EXPRESSION PATTERN OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) AND VON WILLEBRAND FACTOR (VWF) IN RELATION TO ANGIOGENESIS IN CANINE UTERUS

Devendra Pathak[1], Kaveesh Chahal, Opinder Singh and Varinder Uppal
[1]Senior Scientist, Department of Veterinary Anatomy, College of Veterinary Science Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab.
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Vascular endothelial growth factor (VEGF) stimulates endothelial cell propagation and is essential to angiogenesis. The uteri from female canines (n = 54) were presented to the teaching Veterinary and Clinical Complex, College of Veterinary Science, Ludhiana, for neutering and disease conditions, where ovariohysterectomy was performed. A strongly positive reaction for VEGF was observed in the endometrium and blood vessels of all three compartments of the uterus in the prepubertal, cyclical, and gravid uterus. The immunoreactivity of VEGF was cytoplasmic in nature; however, some extracellular reaction of VEGF was also observed, indicating its paracrine role. It was concluded that the localization of these proteins was higher in the epithelium and glands of pubertal and pregnant animals than in the prepubertal group. At the same time, the differential reactivity was observed in the uterus of diseased animals. Immunolocalization of VEGF and vWF revealed a positive linear correlation. Specifically, as VEGF expression increased, the number of vWF-positive blood vessels increased, and vice versa.
Keywords: Canine, expression, Uterus, VEGF, Vimentin.

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In the canine uterus, angiogenesis is essential for establishing and maintaining pregnancy, providing the necessary blood supply to support the developing fetus (Rizov *et al*., 2017). Vascular endothelial growth factor (VEGF) is a multi-functional factor primarily involved in regulatingproliferation, differentiation, migration, survival of endothelial cells, vascular permeability and angiogenesis. This protein promotes uterine blood flow, which is necessary for providing oxygen and nutrients to support the growth and development of embryos. It also supports placental development and regulates cyclical changes. von Willebrand Factor (vWF) is a blood glycoprotein essential for hemostasis. This protein is a marker for identifying blood vessels (Tiulienieva *et al*., 2019).It has been increasingly recognized for its role in angiogenesis. It promotes endothelial cell survival and angiogenesis under hypoxic conditions, prevalent in the rapidly growing and remodelling uterine tissue during pregnancy. In the uterus, vWF facilitates implantation, maintains hemostasis, supports endothelial cell function, and aids in properly functioning endothelial cells, which are critical for creating a vascular network in the uterus during pregnancy (Möller *et al*., 2022.).

The orchestration of this complex process is tightly synchronized by numerous factors, among which the Vascular Endothelial Growth Factor (VEGF) and von Willebrand Factor (vWF) play critical roles. This study aimed to examine the expression of VEGF and vWF immunohistochemically in the uterus of female dogs, shedding light on their synergistic roles in driving angiogenesis during prepubertal, cyclical, and diseased animals.

Materials and Methods

Samples of uteri collected from female canines (n=48) were brought to the Teaching Veterinary Clinical complex of the College of Veterinary Science (Ludhiana) for ovariohysterectomy and processed for paraffin sectioning.

Tissue processing

The tissue pieces of the horn and the body of the uterus were fixed in 10% neutral buffered formalin and processed for the paraffin sections as per the protocol established in the laboratory. Briefly, tissues were dehydrated using alcohol, acetone, and benzene. Infiltration and embedding were done with paraffin wax. 5µ thick sections were cut using a rotary microtome and picked on positively charged glass slides. These sections were used for immunolocalization.

Immunohistochemistry

For immunohistochemical staining, we followed our previous article's instructions. To improve the paraffin sections' adherence to the slides, they were placed on positively charged slides and incubated for an hour at 60°C. The sections were rehydrated to water using descending alcohol grades after being deparaffinized with xylene. Heat-induced antigen retrieval was carried out in a citrate buffer solution at 95–98 degrees Celsius in the microwave for ten minutes. Sections were treated with 3% (v/v) H_2O_2 in methanol for 20 min to decrease the endogenous peroxidase activity, following washing in 0.1M phosphate buffer saline (PBS). 2.5% Normal horse serum was employed for 30 minutes to avoid the non-specific binding of antibodies. Ready-to-use primary antibodies (predefined dilutions) from Biogenex were applied to the sections for an overnight treatment at 4^0C. Universal secondary antibodies conjugated with the enzyme horseradish peroxidase (Vector Laboratories) were utilised to detect bound antibodies. Gill's III hematoxylins and 3, 3'-diaminobenzidine tetrahydrochloride (DAB) were the chromogens utilized for nuclear counterstaining. The slides were washed under running tap water and dehydrated in alcohol. After being cleared with xylene, sections were mounted using DPX mounting material.

