LUTEAL BLOOD FLOW AND GROWTH IN CORRELATION TO CIRCULATING ANGIOGENIC HORMONES AFTER SPONTANEOUS OVULATION IN MARES

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Summary

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For evaluating corpus luteum (CL) function, blood flow, circulating nitric oxide (NO), estradiol (E₂), progesterone, leptin and insulin like growth factor-I (IGF-1) concentrations were measured for 11 days that were equally divided into early and mid-luteal phases. Five mares underwent blood sampling and rectal Doppler ultrasound examination for 18 days for two estrous cycles. CL diameter, circumference, area and volume increased till day 11. Both blood flow blue area and power area increased from day 1 to 11 but colour blood flow red area declined till day 11. NO concentration increased till day 5 then decreased till day 11. Leptin concentrations declined from day 1 to 11. Power blood flow area and colour blood flow blue area exhibited a significant negative correlation with E₂ ($r = -0.79$; -0.75), leptin ($r = -0.73$; -0.51), and IGF-1 ($r = -0.56$; -0.60), but had a positive one with progesterone ($r = 0.47$; 0.52). Days after ovulation and luteal phase affected significantly luteal blood flow and hormone concentrations. The relatively high E₂, leptin, IGF-1 and NO during growth compared to static phase indicated a role enhancing angiogenesis and early CL development.

Key words: Doppler, IGF-1, leptin, luteal vascular perfusion, mare, nitric oxide

INTRODUCTION

The corpus luteum (CL) is a small, transient endocrine gland formed following ovulation from the secretory cells of the ovarian follicles. The main secretory product of CL is progesterone, which regulates various reproductive functions (Khanghah & Kor, 2013). The extremely rapid CL tissue growth and angiogenesis

rival those of even the fastest growing tumours (Reynolds *et al.*, 2000). The luteal vascular tree is composed of arterioles and venules in the periphery of the CL; however, most of its area is composed of capillaries (Smith & Meidan, 2014). During the CL life span, endocrine and vascular changes are dependant on the

luteal angiogenesis in the mare (Ferreira-Dias *et al.*, 2006a,b). Doppler ultrasonography was used to determine normal and irregular alterations in the mare reproductive tract (Ignácio *et al.*, 2011). In mares, the power Doppler display was used to evaluate CL (Bollwein *et al.*, 2002a) and colour Doppler was also used to study luteal blood flow after induction of luteolysis (Ginther *et al.*, 2007b). In horses, a close relationship between luteal blood flow and progesterone during the entire estrous cycle was found (Bollwein *et al.*, 2002a; Ginther *et al.*, 2007a). Ovarian artery of the mare was also used to evaluate CL which can be also done with the measurement of the CL area with coloured signals as an indicator of the extent of blood flow (Ignácio *et al.*, 2011).

One of the growth factors that the CL produces and secretes is the insulin-like growth factor and one of the vasoactive factor that regulate luteal formation and development is nitric oxide (NO) (Miyamoto *et al.*, 2010). In the ovary, insulin-like growth factor (IGF) is one of the numerous local factors that regulate the development of new capillaries from pre-existing ones (Berisha *et al.*, 2016). Components of the IGF system were found within equine ovarian follicle (Donadeu & Ginther, 2002) and played an important role in stimulating luteal angiogenesis (Chouhan *et al.*, 2015). Insulin-like growth factor (IGF)-I had a stimulatory effect on leptin receptor expression of luteal cells (Gregoraszczuk *et al.*, 2007).

NO synthesis has been localised to granulosa luteal cells identified by immunocytochemistry (Zackrisson *et al.*, 1996). NO is a potential mediator of luteal development, maintenance, angiogenesis, and blood flow (Ferreira-Dias *et al.*, 2011). The direct role of NO on CL blood flow as a local vasodilator depended on

the CL stage (Acosta & Miyamoto, 2004). In the mare, NO seems to be involved in CL development via stimulation of angiogenesis and progesterone secretion (Ferreira-Dias *et al.*, 2011).

