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MORPHOMETRIC AND ANGIOGENIC CHANGES IN THE CORPUS LUTEUM OF NILI-RAVI BUFFALO (*Bubalus bubalis*) DURING ESTROUS CYCLE

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The present study was conducted to evaluate the morphological and vascular changes of corpus luteum in relation to functional status of reproductive system in Nili-Ravi buffalo (*Bubalus bubalis*). Blood samples and ovaries of 24 adult cyclic Nili-Ravi buffaloes were collected from the local abattoir of Faisalabad for this study. After the morphometry of both ovaries, corpora lutea were enucleated and luteal tissue was processed by routine paraffin tissue technique and stained with Hematoxylin-Eosin and Periodic Acid-Schiff (PAS) techniques. Mean weight of corpus luteum was 1.23±0.22, 3.15±0.10, 2.25±0.32 and 1.89±0.31g during the metestrus, early diestrus, late diestrus and proestrus/estrus, respectively. The plasma progesterone concentration was 1.68±0.37, 4.29±0.22, 3.89±0.33 and 0.34±0.14ng/ml while mean vascular density (mean number of vessels/10 microscopic fields at 400x) in corpus luteum was 6.33±0.99, 18.00±0.86, 11.50±0.76 and 2.83±0.60 during the metestrus, early diestrus, late diestrus and proestrus/estrus, respectively. Positive correlation ($r=0.936$, $p<0.05$) was ascertained between plasma progesterone concentration and vascular density. In conclusion, morphology of corpus luteum, its angiogenic changes and plasma progesterone concentration are positively correlated. Moreover, the ovary offers an excellent system to study physiologic angiogenesis and vascular regression. Clinical veterinary medicine may profit from understanding these control mechanisms. As a result, new methods to regulate fertility and new therapeutic options for angiogenesis-dependent diseases may be created.
Keywords: Buffalo, corpus luteum, angiogenesis, histomorphometry, estrous cycle, progesterone

INTRODUCTION

Buffaloes play a prominent role in rural livestock production, particularly in Asia. Poor reproductive efficiency is the primary factor affecting productivity in female buffalo by factors like inherent late maturity, poor estrus expression especially in summer, distinct seasonal reproductive patterns, and prolonged inter-calving intervals. The urgent need for improving the reproductive performance of buffalo necessitates a better understanding of the mechanisms controlling cyclical ovarian changes. Corpus luteum is a transitory ovarian endocrine gland which plays a central role in regulating estrous cycle and maintenance of pregnancy by secreting progesterone (Stocco *et al*., 2007) and a high number of progesterone receptors were observed on days 6 and 10 of the estrous cycle (Martin *et al.,* 2013). After ovulation the folliclular theca and granulose cells are transformed into a functional corpus luteum (CL) that becomes highly vascularised for its formation, maintenance and progesterone production. For the proper supply of blood, angiogenesis i.e. the formation of new blood vessels from pre-existing blood vessels is necessary (Plendl *et al*., 2002; Tamanini and Ambrogi, 2004). This bovine Corpus Luteum produces many vital angiogenic and vasoactive factors like the vascular endothelial growth factor (VEGF), the angiopoietin-1 and -2 (ANPT-1 and -2), basic fibroblast growth factor (bFGF), prostaglandin F2 ($PGF_2\alpha$), endothelin-1 (EDN1), angiotensin II (Ang II) and nitric oxide (NO) which regulate the secretion of Progesterone directly or indirectly (Miyamoto *et al.* 2009).

Corpus luteum maintenance and function are mainly dependent on angiogenesis, while the regression of CL is associated with its inhibition (Fraser *et al.,* 2005). The process of angiogenesis is regulated by the interaction of stimulatory, modulatory and inhibitory factors like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor (TGF), platelets derived growth factor (PDGF), interleukin-8 and angiopoieten-1 (Augustin, 2000; Stocco *et al.,* 2007). This (VEGF) system has the possible role in the regulation of luteal angiogenesis and its proliferation along with endothelial cells through their non-angiogenic property (Chouhan *et al*., 2013). There is a correlation between vascularization, and hormone production in the corpus luteum.

This study was planned with the hypothesis that morphological changes in the ovary, corpus luteum and plasma progesterone level reflect the functional status of reproductive system in buffalo. To check the hypothesis, correlation was determined among gross structural characteristics of ovary, vascular density of corpus luteum and plasma progesterone concentration during various stages of estrous cycle in the buffalo.