Microphotography and quantification

Photomicrographs were taken at different magnifications for each section by a bright-field microscope with an attached camera and photography unit (Eclipse 80i, Nikon, Japan). 6-10 photomicrographs at 400 magnifications were taken to analyze the results.

Results and Discussion

The present research is the first to compare VEGF immunolocalization in the uterus during prepubertal, cyclical, and diseased female dogs. The immunolocalization was cytoplasmic in nature in all the stages. The reaction was granular in appearance. In all the stages, localization of VEGF was in all three compartments of the uterus, i.e., endometrium, myometrium, and perimetrium. The results of prepubertal dogs are represented in Figure 1. In the endometrium, moderate to strong reaction was observed in the lining epithelium of endometrium, lining epithelium of endometrial glands, and endothelial cells of blood vessels (Fig.1A).At higher magnification, the VEGF was visualized in the cytoplasm ofthe lining mucosa of glands, more towards the area of the cell-to-cell junctions and towards the periphery of cells. Strong reactions in the endothelial cells of blood vessels located near these glands were observed (Fig.1B). The blood vessels in the endometrium were of varied diameters and appeared branched, with strong reactions in the endothelium observed (Fig.1C)). Tunica muscularis revealed moderate reactions in muscle bundles, and endothelial cells of blood vessels showed strong reactions (Fig.1D). During the prepubertal period, the localization of VEGF in different compartments of the uterus indicated its role in angiogenesis, which might be required for the growing size of the

uterus with age. It is required for uterine vascular adaptation in uterus. VEGF is required for angiogenesis in any organ. VEGF ligands and their receptors were identified by immunohistochemistry in the endometrial stroma, luminal epithelium, glandular epithelium, and blood vessels.

The immune localization of VEGF in the uterus of cyclical female dogs showed a strong reaction in lining epithelium (E), lining epithelium of glands (G), and endothelial cells of blood vessels. Immune reaction products were accumulated towards the luminal side of the endometrial glands (Fig.2A). At higher magnification, a substantial accumulation of the reaction product was seen both towards the luminal and peripheral sides. The blood vessels around the glands showed signs of intense reactions in the endothelial cells. These vessels showed branching buds around. The stromal cells in the endometrium also revealed a strong reaction to the VEGF protein (Fig.2B). Numerous capillaries had strong reactions in the endothelium and stromal cells (S) in the endometrium between the glands (Fig.2C). In the tunica muscularis layer, VEGF was localized muscle bundles of both circular and longitudinal layers. The endothelial cells of muscular and smaller arteries were strongly positive for VEGF protein. The myometrium of the uterus suffers substantial changes in size and cellular properties during the estrous cycleand requires several growth factors, and VEGF is one among them. In the full-thickness rat uterus damage model, the collagen-VEGF combination enhanced uterine function and encouraged remodelling of the scarred