Leptin is secreted by adipocytes and might actively participate in the regulation of NO production (Tsuda & Nishio, 2004). Leptin has been implicated in ovarian function, has immunoregulatory and angiogenic effects (Sierra-Honigsmann *et al.*, 1998). Physiological doses of leptin had induced endothelial nitric oxide synthase (eNOS) expression, which compensates, in part, for a lack of NO production by eNOS to maintain endothelium-dependent relaxation and leptin increased the plasma levels of nitrite, a marker for NO (Benkhoff *et al.*, 2012). Leptin receptors are expressed not only in vascular but also in circulating cells (Benkhoff *et al.*, 2012).

This study aimed to characterise the CL function and vascularity by evaluating CL dimensions (diameter, area, volume, and circumference), circulating leptin, NO, progesterone, estradiol, insulin like growth factor-I and vascularisation using colour and power Doppler ultrasound in spontaneously ovulated mares.

MATERIALS AND METHODS

Animals

Five non-lactating European × Egyptian crossbred broodmares (3–12 years old) of moderate body condition were subjected to ultrasonographic examination throughout two estrous cycles for each first 11 days after ovulation. Mares were given one week off rectal examinations between the two estrous cycles. Mares were granted from the Training Department (El-Basateen Horsley, Ministry of interior) and kept in an indoor paddock with partition individually with a pubertal colt

at the end of the same stable to confirm estrus signs. Mares were kept under natural light and temperature and artificial light was used at night within the paddock. They were maintained on a commercial pelleted ration and hay with free access to water. Before directing this work, mares were examined 3 times at weekly interval by ultrasound to affirm ovarian cyclicity and ovulation. This study was conducted at the Department of Theriogenology, Faculty of Veterinary Medicine, Cairo University (30.0276°N, 31.2101°E) from June 15 to July 28, 2014. The experiment was conducted in accordance with the Institutional Animal Care and Use Committee.

Ultrasound scanning

A pulsed-wave Doppler ultrasound scanner equipped with 12 MHz linear-array transrectal transducer (Sonovet R3, Medison, Samsung, South Korea) was used for the examination of ovaries, by scanning follicles with a diameter > 1 cm in both ovaries for deciding the ovulatory one and the developing CL thereafter. Developing CL at the site of the ovulatory follicle was scanned in colour- and power Doppler modes. All scans were performed by the same operator during the study. All examinations were carried out at the same time (8:00–10:00 h a.m.) to avoid the high temperature and humidity. Days from 1 to 11 corresponded to days after ovulation when the CL was dominant as the disappearance of the large ovulatory follicle was considered the day of ovulation. After day 11, the next ovulatory follicle attracted all the interest.

Measurement of CL blood flow

Whenever luteal tissue could be visualised, three different intersecting diameters of the CL were measured to estimate vo-

lume ($4/3\pi \cdot D_1/2 \cdot D_2/2 \cdot D_3/2$) and area ($\pi \cdot D_1/2 \cdot D_2/2$) using mathematical equations for ellipsoids assuming that the CL is a sphere with longer and thinner shape form ellipsoid (Bollwein *et al.*, 2002a). Several scans of Doppler colour mode and the power mode of the luteal tissue were stored at the recording, with the maximum area, without flash artifacts, and with the maximum number of colour pixels in the CL. When the image on the screen was free of artifacts, the entire area, with a careful slow motion of the probe was recorded (Bollwein *et al.*, 2002a; Ginther *et al.*, 2007a). Either colour or power Doppler mode was activated; the blood flow area within the CL was quantified from the colour images. All scans were done at a pulse-repetition frequency and identical colour gain settings were used.

Areas of colour represented regions with a flow velocity more than 10 mm/s. The colour mode was used to determine the blood flow area within CL. The angle of insonation was not computed as a result of the small diameter of vessels, so care was taken to obtain maximum colour intensity.

Image analysis

Digital video recording was exported from the hard disk of the ultrasound machine to a PC. Three good pictures of CL (both in B-, colour- and Power-Mode) were chosen. Afterward, the chosen pictures were processed using image analysis software (Adobe PhotoShop CC software (1990–2013, Adobe Systems) for further detailed digital image analysis. The region of each CL was manually selected by the magnetic lasso tool in Photoshop and the following parameters were analysed: luteal blood flow (LBF) area away from the probe, which coloured blue (pixels), blood flow area toward the probe, which coloured red

(pixels) and single colour power blood flow area (pixels). The mean values of the three selected images were calculated and one mean value was used as referential. Percents of the coloured pixels were calculated by dividing coloured power area by the total CL area (in pixels) and was used as a quantitative index of the changes in blood flow within the luteal tissue.

