarian plexus (VSP) of uterine broad ligament during the follicular (**A, E, I**), early luteal (**B, F, J**), mid luteal (**C, G, K**) and late luteal (**D, H, L**) phases of the estrous cycle in pig. Scale bar: 100 µm.

Comparing results for VEGF with those obtained for VEGFR-1, we found that the receptor's signal during the follicular phase was the most intense (Figs. 1E, 3A) and at the similar level as VEGF. In contrast to VEGF, VEGFR-1 displayed strong immunoreactivity in the early luteal phase (Figs. 1F, 3A). In the mid luteal phase (Figs. 1G, 3A), the immunostaining was the weakest.

Figures 1I-L present the immunoreactivity of VEGFR-2 in arterial endothelial cells. The strongest immunostaining was observed during the follicular phase of the estrous cycle (Figs. 1I, 4A), similarly to VEGF and VEGFR-1, although the differences between all phases were generally weak and displayed less significance.

Muscular layer

Immunostaining for all the examined factors in the muscular layer of VSP arteries was definitely weaker but optically visible through all the studied stages. VEGF displayed the strongest immunoreactivity during the follicular phase (Figs. 1A, 2B) and it was significantly different from other stages of the estrous cycle. The weakest immunoreaction for VEGF was observed during early luteal phase (Figs. 1B, 2B). This result corresponds with VEGFR-1 immunoreactivity because this receptor showed also the weakest reaction during early luteal phase (Figs. 1F, 3B), whereas the strongest immunostaining was displayed during mid luteal phase (Figs. 1G, 3B). The immunostaining revealed by VEGFR-2 during follicular, early luteal and late luteal phases (Figs. 1I, J, L, 4B) was almost identical if expressed as values, while the staining in the mid luteal phase was weaker and significantly different from the other three.

Taken together, VEGF and both receptors displayed the strongest immunoreactivity in the follicular phase in endothelial cells, whereas in the smooth muscle cells only the pattern of VEGF immunostaining was similar. In all secretory phases, the immunoreactivity of VEGF and its receptors were different for endothelial and smooth muscle cells. The probable explanation is that they play different roles in the endothelium and muscular layer. Most biological functions of VEGF are mediated via VEGFR-2 and the role of VEGFR-1 is currently still to be discovered.

Discussion

This study presents the cellular localization of VEGF, VEGFR-1 and VEGFR-2 in the porcine broad ligament of the uterus. We have demonstrated the presence of these factors in the endothelial cells and muscular layer of the arteries of vascular subovarian plexus during the estrous cycle suggesting that their immunohistochemical response might be phase-related. Expression of VEGF, VEGFR-1 and VEGFR-2 in the reproductive vascular system still remains poorly understood, although it is proven that these factors play a crucial role in the female reproductive tract. Our study was focused on the uterine broad ligament vasculature supplying all reproductive organs with blood, oxygen, vasoactive molecules, hormones and other active substances. There is no data in the literature describing similar studies. Most of papers refer to reproductive organs: ovary and uterus of various animals and of humans [3, 23, 28, 30, 32, 42, 57] and these organs are particularly interesting in terms of VEGF localization and steroid hormones influencing this growth factor. Cooper and coworkers [9] described localization of VEGF and VEGFR-1 in human placenta and decidua. VEGF immunoreactivity was localized to placental macrophages and glandular epithelium and also maternal macrophages in decidua. The immunostaining of VEGFR-1 was observed in extravillous trophoblast. In human endometrium immunoreactivity of VEGF in glandular and stromal cells was significantly higher during the proliferative phase of the menstrual cycle [34]. The authors concluded that VEGF expression appeared to be under the influence of estrogens rather than progesterone. Similar results were demonstrated by Li *et al.* [27], who also immunolocalized VEGF in human endometrium. They detected no staining around the endometrial blood vessels throughout the secretory phase. The intense immunostaining was observed in stromal cells in the mid to late proliferative endometrium, which corresponds to the increase in the estradiol concentration. Observations by Winther and coworkers [57] confirmed the immunolocalization of VEGF and its both receptors in uterine luminal and glandular epithelium, trophoblast cells, endothelial cells and, for the first time, in smooth muscle cells of the vessel walls in pregnant and non-pregnant pigs. Endothelial cells of arteries displayed intense immunostaining for VEGFR-1 and VEGFR-2 during the late luteal phase of the estrous cycle. Evidence suggests that VEGF activity is spatially and temporally expressed during the cycle in ovary and uterus [39].