MATERIALS AND METHODS

Twenty four adult cyclic Nili-Ravi (*Bubalus bubalis*) buffaloes ranging from 4 to 8 years of age having normal clinical parameters (temperature, pulse rate and breathing rate) were selected from the local abattoir. Blood samples of 10 ml from each animal were collected at the time of slaughter in test tubes containing EDTA solution to determine the plasma progesterone concentration. The animals were slaughtered and reproductive organs were collected immediately after evisceration. The stage of estrous cycle was determined by the gross examination of the reproductive organs such as tonicity of uterus, size and consistency of the follicles and corpora lutea present on the ovaries as described previously (Sarkar *et al.,* 2010). Both ovaries from the 24 reproductively cyclic animals were collected and classified into four groups according to the stages of estrous cycle (proestrus/estrus, metestrus, early diestrus and late diestrus). The proestrus/estrus was from day 17 to 21 and day 1, metestrus was from day 2 to 5, early diestrus was from day 6 to 12 and late diestrus was from day13 to 16 of the cycle. Each group contained ovaries of 6 animals containing corpus lutea of various stages, i.e. developing corpus luteum, early corpus luteum, mature corpus luteum and regressing corpus luteum).

Corpora lutea were enucleated from the ovaries of each group by surgical method and the measurements were taken to determine weight, length, width, diameter and circumference by Vernier's caliper and electrical weighing balance. The volume was measured by graduated cylinder containing water.

Corpora lutea were processed for histological studies using hematoxylin and eosin and PAS. Briefly, after enucleation from the ovaries, the CLs were dissected into pieces of 1-2 cm size and fixed in 10% phosphate buffered formalin, subjected to dehydration in ascending grades of ethanol followed by clearing in xylene and embedding in paraffin. About 5µm thick sections were cut for staining.

The walls of blood vessels were marked using PAS technique. This reagent strongly reacts with the carbohydrates in the micro-vascular basement membrane and has been widely used as a marker for endothelial cells. For each buffalo, number of vessels was determined in 10 randomly chosen microscopic fields in each histological section, using a light microscope at 400X, connected to a computerized cell analysis system. All blood vessels in luteal tissue were evaluated equally without distinguishing their nature (arterioles, venules and capillaries).

The plasma samples extracted via centrifugation of collected blood samples were stored at -20ºC until analysis by radio-immune assay (RIA).

Statistical Analysis**:** The data were expressed as mean ± standard error of mean (SEM) values. Statistical comparison was done using one-way ANOVA. Difference at $P<0.05$ was accepted as significant. The 'r' values between size of the ovary, the corpus luteum, the vascular density and progesterone concentration were determined using Pearson's correlation coefficient.

RESULTS

Morphology and morphometry of corpora lutea**:** The mean weight of corpus luteum was 1.23±0.22, 3.15±0.10, 2.25±0.32 and 1.89±0.31gduring the metestrus, early diestrus, late diestrus and proestrus/estrus phases, respectively. The weight of corpus luteum of proestrus/estrus phase (1.23±0.22g) increased significantly ($P<0.05$) during early diestrus stage (3.15±0.10g) which gradually reduced in later stages of estrous cycle. It was actually due to the regression of corpus luteum that normally occurs at the end of diestrus (Table 1).

Table 1. Mean±SEM values of morphometric parameters of corpus luteum in the Nili-Ravi buffalo (*Bubalus bubalis*) during various phases of estrous cycle.

Parameter	Stage	Corpus luteum
Weight (g)	M	1.23 ± 0.22 c
	ED	3.15 ± 0.10 a
	LD	2.25 ± 0.32 ab
	P/E	1.89 ± 0.31 bc
	Mean	2.13 ± 0.19
Length (cm)	M	1.31 ± 0.13 b
	ED	1.78 ± 0.10 a
	LD	1.77 ± 0.13 ab
	P/E	1.60 ± 0.10 ab
	Mean	1.61 ± 0.07
Diameter (cm)	M	0.96 ± 0.14 b
	ED	1.44 ± 0.10 a
	LD	1.40 ± 0.08 a
	P/E	1.32 ± 0.07 ab
	Mean	1.28 ± 0.06
Circumference ($cm^{3)}$)	M	3.49 ± 0.36 b
	ED	4.95 ± 0.34 a
	LD	4.78 ± 0.22 a
	P/E	4.53 ± 0.25 ab
	Mean	4.44 ± 0.18

M = metestrus; ED: early diestrus; LD = late diestrus P/E proestrus/estrus. Different superscripts in each category indicate difference at $P<0.05$.

