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Immunolocalization of von Willebrand Factor and Vascular Endothelial Growth Factor during Follicular Atresia in the Swamp Buffalo Ovary

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Abstract. The aim of this study was to investigate the distribution pattern of von Willebrand factor (vWF) and vascular endothelial growth factor (VEGF) in the healthy antral and atretic follicles of Philippine swamp buffaloes (SB) in comparison with Holstein-Friesian cows (HF). Paraffin sections of healthy follicles and atretic follicles at various stages were immunostained with vWF antibody and VEGF antibody. The density of vWF-positive capillary vessels in the theca interna significantly increased as atresia progressed in SB, whereas the density significantly decreased in late atretic follicles compared with advanced ones in HF. On the other hand, the area of vWF-positive capillary vessels in the theca interna significantly increased as atresia progressed in both SB and HF. Immunoreactions of VEGF in the granulosa cells (in all follicle types) were observed in both SB and HF. In the granulosa layer, a reduction in the VEGF immunoreaction was noted as follicles progressed from healthy to advanced atretic follicles in both animals. Granulosa cells (in both SB and HF) showed a higher immunopositive staining than theca cells. In the theca interna, VEGF immunostaining diminished as follicles progressed to the late atretic follicles in both animals. These results indicate that during atresia, changes of vWF expression are the opposite of VEGF expression in SB. Both vWF and VEGF are suggested to be associated with follicular atresia in SB.

Key words: Atretic follicles, Philippine swamp buffaloes, Thecal microvasculature, Vascular endothelial growth factor, von Willebrand factor

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The von Willebrand factor (vWF), a multimeric glycoprotein, is present in the plasma and subendothelium [1] and mediates adhesion of platelets to the subendothelium at the sites of vascular injury and disorder [2, 3]. The vWF is synthesized by endothelial cells (EC) and megakaryocytes, and then stored in EC (Weibel-Palade bodies) and platelets (α -granules) [4–6]. It is

known that increased expression of vWF in blood vessel endothelial cells is associated with endothelial damage [7, 8], blood vessel maturation [9] and regression [10]. It has been reported that the regression process in atretic follicles coincides with an increased expression of vWF [11, 12].

Conversely, the vascular endothelial growth factor (VEGF) is considered to be a major angiogenic factor in the ovary [13], and its production declines in atretic follicles [14]. VEGF plays a crucial role in endothelial cell proliferation

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[15], chemotaxis [16], and protection from apoptosis [17–19]. VEGF exerts its cellular effects through interaction with its tyrosine kinase receptors Flt-1 (VEGF-R1) and Flk-1/KDR (VEGF-R2), which bind the ligand with high affinity [20].

The relationship between thecal vasculature distribution and follicular development or atresia has been shown in domestic animals: cattle [21, 22], sheep [23, 24], and pigs [24, 25]. However, literature concerning the vasculature surrounding follicles in the buffalo is limited, except for a recent study reporting dynamic changes (using *Bandeiraea simplicifolia*-I lectin) in the ovarian thecal vasculature of the swamp buffalo associated with follicular atresia [26]. We hypothesized that vWF expression will tend to increase while VEGF expression will tend to decrease as follicular atresia progresses in the buffalo ovary. Hence, the aim of this preliminary study was to determine the maturity (aging) of microvessels in the theca layers during the atretic process in the buffalo. For this purpose, the distribution of vWF was compared among types of follicle and between buffaloes and cattle. We also investigated the involvement of microvessel maturity in follicular VEGF expression.

Materials and Methods

Collection and preparation of tissue

Fifteen ovaries from healthy, non-pregnant swamp buffaloes (SB) and ten ovaries from Holstein-Friesian cows (HF) were collected from slaughterhouses in the Philippines and Japan, respectively. The estimated age of SB was 5–9 years, determined by dental examination [27]. Blood samples were also collected from SB before slaughter for determination of plasma progesterone level [28–30] to estimate whether the animals were in the luteal phase (>1 ng/ml) or not. However, age and progesterone concentration of HF were not investigated due to restrictions in the slaughterhouse. No macroscopic abnormalities were observed in the reproductive tracts of animals. Ovaries were immediately fixed in 10% formalin in phosphate buffered saline (PBS, pH 7.4) and processed for paraffin sections (3- μ m thick), which were air dried on slides treated with 3-aminopropyltriethoxysilane (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) for the vWF immunostaining or on MAS coated slides

(Matsunami Glass Industries Ltd., Kishiwada, Japan) for immunostaining of VEGF.