There are many studies supporting regulatory influence of estrogens on VEGF mRNA expression. Cullinan-Bove and Koos [10] have shown that VEGF is regulated by estradiol in the rat uterus *in vivo* inducing increase in uterine capillary permeability and growth. Ochoa *et al.* [37] investigated VEGF in the rat pituitary and concluded that VEGF in the cyclic uterus are under the control of estrogens. Reynolds *et al.* [42] documented rapid uterine response of VEGF to estradiol 17β in ovariectomized ewes.

Many investigations were also conducted *in vitro* on human uterine stromal cells [51], human carcinoma cell lines [6], and in breast cancer cells [48]. Isolated human endometrial cells treated with estradiol (E2) revealed significant, 3.1-fold increase in VEGF mRNA expression over the control value [51]. VEGF and its receptors as estrogen-responsive factors play an important role in endometrial angiogenesis, in the increase in microvascular permeability and hormone-dependent vascular growth. The study of Meduri *et al.* [32] was directed at determining the expression and modulation of VEGFR-1 and VEGFR-2 and at seeking their relation to the phase of the cycle. The immunohistochemical investigation revealed that the number of VEGFR-2-stained capillaries was maximal in the proliferative phase, whereas about 2-fold higher number of VEGFR-1 stained capillaries was observed in the secretory phase of the menstrual cycle.

Immunoreactivity of VEGFR-2 was higher in the midsecretory stage of the cycle, whereas VEGFR-1 staining was intense during the late proliferative

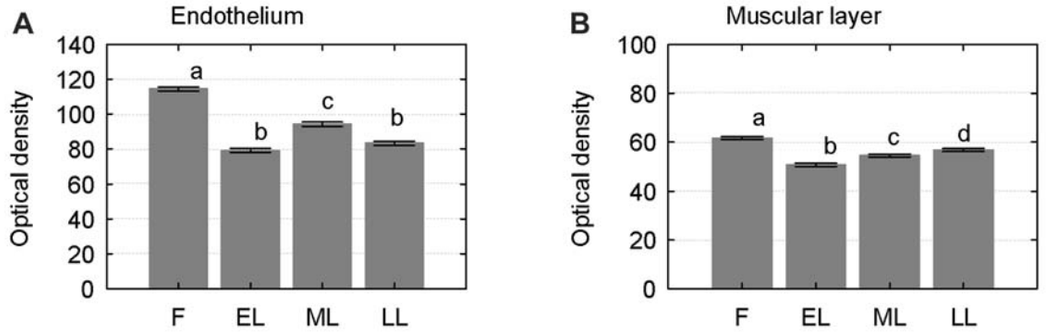

Fig. 2. Comparison of the intensity (optical density) of VEGF immunostaining in the endothelium (**A**) and muscular layer (**B**) of VSP arteries at various stages of the estrous cycle presented graphically. Different small letters indicate significant differences (P≤0.001). Each value is the mean ± SEM. Abbreviations for figures 2-4: F - follicular, EL - early luteal, ML - mid luteal, LL - late luteal phases of the estrous cycle.