The mean length of the corpus luteum was 1.31±0.13, 1.78±0.10, 1.77±0.13 and 1.60±0 cm during the metestrus, early diestrus, late diestrus and proestrus/estrus, respectively (Table 1). The maximum length of the corpus luteum was during the early diestrus stages. The length of corpus luteum showed significant ($P<0.05$) increase from proestrus/estrus (1.31±0.13cm) to early metestrus (1.78±0.10cm) phase.

Like weight and length, the mean diameter (cm) of corpus luteum increased significantly from metestrus (0.96±0.14) to early diestrus (1.44±0.10) and later decreased in subsequent phases (1.40+0.08 in late diestrus and 1.32+0.07 in proestrus). However, these changes were non-significant (Table 1). The mean circumference (cm^3) of the corpus luteum was 3.49±0.36, 4.95±0.34, 4.78±0.22 and 4.53±0.25 during the metestrus, early diestrus, late diestrus and proestrus/estrus, respectively (Table 1). This increase in the circumference in the corpus luteum was found to be significant ($P<0.05$) from metestrus (3.49±0.36) to late diestrus (4.95±0.34).

Plasma Progesterone: Mean plasma progesterone concentration was 1.68±0.37, 4.29±0.22, 3.89±0.33 and 0.34±0.14 ng/ml during the metestrus, early diestrus, late diestrus and proestrus/estrous, respectively. It was found to be reasonably different in Nili-Ravi buffalo at various stages of estrous cycle (Fig. 1).

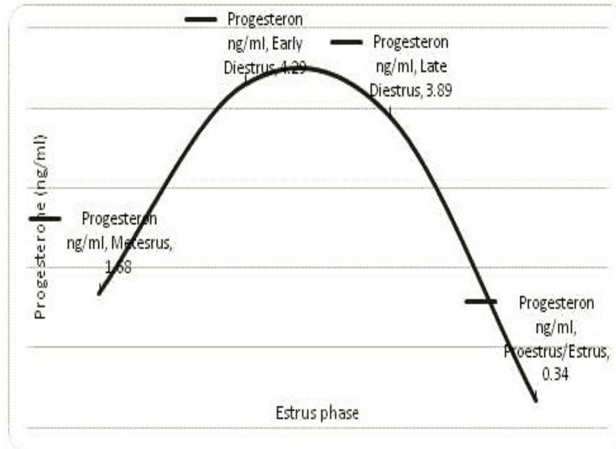

Figure 1. Mean±SEM values of plasma progesterone conc.(ng/ml) during various phases of estrous cycle in Nili-Ravi buffaloes (*Bubalus bubalis*).

The maximum level of plasma progesterone concentration was found to be during early diestrus stage and minimum at proestrus/estrus stage. From metestrus to early diestrus, plasma progesterone concentration suddenly increased and then it decreased gradually in late diestrus and proestrus/estrus stages (Fig. 1).