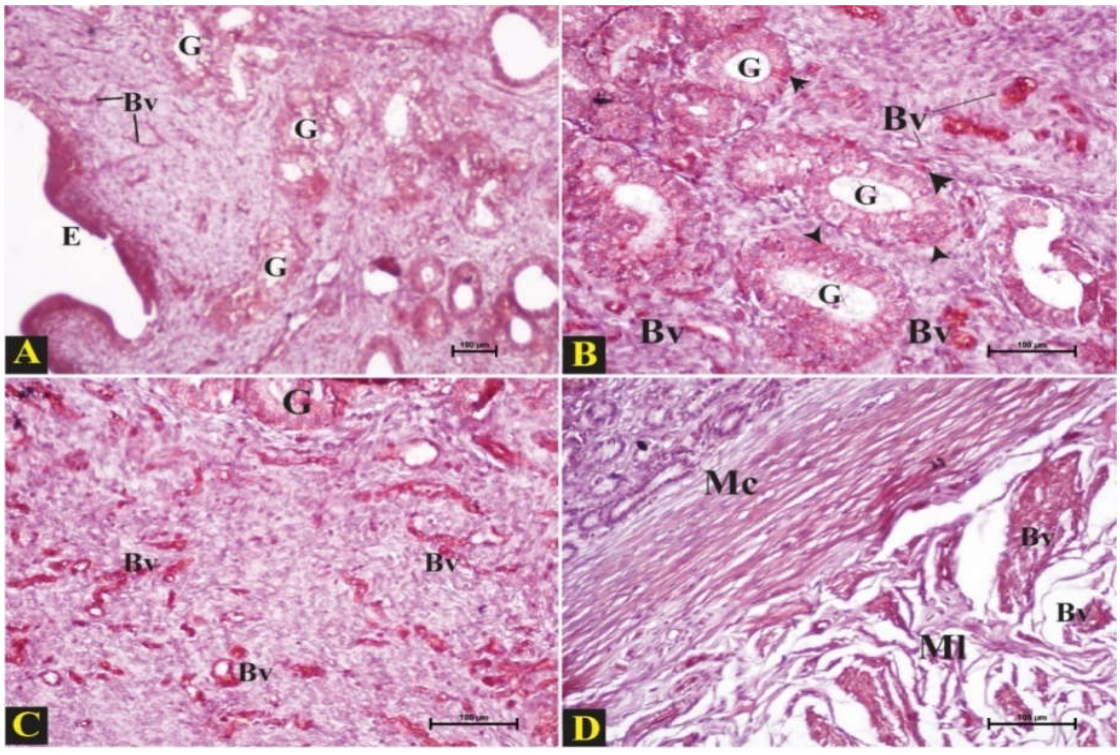

Figure 1.Representative photomicrographs of immunolocalization of VEGF in the uterus of prepubertal female dogs showing A) moderate to a strong reaction in lining epithelium (E), lining epithelium of glands (G), endothelial cells of blood vessels (Bv); B) moderate to strong reaction ofVEGF in lining mucosa of glands (G), towards the periphery of cells (arrowheads) and blood vessels (Bv); C) numerous capillaries with strong reaction in the endothelium (Bv) in the endometrium in between the glands (G); D) immunolocalization in the inner circularmuscular layer (Mi), outer longitudinal (Ml) and in blood vessels (Bv); Poly HRP method, scale bar 100 µm.

uterus as also mentioned by Hanuman *et al.,* 2023. There was a contrasting difference from the prepubertal uterus for localization of VEGF. Here, we observed VEGF on either side of the cells. The presence of VEGF towards the luminal and peripheral sides suggested that blood flow is high due to the hormonal changes in the endometrium. It is postulated that the secretion of VEGF is