Blood sampling and hormonal assaying

Daily blood samples were obtained from the jugular vein shortly after ultrasound examination using vacutainer tubes with anticoagulant (K_3 EDTA), then centrifuged at 2000 rpm for 10 min. Plasma was harvested and stored at -18°C until hormone assaying. All hormones were assayed using commercial ELISA (DRG, International, Inc., USA). Progesterone sensitivity was 0.045 ng/mL. Intra- and inter-assay coefficients of variation of progesterone assay were 6.86 and 5.59%. Estradiol sensitivity was 9.714 pg/mL but respective intra- and inter-assay coefficients of variation were 2.71 and 6.72%. Leptin sandwich sensitivity was 1.0 ng/mL, with intra- and inter-assay coefficients of variation of 3.1 and 9.7%. Insulin like growth factor-I (IGF-I) assay sensitivity was 1.29 ng/mL (intra- and inter-assay coefficients of variation of 6.62% and 7.79%). Nitric oxide metabolites (NOMs) were measured by mixing equal volume of serum samples with freshly prepared Griess reagent, incubation for 10 min at room temperature; then absorbance was read at 540 nm against nitrite (NO_2) standards (0–50 μM) using a microtiter plate reader as previously measured in our laboratory (Abdelnaby *et al.*, 2016).

Statistical analysis

Data are presented as mean $\pm$ SEM (standard error of mean) using SPSS software

(2007). Simple one-way ANOVA was used to study the influence of days after ovulation on CL area, volume, hormones and blood flow parameters (area of red colour toward the probe, blue colour away from the probe and power- flow Doppler mode). Data are presented in plots with error bars. The effect of luteal phase was also evaluated using independent sample *t*-test where early growth luteal phase (days 1 to 5) and mid-luteal static phase (days 6 to 11) after ovulation were compared. The Pearson correlation coefficient was also calculated.

RESULTS

Days after ovulation changed significantly ($P=0.0001$) both area (cm^2) and volume (cm^3) of the corpus luteum (CL). From day 1 after ovulation until day 11, area (9.30 ± 0.46 to 16.43 ± 1.35) and volume (14.47 ± 1.11 to 33.97 ± 4.21) of CL increased significantly (Fig. 1). Values of CL circumference increased linearly from day 1 to 11.

The area of colour signal away probe (blue/pixel) and power mode significantly ($P<0.0001$) increased during mid-luteal static phase but the area of the colour signal toward probe (red/pixel) and % of coloured pixels significantly ($P<0.01$) decreased (Table 1). Days after ovulation affected significantly ($P<0.0001$) vascular perfusion of the CL, at which blood flow area away from the probe (Fig. 2) increased significantly from day 1 (1621 ± 39.8) reaching a maximum on day 11 (3355 ± 189). The blood flow area toward the probe (red) decreased significantly ($P=0.0001$) from day 1 (1417 ± 57.8) until day 11 (1021 ± 76.1). Contrary, the power blood flow area/pixel significantly ($P=0.001$) increased from day 1 post ovulation (2626 ± 319.1) reaching a

Fig. 1. Area (cm²), volume (cm³), colored pixels (%) and nitric oxide concentrations (µmol/L) during the first 11 days after ovulation. Data are presented as mean ± SEM, n=10.

Table 1. Area of colour blood flow away from the probe, toward the probe, power blood flow and percent of coloured pixels for power blood flow mode of mare CL during the early and mid-luteal phase. Data are presented as mean ± SEM, n=10

Blood flow area (px)	Early (growth) phase: days 1 to 5	Mid (static) phase: days 6 to 11	Total: days 1 to 11	P value
Away from the probe (blue)	1808.13±41.04	2623.24±81.75	2241.16±86.75	0.0001
Toward the probe (red)	1354.47±24.31	1141.65±24.37	1241.41±25.53	0.0001
Power-mode	2678.20±71.99	3626.06±102.99	3181.75±105.94	0.0001
Coloured pixels %	13.39±0.39	11.89±0.36	12.59±0.29	0.007

maximum on day 11 (3981±216.5) with a slight decline on day 2 (2491±31.9). The percent of coloured pixels of power blood flow mode was significantly affected by days after ovulation (P=0.03). It was significantly high on day 1, but the lowest coloured pixels percent was observed on day 10 (Fig. 1).