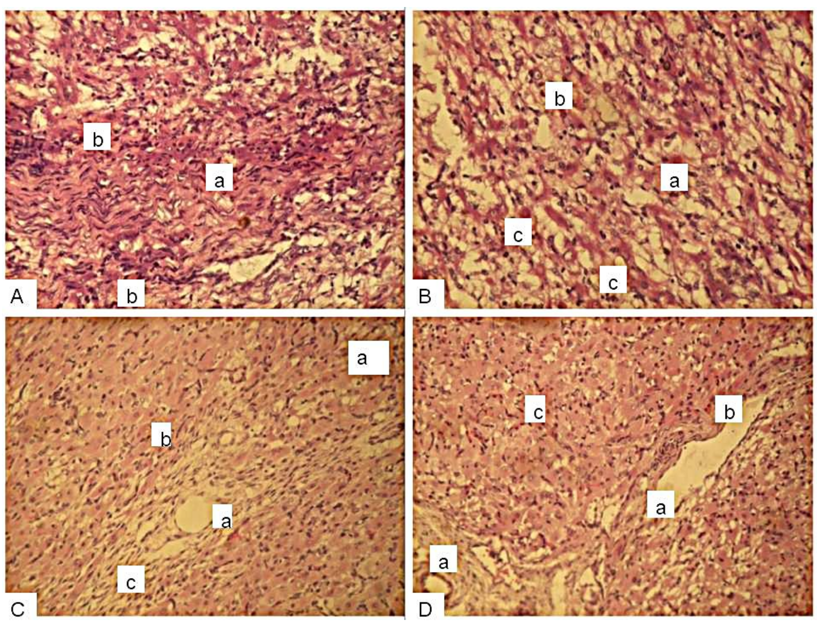

Figure 2. Corpus luteum of Nili-Ravi buffaloes (*Bubalus bubalis*) at various stages of estrus stage of estrous cycle (400X; H&E): A. Estrus stage: connective tissue fibers present in abundant quantity (a), apoptotic cells having no nucleus (b); B. Postestrus stage. Fibroblast cells of connective tissue (a), apoptotic cells having no nucleus (b), large luteal cells (c); C. Early diestrus stage. Blood vessel in corpus luteum (a) Large luteal cells with large spherical nucleus (b) Small luteal cells with small elliptical nucleus (c); D. Late diestrus stage: blood vessel in corpus luteum (a), large luteal cells with large spherical nucleus (b),small luteal cells with small elliptical nucleus (c).

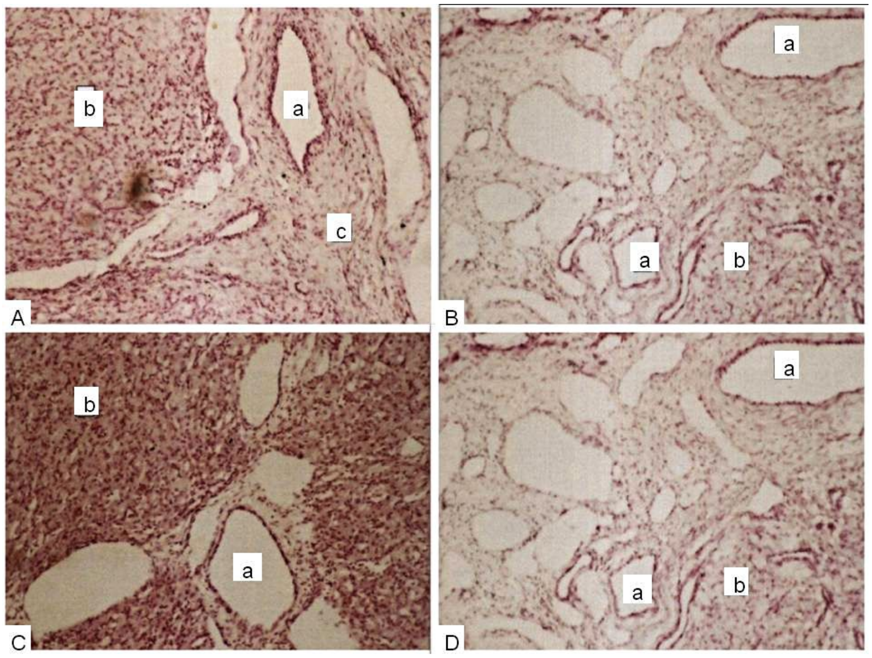

Figure 3. Corpus luteum of Nili-Ravi buffaloes (*Bubalus bubalis*) at various stages of estrus cycle 200X; PAS's reagent. A. Metestrus stage: blood vessels (a) luteal cells (b) fibers of connective tissue (c); B. Early diestrus stage: blood vessels (a) luteal cells (b); C. Late diestrus stage of estrous cycle. (a) blood vessels (b) luteal cells; D. Proestrus stage (a) blood vessels (a), luteal cells (b), fibers of connective tissue(c).

***Histology of corpus luteum*:** Corpus luteum of Nili-Ravi buffalo *(Bubalus bubalis)* at various stages of estrus stage of estrous cycle are presented in Fig 2-3. The corpus luteum of metestrus was observed to be consisted of well-defined lobular structure (Fig. 3A). Each lobe of corpus luteum consisted of newly forming luteal cells. The lobes were separated by a clear cut septa made of connective tissue. The blood vessels were also observed mostly in this area. The whole corpus luteum was surrounded by a capsule made of connective tissues.