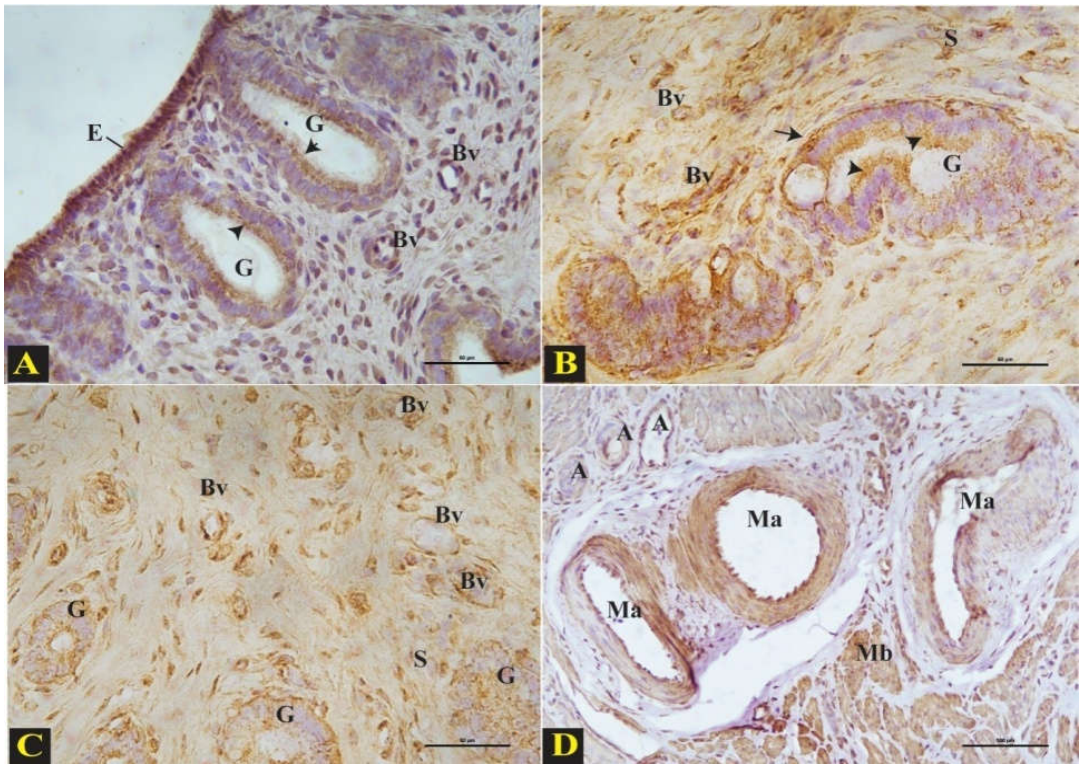

Figure 2. Representative photomicrographs of immunolocalization of VEGF in the uterus of cyclical female dogs showing A) strong reaction in lining epithelium (E), lining epithelium of glands (G) with a visible accumulation of reaction products towards the luminal side (arrowheads), endothelial cells of blood vessels (Bv); B) strong to the intense reaction ofVEGF in lining mucosa of glands (G), towards the luminal surface of cells (arrowheads) and the periphery of cells (arrow) and blood vessels (Bv) and stromal cells (S); C) numerous capillaries with strong reaction in the endothelium (Bv) and stromal cells (S) in the endometrium in between the glands (G); D) immunolocalization in the inner circular muscular layer (Mi), outer longitudinal (Ml) and in blood vessels (Bv); Poly HRP method, scale bar 50 µm.

from both the compartments of endometrial cells, i.e., basal and luminal. The release of VEGF through the peripheral compartment indicated that it might act through the local paracrine mechanism, motivating the nearby blood vessels to form new blood vessels. The presence of VEGF towards the luminal side indicated that it might be secreting along with other endometrial proteins. These two-sided secretions might be meant to meet the growing demand for angiogenesis during the estrous cycle. According to Guo *et al.* (2021) also, VEGF promoted embryo development, enhanced endometrial receptivity, and facilitated interactions between the developing embryo and the endometrium, all of which led to embryo implantation. Reproductive failure, such as recurrent implantation failure (RIF) and recurrent miscarriage (RM), is correlated with altered VEGF expression.

The immunolocalization of VEGF in the uterus of diseased female dogs varied with the type of disease. A moderate reaction

was observed in the wall of the cyst in cystic endometritis cases (Fig.3A,B, C&D). Moderate to strong reaction in papillary growth around cysts, infiltrating cells were seen accumulated inside a cyst-like structure (Fig.3A, B &D). Strong reaction in the stromal layer and epithelial cells in papillary growths in endometrial hyperplasiacases (Fig.3C).The lining of varied-sized cysts and blood vessels locatedbetween them revealed a strong reaction. At higher magnification, infiltrating cells were seen inside cystic structures implicating pyometra (Fig.3E). In cystic endometrial hyperplasia cases, hyperplasia with immunolocalization of VEGF was observed (Fig.3F). The results showed a differential expression pattern in diseased conditions. The disease conditions that involved hypertrophy have higher expression of VEGF. The disease conditions that involved the cystic condition had a lesser expression of the angiogenic protein