NO (µmol/L) concentration (Fig. 1) increased significantly (P=0.0001) from day 1 (26.52±1.82) reaching a maximum level on day 5 (56.53±4.69) then de-

creased again to a minimum on day 11 (22.47±0.80). NO decreased significantly (P=0.007) during the luteal static phase compared to the luteal growth phase (Table 2). In contrast, leptin (ng/mL) concentration significantly (P=0.003) decreased gradually from day 1 (17.51±0.27) to reach a minimum level on day 10 (6.55±3.04). Leptin (P=0.0001) concentrations were significantly lower during the luteal static phase compared to the luteal growth phase (Table 2). Progester-

rone (P₄; ng/mL) levels (Fig. 2) significantly increased ($P=0.005$) from day 1 post ovulation from 8.65 ± 2.58 reaching 15.70 ± 0.56 on day 10. Progesterone levels (ng/mL) were significantly ($P=0.0001$) high during static phase than during the luteal growth phase. Days after ovulation significantly affected IGF-1 ($P=0.007$) and estradiol (E₂; $P=0.0001$) levels.

Estradiol decreased significantly in a linear fashion from similar levels on days

1 and 2 post ovulation to significantly low concentrations on day 7 (Fig. 3). Then after a short increase on day 8 its levels decreased again to low values on day 10. Concentrations of insulin like growth factor-1 (IGF-1) were significantly high from day 1 to day 6 then started to decline linearly from day 7 to day 11 (Fig. 3). IGF-1 ($P=0.0001$) and estradiol ($P=0.0001$) levels were significantly low during the

Fig. 2. Blood flow areas (colour-flow mode, power-flow mode) in pixels, progesterone (ng/mL) and leptin (ng/mL) concentrations during the first 11 days after ovulation. Data are presented as mean $\pm$ SEM, $n=10$.

Table 2. Circulating progesterone, leptin, nitric oxide metabolites, estradiol and insulin-like growth factor-1 (IGF-1) of mare during the early and mid-luteal phases. Data are presented as mean $\pm$ SEM, $n=10$

	Early (growth) phase: days 1 to 5	Mid (static) phase: days 6 to 11	Total: days 1 to 11	P value
Progesterone (ng/mL)	11.91 $\pm$ 0.72	14.88 $\pm$ 0.30	13.49 $\pm$ 0.45	0.0001
Leptin (ng/mL)	16.30 $\pm$ 0.36	9.83 $\pm$ 1.08	12.87 $\pm$ 0.83	0.0001
Nitric oxide (μ mol/L)	39.08 $\pm$ 3.18	29.74 $\pm$ 1.149	34.12 $\pm$ 1.79	0.007
Estradiol (pg/mL)	190.11 $\pm$ 4.72	137.99 $\pm$ 5.57	163.21 $\pm$ 5.97	0.0001
IGF-1 (ng/mL)	327.41 $\pm$ 13.90	256.32 $\pm$ 12.71	290.72 $\pm$ 11.29	0.001

Fig. 3. Corpus luteum circumference (px), estradiol (E2, pg/mL) and insulin like growth factor-I (IGF-1, ng/mL) concentrations during the first 11 days after ovulation. Data are presented as mean $\pm$ SEM, n=10.

mid-luteal static phase compared to the luteal growth phase (Table 2).

Correlations between CL dimensions, blood flow parameters and circulating hormones are presented in Table 3.

DISCUSSION

The day after ovulation significantly affected the area of CL in the present study and CL area of pony mares (Ginther *et al.*, 2007a). After spontaneous ovulation, the progressive increase of CL area from day 1 to day 11 was also observed after induced ovulation from day 0 to day 8 (Romano *et al.*, 2015). In contrast to the steady increase of luteal area and volume from day 1 till day 11 recorded during this study, CL area increased after ovulation and reached their maximum on day 2 (Bollwein *et al.*, 2002a), day 4 (Ginther *et al.*, 2007a) and mid-luteal phase (Ferreira-Dias *et al.*, 2006b) followed by a slow decrease in cross-sectional area up to day 15 (Bollwein *et al.*, 2002a). The positive correlation between CL area, volume and

progesterone in mares in this study was also reported in pony mares (Ginther *et al.*, 2007a).