Immunohistochemistry

After deparaffinization with xylene, the sections were rehydrated through a series of graded volumes of ethanol in distilled water (100, 95, 90, 80, and 70%; 15 sec each) and washed in PBS for 5 min. Antigen retrieval for vWF was done by incubating sections with 20 μ g/ml proteinase K (Sigma) in 10 mM Tris-HCl, pH 7.4, at 35 C for 20 min in a humidified chamber. For VEGF, antigen retrieval was conducted by heating sections in a solution of 10 mM sodium citrate at 121 C for 1 min followed by cooling for 20 min at room temperature. The sections were then incubated for 10 min with 1% hydrogen peroxide to remove endogenous peroxidase activity. After washing with PBS (2 times), the sections were incubated with normal goat serum for blocking (1:1,200 dilution) for 30 min. For vWF, sections were incubated with anti-human vWF antibody (reacts specifically with the cytoplasm of endothelial cells; rabbit monoclonal; 1:750 dilution; F3520, Sigma) for 30 min at room temperature. For VEGF, sections were incubated overnight at 4 C with anti-human VEGF antibody (reacts specifically with the 165, 189, and 121 amino acid splice variants of VEGF; rabbit polyclonal; 1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Control slides were prepared in an identical manner, except that the first antibody was replaced with normal 1 μ g/ml rabbit IgG. After washing with PBS twice, immunoreactions were detected by a MAX (PO) MULTI kit (Nichirei, Tokyo, Japan) according to the manufacturer's protocol. The sections were incubated with a second antibody conjugated with peroxidase and an amino acid polymer for 30 min at room temperature. Then, they were incubated with substrate solution consisting of 0.2 mg/ml 3,3'-diaminobenzidine-4HCl and 0.005% H₂O₂ in 0.05 M Tris-HCl (pH 7.5). The slides were rinsed with water and counterstained with hematoxylin. After washing with running tap water, the slides were dehydrated, cleared, and covered.

Classification of follicles

Follicles were histologically classified into healthy, early atretic, advanced atretic, and late atretic, as previously reported [26, 31, 32]. Healthy follicles had an intact granulosa layer with a

compact and well-organized arrangement. Atretic follicles were classified based on the criteria described by Xu *et al.* [33]. Briefly, early atretic follicles had a minimal number of pyknotic cells, degenerated cells, and/or apoptotic bodies distributed along the antral border of the granulosa layer. Advanced atretic follicles contained numerous pyknotic cells, degenerated cells, and/or apoptotic bodies in the granulosa layer, while the granulosa layer disappeared in late atretic follicles. The numbers of follicles examined, for SB and HF respectively, were as follows: healthy, 12 and 15; early atretic, 5 and 14; advanced atretic, 4 and 4; and late atretic, 7 and 6.

Analysis of vWF-positive capillary vessels and VEGF immunostaining

All available follicles with a diameter of 3 to 5 mm at the largest cross-sectional area were included in the analysis. Observation was conducted at 5 equidistant areas of the theca layer in each follicle (approximately 10,000 to 50,000 μm^2). The vWF-positive capillary vessels were counted using an image analyzer with a computer system (Mac Aspect, Mitani Co., Fukui, Japan) [34, 35]. The capillary vessel area contained both endothelial cells and the vessel lumen. The capillary vessel density and capillary vessel area in the theca interna and externa were expressed as values per 20,000- μm^2 area. The density and area of follicles was expressed as the average of 5 counts. The pattern of VEGF immunostaining was graded quantitatively as follows: partial, "+/-"; half, "+"; and whole "++". Observation was conducted at 5 equidistant areas of each follicle at 400 $\times$ magnification (10 $\times$ ocular lens, 40 $\times$ objective lens).