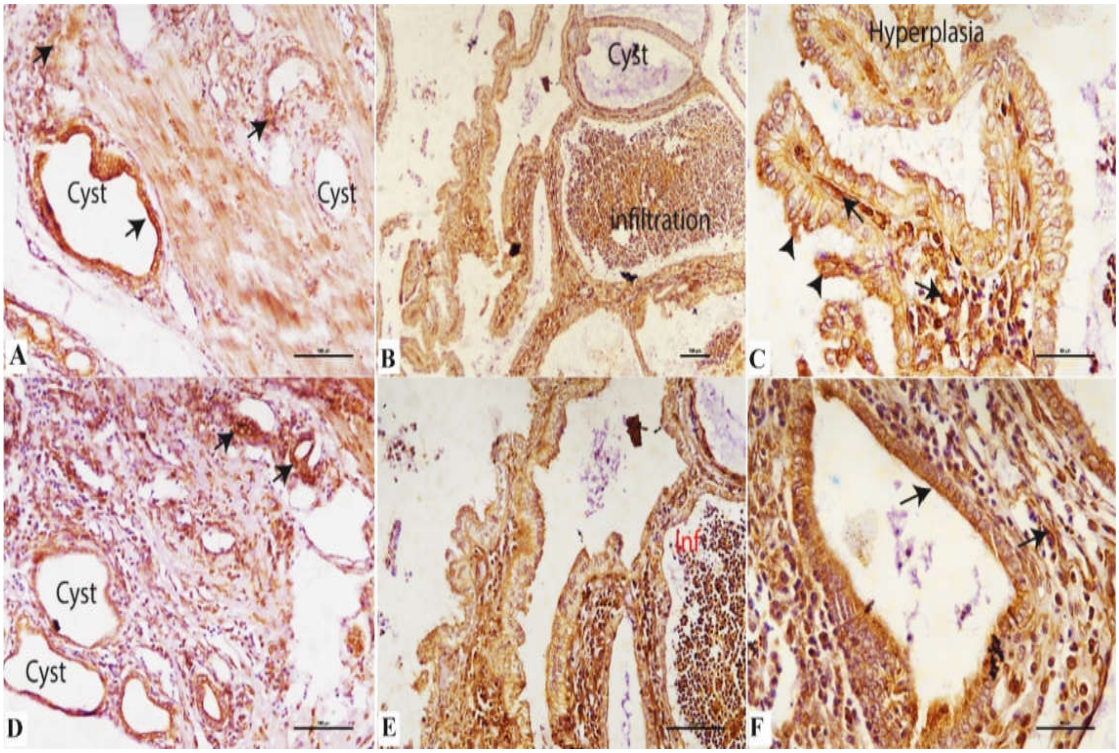

Figure 3. Representative photomicrographs of immunolocalization of VEGF in the uterus of diseased female dogs showing A)moderate reaction in the wall (arrow) of cyst in cystic endometritis; B) moderate to a strong reaction in papillary growth around cystsand infiltrating cells were seen accumulated inside a cyst-like structure; C) strong reaction in the stromal layer (arrow) and epithelial cells (S) in papillary growths; D)strong reaction in lining of varied sized cysts (arrow) and blood vessels in between them;E) higher magnification of infiltrating cells and papillary growths, F) higher magnification of cyst and hyperplasia with immunolocalization (arrows), Poly HRP method, scale bar for A, B, D & E 100 µm and C& F 50 µm.

The correlation between the expression of VEGF and the number of blood vessels was established in the current study. The blood vessels were marked by the presence of vWF in the endothelial cells. The immune localization of vWF in the uterus of female dogs showed a strong reaction in the lining endothelial cells of blood vessels of all calibres, i.e., capillaries to muscular arteries (Fig. 4).In the endometrium, most capillaries were seen lined with vWF-positive endothelium (Fig. 4A).While the blood vessels in tunica muscularis were smaller and muscular arteries and the endothelium lining

showed immune reaction for vWF (Fig. 4B). In the cases of endometrial hyperplasia, numerous vWF-positive blood vessels were seen (Fig. 4C), while the blood vessels were few in cystic endometritis cases (Figure 4D).According to recent research, vWF regulates angiogenesis, the proliferation of smooth muscle cells, the spread of tumour cells, and immune system interaction.