Similarly, the day after ovulation had significantly affected the percentage of luteal area with colour signals of pony mares (Ginther *et al.*, 2007a). In spontaneously ovulated mares of the current study, the increase of luteal vascularisation from day 1 to day 11 was also observed from day 0 to day 8 after induced ovulation (Romano *et al.*, 2015). Based on histologic sections of all blood vessels in luteal tissue, the microvascular density increased during early and mid-luteal phase of equine corpora lutea (Ferreira-Dias *et al.*, 2006b). Similar to the acute increase in luteal blood flow in mature CL periphery which correlated directly to progesterone, cyclic changes and individual differences in CL vascularisation were previously recorded in mares and the number of coloured pixels using power mode rose distinctly after ovulation until day 5, then remained at a constant high level until day 7 (Bollwein *et al.*, 2002a). The cyclic changes of luteal vascularisa-

Table 3. Pearson correlations coefficients between corpus luteum (CL) dimensions, blood flow (BF) areas and estradiol (E₂); progesterone (P₄); insulin-like growth factor (IGF-1); leptin and nitric oxide (NO) between days 1 to 11 of the estrous cycle (day 0 = ovulation)

	CL volume	CL area (px)	CL area (cm ²)	CL circumference	Power BF area	Blue BF area	Red BF area	E ₂	P ₄	IGF-1	Leptin	NO
CL diameter	0.98 ^a	0.77 ^a	0.98 ^a	0.87 ^a	0.70 ^a	0.60 ^a	-0.78 ^a	-0.63 ^a	0.63 ^a	-0.43 ^b	-0.89 ^a	-0.33
CL volume	1	0.81 ^a	0.99 ^a	0.91 ^a	0.68 ^a	0.64 ^a	-0.82 ^a	-0.65 ^a	0.60 ^a	-0.52 ^b	-0.92 ^a	-0.39 ^c
CL area (px)		1	0.81 ^a	0.72 ^a	0.80 ^a	0.83 ^a	-0.87 ^a	-0.85 ^a	0.64 ^a	-0.70 ^a	-0.74 ^a	-0.44 ^b
CL area (cm ²)			1	0.92 ^a	0.66 ^a	0.63 ^a	-0.80 ^a	-0.64 ^a	0.63 ^a	-0.50 ^b	-0.91 ^a	-0.39 ^c
CL circumference				1	0.45 ^b	0.52 ^b	-0.77 ^a	-0.54 ^b	0.62 ^a	-0.52 ^b	-0.84 ^a	-0.33
Power BF area					1	0.63 ^a	-0.75 ^a	-0.79 ^a	0.47 ^b	-0.56 ^a	-0.73 ^a	-0.30
Blue BF area						1	-0.68 ^a	-0.75 ^a	0.52 ^b	-0.60 ^a	-0.51 ^b	-0.38 ^c
Red BF area							1	0.79 ^a	-0.49 ^b	0.77 ^a	0.80 ^a	0.26
E ₂								1	-0.64 ^a	0.60 ^a	0.63 ^a	0.28
P ₄									1	-0.18	-0.50 ^b	-0.06
IGF-1										1	0.53 ^b	0.37 ^c
Leptin											1	0.36 ^c

Correlation is significant at ^a P<0.0001, ^b P<0.01, ^c P<0.05.

tion observed in mares of our study using colour mode were synchronous to the cyclic changes of uterine vascularisation of the same mares (Abdelnaby *et al.*, 2015) and the cyclic changes of uterine artery measured by pulsatility index (PI) during untreated and estrogen-treated cycles (Bollwein *et al.*, 2004). The increased resistance index (RI) indicated low blood flow and the reverse is true (Ferreira-Dias *et al.*, 2011). Contrary to the changes of vascular perfusion determined using colour Doppler, the high PI values observed on day 1, decreased until day 5, and re-increased after that till day 15 as reported by Bollwein *et al.* (2004) whereas the luteal area of coloured pixels of mares of this study transiently declined during day 2 but continued increasing after that until day 11. Mean percentage of CL with colour-power Doppler signals for blood flow was high on days 1 and 6 but was maximum on day 10 in pony mares (Ginther *et al.*, 2007a).