Statistical analysis

Differences in the vWF-positive capillary vessel density and area among different breeds, as well as among different follicular stages of atresia were analyzed by two-factor ANOVA, followed by Duncan's multiple range test (EXCEL Statistics Ver. 4; Esumi Co., Tokyo, Japan). Differences were considered to be significant at $P < 0.05$.

Results

Immunoassay of SB plasma samples showed that 60% of the animals were in the luteal phase

(progesterone level above 1 ng/ml). Due to a limited number of follicles available for examination, comparison of immunolocalization between animals with a progesterone level of <1 and >1 ng/ml was not performed. Likewise, the frequency distribution of the different follicle sizes in the different follicle types was not investigated.

In both SB and HF, the endothelial cells of the thecal capillary vessels of atretic follicles showed a greater affinity to anti-vWF antibody than healthy follicles (Fig. 1). Capillary vessels without staining for vWF were observed in the healthy follicles (Fig 1A and E). Atretic follicles also showed pyknosis of granulosa cells, and apoptotic body-like debris were present, indicating follicular atresia. Control slides showed a negative reaction, confirming the specificity of the antibody used in this study (pictures not shown).

In the theca interna, SB showed a significant increase in the density of vWF-positive capillary vessels as follicles progressed from healthy to late atretic follicles, while HF showed a plateau in early and advanced atretic follicles, which significantly declined as they progressed to the late atretic follicles (Fig. 2A). There were significant differences in density of vWF-positive capillary vessels in advanced and late atretic follicles between SB and HF. However, the area of vWF-positive capillary vessels significantly increased as atresia progressed in both breeds (Fig. 2B).

In the theca externa, density of vWF-positive capillary vessels significantly increased in the early atretic follicles of both breeds compared to those of healthy follicles (Fig. 2C). Moreover, late atretic follicles had a significantly higher number of vWF-positive capillary vessels than in the early atretic follicles of both SB and HF. HF late atretic follicles contained a significantly high number of vWF-positive capillary vessels as compared to SB. The area of vWF-positive capillary vessels significantly increased as follicles progressed to late atretic follicles in SB and advanced atretic follicles in HF (Fig. 2D).

Table 1 shows the distribution pattern of VEGF staining in the different stages of follicular atresia in SB and HF. A decrease in immunopositivity (granulosa and theca interna layers) was noted as follicles progressed from healthy to atretic follicles in both breeds. Both breeds gave a positive immunoreaction for anti-VEGF antibody in all atretic stages of follicles, with the granulosa layer

Fig. 1. The von Willebrand factor (vWF) immunostaining in the various stages of follicular atresia in buffaloes (A: healthy; B: early atretic; C: advanced atretic; D: late atretic) and Holstein-Friesian cows (E: healthy; F: early atretic; G: advanced atretic; H: late atretic). Solid arrows indicate a positive immunoreaction and arrowheads indicate a negative immunoreaction. G: Granulosa layer; Ti: Theca interna; Te: Theca externa. Scale bar = 10 μ m.

showing a better affinity for immunostaining than the theca layers (Fig. 3). In healthy follicles of both breeds, the entire granulosa layer was positively stained for VEGF antibody. However, VEGF reactivity decreased in atretic follicles, resulting in only a partial staining with VEGF antibody in advanced atretic follicles. In the theca interna of both SB and HF, half the layer was immunostained with VEGF antibody in the healthy follicles, whereas only a partial VEGF-positive layer was observed in all atretic follicles. Control slides showed a negative reaction, confirming the specificity of the antibody used in this study (data not shown).

Discussion

The preliminary findings from the present work are presented in this report. In SB, both density and area of vWF-positive capillary vessels in the theca interna increased significantly as follicles progressed from healthy to late atretic follicles. Increased expression of vWF can be associated in part to maturation and/or aging of blood vessels [9]. Therefore it is suggested that the atretic process is accompanied by an increase in matured or aged capillary vessels in the theca interna of SB. The present results for SB are similar to previous data we obtained from cattle [11]. The density of vWF-positive capillary vessels in the theca interna of SB increased continuously as follicles progressed from healthy to late atretic follicles, while HF showed a plateau in early and advanced atretic follicles, which significantly declined as they progressed to late atretic follicles. This suggests a difference in the changes of matured/aged capillary vessel density in the theca interna during the atretic process between SB and HF. Since the total number of capillary vessels was not altered during the atretic process [26], follicular progression to late atresia in HF reduces the density of only matured/aged capillary vessels. In the theca interna of late atretic follicles, a significantly increased density of vWF-positive capillary vessels was attained in SB compared with HF. However, there was no difference in the total number of capillary vessels between SB and HF in the theca interna of late atretic follicles [26]. These results indicate a greater ratio of matured/aged capillary vessel in SB than HF.