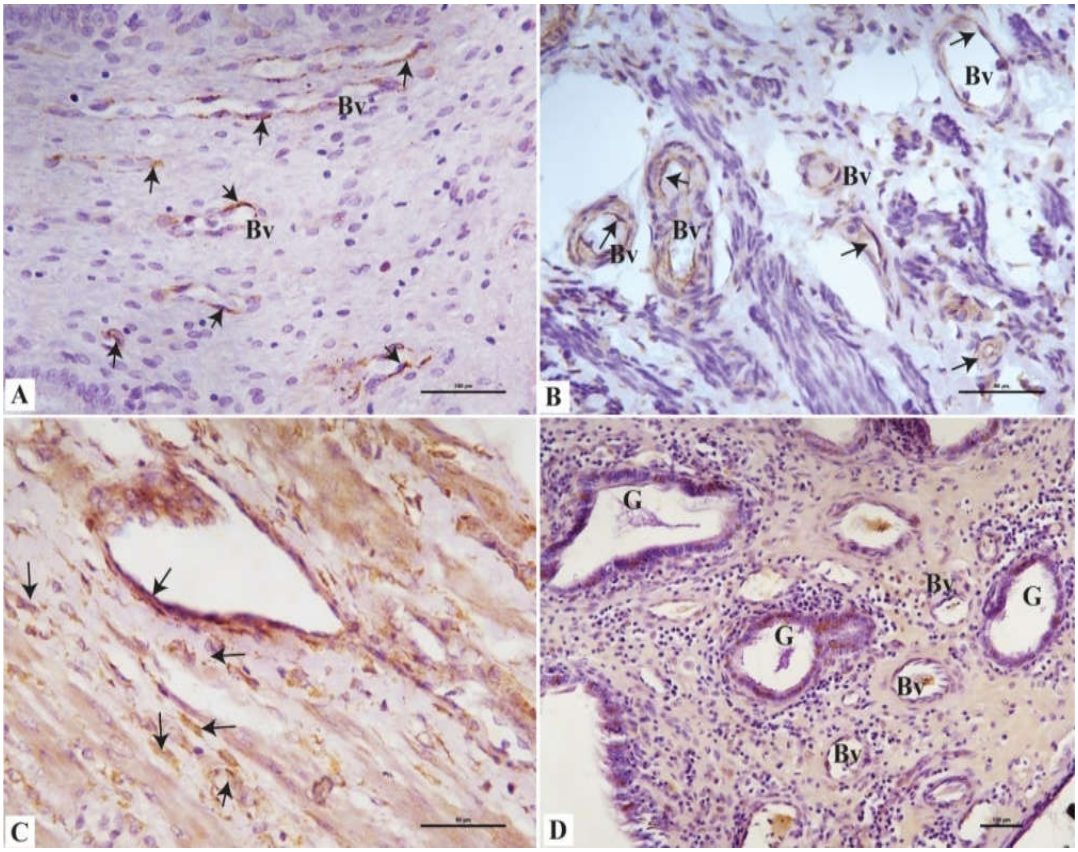

Figure 4. Representative photomicrographs of immunolocalization of vWF in the uterus of female dogs showing A) strong reaction in lining epithelium (E), lining epithelium of glands (G) with visible accumulation of reaction products towards luminal side (arrow heads), endothelial cells of blood vessels (Bv); B) strong to intense reaction ofVEGF in lining mucosa of glands (G), towards the luminal surface of cells (arrowheads) and towards the periphery of cells (arrow) and blood vessels (Bv) and stromal cells (S); C) numerous capillaries with strong reaction in the endothelium (Bv) and stromal cells (S) in the endometrium in between the glands (G); D) immunolocalization in the inner circular muscular layer (Mi), outer longitudinal (Ml) and in blood vessels (Bv); Poly HRP method, scale bar 50 µm.

Conclusion

A cytoplasmic reaction for VEGF was observed in the endometrium and blood vessels of all three compartments of the uterus in the prepubertal, cyclical, and gravid uterus. It was concluded that the localization of these proteins was higher in the epithelium and glands of pubertal and pregnant animals than in the prepubertal group. The differential reactivity was observed in the uterus of diseased animals. Immuno localization of VEGF and vWF revealed a positive linear correlation.

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