***Angiogenesis in corpus luteum*:** Mean vascular density (no. of vessels/10 microscopic fields) of 6.33±0.99, 18.00±0.86, 11.50±0.76 and 2.83±0.60 in the CL during the metestrus, early diestrus, late diestrus and proestrus/estrus, respectively was found to be influenced by the stages of estrous cycle (Fig. 3). There was gradual increase in the vascular density from metestrus to early diestrus which decreased gradually to late diestrus. Vascular density in corpus luteum of various estrus phases was found to be significantly different (Table 2).

***Correlation between plasma progesterone and vascular density*:** In the present study, plasma progesterone concentration in Nili-Ravi buffalo was found to be highly correlated (r = 0.936; P<0.01) with vascular density in the corpus luteum during different phases of estrous cycle (Table 3).

Table 2. Mean±SEM values of angiogenesis in corpus luteum (mean no. of vessels) of Nili-Ravi buffalo (*Bubalus bubalis*) during various phases of estrous cycle.

Stage	Angiogenesis (Vascular density)
Metestrus	6.33 ± 0.99 c
Early Diestrus	18.00 ± 0.99 a
Late Diestrus	11.50 ± 0.76 b
Proestrus/Estrus	2.83 ± 0.60 d
Overall Mean	9.67 ± 1.67

Different superscripts indicate statistical difference at P<0.05.

Table 3. Pearson's Correlation matrix: showing 'r' values between progesterone concentration, angiogenesis, CL weight (wt), CL diameter (dia), and CL circumference (circ.).

	Prog.Conc.	Angio-	CL-Wt	CL-Dia	CL-Circ.
Prog.	1.000				
Angio-	0.936	1.000			
CL-Wt	-	0.488	1.000		
CL-Dia.	0.360	0.415		1.000	
CL-Circ.	0.447	0.487	-	0.852	1.000

Correlation is significant at 1 percent (**) and 5 percent (*) levels (2-tailed).

DISCUSSION

The values of length of corpus luteum in the present study were smaller than those described by (Noakes *et al*. 2009). The mean weight of corpora lutea in cows (Fields and Fields, 1996) was greater than the weight determined in Nili-Ravi buffaloes in the present study . The greater values of weight of corpus luteum in cow were due to larger size of the ovaries in this species as compared to buffaloes. Similar findings for bovine corpus luteum were also observed and reported that corpus luteum showed rapid weight gain from day 2-12post ovulation (Koos, 1993).

The values of plasma progesterone concentration in the present study were in the range as described earlier by Roy and Prakash (2009) in Murrah buffalo heifers. These values of progesterone in Nili-Ravi buffaloes were smaller than the values of plasma progesterone concentration in cows (Dolaldson *et al.* 1970). The higher values of plasma progesterone conc. in cows might be due to bigger size of bovine CL than that of buffalo. The plasma progesterone concentrations in cows (Fields and Fields, 1996) were also higher than the values determined in the present study . The values of plasma progesterone in cattle were 0.5 ng/ml on day 0 of estrous, cycle 3-7 ng/ml, on day 12 to 16 (Terblanche and Labuschagne, 1981). The values of plasma progesterone in present study were in line with this range. Similar findings were observed in the goats ($r = 0.81$; $P<0.05$). They also found a significant correlation ($r = 0.69$; $P< 0.01$) between size of the ovary and size of corpus luteum during the various stages of the estrous cycle. The values of vascular density in the present study were smaller than those reported for the goat. The values were 24.42±6.66, 36.26±5.61, 8.59±2.2 and 3.97±1.12 vessels/mm^2 for days 2, 12, 16 and 22 days of post ovulation in goats, respectively (Miranda-Moura *et al.* 2010). The smaller values of vascular density in buffaloes might be due to species difference. Similar results have been previously reported in buffalo cows (Moura *et al.* 2003) and cows (Zheng *et al.,* 1994). It is conceivable that morphology of corpus luteum, its angiogenic changes and plasma progesterone concentration are positively correlated. Moreover, the ovary offers an excellent system to study physiologic angiogenesis and vascular regression. Clinical veterinary medicine may profit from understanding these control mechanisms. As a result, new methods to regulate fertility and new therapeutic options for angiogenesis-dependent diseases may be created

REFERENCES

Augustin, H.G. 2000. Vascular morphogenesis in the ovary. Clin. Obstet. Gynecol. 14: 867-882.