Fig. 2. Density (A and C) and area (B and D) of von Willebrand factor positive-capillary vessels in the theca interna (A and B) and externa (C and D) for various follicular stages of atresia. Bars represent the mean $\pm$ SE. The closed and open bars represent swamp buffaloes and Holstein-Friesian cows, respectively. Means with different letters (a, b, c, and d) represent a significant follicle type difference in the same breed and asterisks (*) represent a significant breed difference in the same follicle type. The level of significance is $P < 0.05$.

Table 1. Distribution patterns of VEGF immunolocalization in the various stages of follicular atresia. +/- partial; + half; ++ whole. (Gc: Granulosa cells, Ti: Theca interna cells)

Follicle type	Swamp buffalo		Holstein-Friesian	
	Gc	Ti	Gc	Ti
Healthy antral	++	+	++	+
Early atretic	+	+/-	+	+/-
Advanced atretic	+/-	+/-	+/-	+/-
Late atretic		+/-		+/-

In the theca externa, late atretic follicles of both SB and HF had a significantly higher density of vWF-positive capillary vessels than healthy and early atretic follicles. However, HF showed significantly higher density of vWF-positive capillary vessels than SB. This indicates a dramatic increase in the number of matured/aged capillary

vessels in late atretic follicles, especially in HF, which is quite different from SB. Since there was no difference in the total number of capillary vessels (matured and immature) in late atretic follicles between SB and HF [26], the ratio of matured capillary vessels to the total number of capillary vessels is higher in HF than SB. This result seems to be opposite to the case of the theca interna, suggesting a physiological difference in capillary vessel function in late atretic follicles between SB and HF.

In the present study, we demonstrated that VEGF was localized in the granulosa and theca layers in both SB and HF. This localization was high in the granulosa of healthy follicles in SB and HF. A similar observation was noted in bovine [36] and rat [37] ovarian follicles. These previous studies also noted that an increased ligand expression resulted in enhanced capillary

Fig. 3. Vascular endothelial growth factor (VEGF) immunostaining for the various stages of follicular atresia in buffaloes (A: healthy; B: early atretic; C: advanced atretic; D: late atretic) and Holstein-Friesian cows (E: healthy; F: early atretic; G: advanced atretic; H: late atretic). Arrows indicate a positive immunoreaction. G: Granulosa layer; Ti: Theca interna; Te: Theca externa. Scale bar = 10 μ m.

proliferation. Recently, it was reported that a close temporal association between VEGF expression and angiogenesis occurs in the equine corpus luteum [38]. Moreover, VEGF mRNA expression is temporally and spatially related to the proliferation of blood vessels in the ovary [39]. These findings support the concept that VEGF plays a major role as a potent regulator of angiogenesis.

Our immunohistochemical localization of VEGF suggests that the capacity of atretic follicles to produce VEGF is reduced as compared to healthy ones, which supports previous reports in other species [14, 40]. The density and area of vWF-positive capillary vessels increased in the SB theca interna, suggesting that vWF expression is negatively correlated to VEGF expression.

We observed that alteration of VEGF expression in the theca interna was slighter (between partial and half) than in the granulosa (between partial and whole) as follicles progressed from healthy to atretic follicles in SB, as well as in HF. The formation of vasculature depends on VEGF expression [13], and vasculogenesis in the theca interna may be attributable to the VEGF secreted by granulosa cells.

In conclusion, this study demonstrates for the first time in SB ovary that matured capillary vessels (vWF-positive) increase in the process of follicular atresia. Furthermore, VEGF is expressed in the granulosa cells as well as in the theca interna of SB, and this expression tends to decrease in atretic follicles. It is suggested that vWF and VEGF are associated with follicular atresia in a converse relationship.