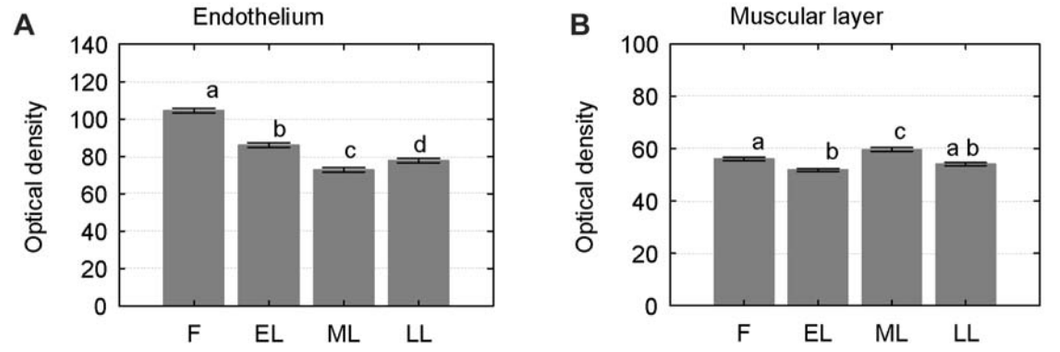

Fig. 3. Comparison of the intensity (optical density) of VEGFR-1 immunostaining in the endothelium (**A**) and muscular layer (**B**) of VSP arteries at various stages of the estrous cycle presented graphically. Different small letters indicate significant differences (P≤0.001). Each value is the mean ± SEM.

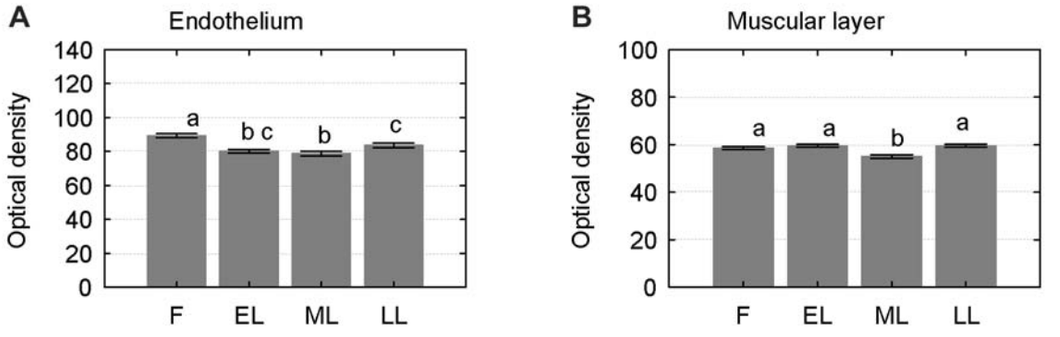

Fig. 4. Comparison of the intensity (optical density) of VEGFR-2 immunostaining in the endothelium (**A**) and muscular layer (**B**) of VSP arteries at various stages of the estrous cycle presented graphically. Different small letters indicate significant differences (P≤0.001). Each value is the mean ± SEM.

phase. Malamitsi-Puchner *et al.* [30] demonstrated that vascular expression of VEGFR-2 was high during the proliferative phase. These findings, as well as others suggest a relation of VEGF and its receptors immunoreactivity to the phases of the reproductive cycle.

A limited number of studies suggest that VEGF expression is increased not only by estrogens but also by progestins [7, 14, 17 , 25, 35], however, the effects of progestins on expression of VEGF and both receptors is not so clear as those of estrogens [19] because the knowledge of this mechanism is just beginning to

emerge. Greb *et al.* [14] observed intense VEGF immunostaining in the primate endometrial stroma after progestin treatment. According to Hyder and coworkers [17], progesterone increases VEGF protein expression in a human breast cancer cell line. Ancelin *et al.* [1] showed that progesterone selectively increased VEGF (189) expression in the human uterus.

The present study revealed that VEGF, VEGFR-1 and VEGFR-2 are expressed in the endothelial and smooth muscle cells in the area of VSP of cyclic pigs. We have observed a correlation between immunoreactivities of VEGF and its receptors in arterial walls and the estrous phases. Cyclic changes in expression of VEGF and both receptors may suggest that these factors remain under the influence of ovarian steroids. Positive expression of VEGF, VEGFR-1 and VEGFR-2 in the endothelial and smooth muscle cells gives evidence for the hypothesis that they also play a role to act in non-endothelial cells, stimulating different functions in the vasculature. Such results might also suggest that VEGF and both receptors remain under stronger influence of estrogens than progesterone. Still, all these results need further detailed investigation.

This study is the first step leading to further investigations that will demonstrate whether locally synthesized VEGF acting via its receptors is affected by ovarian steroid hormones and undergoes the cyclic changes in the uterine vasculature through the estrous cycle.

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