Chouhan, V.S., R.P. Panda, V.P. Yadav, V. Babitha, F.A. Khan, G. Das, G.K. Gupta, M. Dangi, S.S.G. Singh, S. Bag, G.T. Sharma, B.D. Berisha, S. Schams and M. Sarka. 2013. Expression and localization of vascular endothelial growth factor and its receptors in the corpus luteum during oestrous cycle in Water buffaloes (*Bubalus bubalis*). Reprod. Dom. Anim. 48: 810–818.

Dolaldson, L.E., J.M. Bassett and G.D. Thorburn. 1970. Peripheral plasma progesterone concentration of cows during puberty, oestrous cycles, pregnancy and lactation and the effects of under-nutrition or exogenous oxytocin on progesterone concentration. J. Endocrinol. 48: 599-614.

Fields, M.J. and P.A. Fields. 1996. Morphological characteristics of the bovine corpus luteum during the estrous cycle and pregnancy. Theriogenology 45: 1295-1325.

Fraser, H.M., H. Wilson, K.D. Morris, I. Swanston and S.J. Wiegand. 2005. Vascular endothelial growth factor trap suppresses ovarian function at all stages of the luteal phase in the macaque. J. Clin. Endocrinol. Metab. 90: 5811-5818.

Koos, R.D. 1993: Ovarian angiogenesis. In: E.Y. Adashi and P.C.K. Leung (eds.), The Ovary. Raven Press, New York

Martin, I., M.M.P. Rodrigues, C.J. Fujiharaa, W.C.M. Filhoa, E. Obaa, L. Laufer-Amorimb and J.C.P. Ferreira. 2013. Localization patterns of steroid and luteinizing hormone receptors in the corpus luteum of Nelore (*Bos taurus indicus*) cows throughout the estrous cycle. Livest. Sci.155: 442-453.

Miranda-Moura M.T.M., V.U. Fonseca, N.B. Silva, M.L. Freitas, O.B. Almeida, H.A.O. Rocha, P.C. Papa and C.E.B. Moura. 2010. Morphological features and vascularization study of caprine cyclic corpus luteum. Pesquisa. Vet. Bras. 30: 351-357.

Miyamoto, A., K. Shirasuna and K. Sasahara. 2009. Local regulation of corpus luteum development and regression in the cow: Impact of angiogenic and vasoactive factors. Dom. Anim. Endocrinol. 37: 159–169.

Moura, C.E.B., D.B. Campos, F.T.V Pereira, M.F. Oliveira, M.A. Miglino and P.C. Papa. 2003. Density of Corpus luteum from superovaluted and control buffalo cows. Acta Microsc. 12: 43-44.

Noakes, D.E., T.J. Timothy, G.C. Parkinson and W. England. 2009. Arthur's Veterinary Reproduction and Obstetrics 9th ed. Saunders Elsevier, USA.

Plendl, J., C. Neumueller, A. Vollmar, R. Auerbach and F. Sinowatz. 2002. Isolation and characterization of endothelial cells from different organs of fetal pigs. Anat. Histol. Embryol. 194: 445-456.

Roy, K.S. and B.S. Prakash. 2009. Plasma progesterone, oestradiol-17β and total oestrogen profiles in relation to oestrous behaviour during induced ovulation in Murrah buffalo heifers. J. Anim. Physiol. Anim. Nutr. (Berl.). 93: 486-95.

Sarkar, M., S. Schilffarth, D. Schams, H.H. Meyer and B. Berisha. 2010. The expression of leptin and its receptor during different physiological stages in the bovine ovary. Mol. Reprod. Dev. 77: 174–81.

Stocco, C.C. Telleria and G. Gibori, 2007. The molecular control of corpus luteum formation, function and regression. Endocrinol. 28: 117- 49.

Tamanini, C.M. and M. De Ambrogi. 2004. Angiogenesis in developing follicle and corpus luteum. Reprod Domest. Anim. 39:206-16.

Terblanche, H.M. and J.M. Labuschagne. 1981. Plasma progesterone in cattle. II. Levels during the oestrous cycle, pregnancy and parturition. J. S. Afr. Vet. Assoc. 52:187-89.

Zheng, J., P.M. Fricke, L.P. Reynolds and D.A. Redmer. 1994. Evaluation of growth, cell proliferation, and cell death in bovine corpora lutea throughout the estrous cycle. Biol. Reprod. 51: 623-632.