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References

1. Meyer D, Pietu G, Fressinaud E, Girma JP. von Willebrand factor: structure and function. *Mayo Clin Proc* 1991; 66: 516–523.
2. Soylemezoglu O, Sultan N, Gursel T, Buyan N, Hasanoglu E. Circulating adhesion molecules ICAM-1, E-selectin, and von Willebrand factor in Henoch-Schonlein purpura. *Arch Dis Childhood* 1996; 75: 507–511.
3. Weiss HJ. von Willebrand factor and platelet function. *Annals New York Acad Sci* 1991; 614: 25–37.
4. Wagner DD, Olmsted JB, Marder VJ. Immunolocalization of von Willebrand protein in Weibel-Palade bodies of human endothelial cells. *J Cell Biol* 1982; 95: 355–360.
5. Cramer EM, Meyer D, le Menn R, Breton-Gorius J. Eccentric localization of von Willebrand factor in an internal structure of platelet alpha-granule resembling that of Weibel-Palade bodies. *Blood* 1985; 66: 710–713.
6. Manucci PM. Platelet von Willebrand factor in inherited and acquired bleeding disorders. *Proc Natl Acad Sci USA* 1995; 92: 2428–2432.
7. Woolf AD, Wakerley G, Wallington TB, Scott DG, Dieppe PA. Factor VIII related antigen in the assessment of vasculitis. *Annals Rheumatic Dis* 1987; 46: 441–447.
8. Bowyer SL, Ragsdale CG, Sullivan DB. Factor VIII related antigen and childhood rheumatic diseases. *J Rheumatol* 1989; 16: 1093–1097.
9. Augustin HG. Antiangiogenic tumour therapy: will it work? *Trends Pharmacol Sci* 1998; 19: 216–222.
10. Modlich U, Kaup FJ, Augustin HG. Cyclic angiogenesis and blood vessel regression in the ovary: blood vessel regression during luteolysis involves endothelial cell detachment and vessel occlusion. *Lab Invest* 1996; 74: 771–780.
11. Isobe N, Kawai H, Yoshimura Y, Nakao T. Changes in the localization of immunoreactive von Willebrand factor in microvascular network of bovine ovarian follicles during atresia. *Anim Sci J* 2001; 72: 473–482.
12. Isobe N, Yoshimura Y, Nakao T. Distribution of immunoreactive von Willebrand factor in the microvascular network of bovine cystic follicles. *Anim Sci J* 2002; 73: 123–129.
13. Reynolds LP, Grazul-Bilska AT, Redmer DA. Angiogenesis in the corpus luteum. *Endocrine* 2000; 12: 1–9.
14. Yamamoto K, de Waard V, Fearn C, Loskutoff DJ. Tissue distribution and regulation of murine von Willebrand factor gene expression *in vivo*. *Blood* 1998; 92: 2791–2801.
15. Zeng H, Dvorak HF, Mukhopadhyay D. Vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF) receptor-1 down-modulates VPF/VEGF receptor-2-mediated endothelial cell proliferation, but not migration, through phosphatidylinositol 3-kinase-dependent pathways. *J Biol Chem* 2001; 276: 26969–26979.
16. Matsumoto Y, Tanaka K, Hirata G, Hanada M, Matsuda S, Shuto T, Iwamoto Y. Possible involvement of the vascular endothelial growth factor-Flt-1-focal adhesion kinase pathway in chemotaxis and the cell proliferation of osteoclast precursor cells in arthritic joints. *J Immunol* 2002; 168: 5824–5831.
17. Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit B, Ferrara N. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 1998; 273: 30336–30343.
18. Tran J, Master Z, Yu JL, Rak J, Dumont DJ, Kerbel RS. A role for surviving in chemoresistance of endothelial cells mediated by VEGF. *Proc Natl Acad Sci USA* 2002; 99: 4349–4354.
19. Greenaway J, Connor K, Pedersen HG, Coomber BL, Lamarre J, Petrik J. Vascular endothelial growth factor and its receptor Flk-1/KDR, are cytoprotective in the extravascular compartment of the ovarian follicle. *Endocrinology* 2004; 145: 2896–2905.
20. De Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 1992; 255: 989–991.
21. Marion GB, Gier HT, Choudary JB. Micromorphology of the bovine ovarian follicular system. *J Anim Sci* 1968; 27: 451–465.
22. Jiang JY, Macchiarelli G, Tsang BK, Sato E. Capillary angiogenesis and degeneration in bovine ovarian antral follicles. *Reproduction* 2003; 125: 211–223.
23. Hay MF, Cran DG, Moor RM. Structural changes occurring during atresia in sheep ovarian follicles. *Cell Tissue Res* 1976; 169: 515–529.
24. Cran DG, Osborn JC, Rushton D. Thecal vasculature and oocyte maturation during follicular atresia in the sheep and pig. *Reprod Nutr Dev* 1983; 23: 285–292.
25. Shimizu T, Jiang JY, Sasada H, Sato E. Changes of mRNA expression of angiogenic factors and related receptors during follicular development in gilts. *Biol Reprod* 2002; 67: 1846–1852.
26. Feranil JB, Isobe N, Nakao T. Changes in the thecal vasculature during follicular atresia in the ovary of swamp buffalo. *J Reprod Dev* 2004; 50: 315–321.
27. Maala CP, Domingo R, Ducusin RJ. The anatomy of the temporary and permanent incisors of the

- carabao (*Bubalus bubalis*). *Phil J Vet Med* 1988; 25: 7–14.
28. **Isobe N, Nakao T.** Direct enzyme immunoassay of estrone sulfate in the plasma of cattle. *J Reprod Dev* 2002; 48: 75–78.
  29. **Isobe N, Nakao T, Uehara O, Yamashiro H, Kubota H.** Plasma concentration of estrone sulfate during pregnancy in different breeds of Japanese beef cattle. *J Reprod Dev* 2003; 49: 369–374.
  30. **Isobe N, Nakao T.** Direct enzyme immunoassay of progesterone in bovine plasma. *Anim Sci J* 2003; 74: 369–373.
  31. **Isobe N, Nakao T, Yoshimura Y.** Immunohistochemical localization of 3β -hydroxysteroid dehydrogenase in the granulosa and theca interna layers of bovine cystic follicles. *J Reprod Dev* 2003; 49: 227–233.
  32. **Feranil JB, Isobe N, Nakao T.** Cell proliferation in the atretic follicles of buffalo and cattle ovary. *Reprod Domest Anim* 2004; 39: 405–409.
  33. **Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RA.** Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. *Biol Reprod* 1995; 53: 951–957.
  34. **Isobe N, Yoshimura Y.** Localization of apoptotic cells in the cystic ovarian follicles of cows: a DNA-end labeling histochemical study. *Theriogenology* 2000; 53: 897–904.
  35. **Isobe N, Yoshimura Y.** Immunocytochemical study of cell proliferation in the cystic ovarian follicles in cows. *Theriogenology* 2000; 54: 1159–1169.
  36. **Berisha B, Schams D, Kosmann M, Amselgruber W, Einspanier R.** Expression and localisation of vascular endothelial growth factor and basic fibroblast growth factor during the final growth of bovine ovarian follicles. *J Endocrinol* 2000; 167: 371–382.
  37. **Koos RD.** Increased expression of vascular endothelial growth/permeability factor in the rat ovary following an ovulatory gonadotropin stimulus: potential roles in follicle rupture. *Biol Reprod* 1995; 52: 1426–1435.
  38. **Al-zi'abi MO, Watson ED, Fraser HM.** Angiogenesis and vascular endothelial growth factor expression in the equine corpus luteum. *Reproduction* 2003; 125: 259–270.
  39. **Phillips HS, Hains J, Leung DW, Ferrara N.** Vascular endothelial growth factor is expressed in rat corpus luteum. *Endocrinology* 1990; 127: 965–967.
  40. **Celik-Ozenci C, Akkoyunlu G, Kayisli UA, Arici A, Demir R.** Localization of vascular endothelial growth factor in the zona pellucida of developing ovarian follicles in the rat: a possible role in destiny of follicles. *Histochem Cell Biol* 2003; 120: 383–390.