The steady increase of circulating P_4 levels from day 1 to day 10 in our mares was similar to the progressive increase of plasma P_4 presented after induced ovulation from day 0 to day 8 in mares (Romano *et al.* 2015), pony mares (Ginther *et al.*, 2007a) and till mid-luteal phase (Ferreira-Dias *et al.*, 2006b). P_4 levels depended primarily on the luteal blood flow (Acosta *et al.*, 2002). A close relationship between luteal blood flow and P_4 has also been found in horses during the entire estrous cycle (Bollwein *et al.*, 2002a; Ginther *et al.*, 2007a). In mares, the close correlation between CL coloured pixels with P_4 and area (Bollwein *et al.*, 2002a) emphasises that a high luteal blood supply represents an important precondition for the secretion of P_4 . No indication was found that an acute increase or decrease in luteal blood flow occurred prior to the

precipitous decrease in plasma P_4 in pony mares (Ginther *et al.*, 2007a). The absence of relationship between diameter of the CL and PI of the ipsilateral ovarian artery during diestrus (Bollwein *et al.*, 2002b) was quite different from our results but the negative correlation between PI and P_4 (Bollwein *et al.*, 2002b) was similar to the positive correlation between P_4 and power Doppler vascularisation area that could be referred to the close contact of majority of the steroidogenic cells of the CL with the capillaries (Reynolds *et al.*, 1992). The circulation of the CL depended mainly on the day of the estrous cycle, so vascularisation precedes P_4 synthesis (Acosta & Miyamoto, 2004).

During early luteal development in mares of the present study, the increased circulating NO levels were accompanied by increased luteal blood flow and this could be attributed to NO from large arterioles surrounding CL (Miyamoto *et al.*, 2005) and the role of NO and other factors in the CL blood flow increase (Skarzynski *et al.*, 2008; Miyamoto & Shirasuna, 2009). *In vitro*, NO donor increased the secretion of P_4 and $PGF_{2\alpha}$ in early equine luteal tissue but not in mid-luteal tissue proving that NO may play a role in CL growth during early luteal development when vascular development is more intense (Ferreira-Dias *et al.*, 2011) and indicated an angiogenic effect on CL. Locally eNOS protein was shown to be highly expressed in the mare early CL when NO had stimulated luteal tissue for angiogenic factors production (Ferreira-Dias *et al.*, 2011). The decrease of circulating NO associated with the increase of P_4 during luteal static phase in mares may be referred to increased levels of NO or NO-releasing agents that can inhibit steroidogenesis in granulosa and luteal cells (Van Voorhis *et al.*, 1994), since NO pro-

duced locally inhibits NOS activity in ovarian tissue and has anti-steroidogenic effect, diminishing serum progesterone (Motta *et al.*, 1997). The long-lasting effects of P₄ and its precursor may inhibit angiogenic factor(s) production by equine mid-stage luteal cells, preparing for CL functional and structural regression (Ferreira-Dias *et al.*, 2006a).

Leptin and insulin-like growth factor-I were considered angiogenic factors (Sarkanen *et al.*, 2012). Treatment with leptin led to a concentration-dependent increase in cell number which was associated with an enhanced formation of capillary-like tubes in an *in vitro* angiogenesis assay and neovascularisation in an *in vivo* model of angiogenesis (Bouloumie *et al.*, 1998). Ovaries are able to produce small amounts of leptin which modulate their function during different reproductive conditions (Fruhbeck, 2006). Both circulating and locally produced leptin may be involved in the luteal angiogenic process through regulation of potent angiogenic hormones and both leptin and its receptors had been identified in luteal tissue and were hypothesised to influence luteal vasculature through the regulation of PGF_{2α} (Wiles *et al.*, 2014). The high serum leptin levels during early luteal growth compared to mid-luteal static phase of mares could be attributed to the decreased expression of leptin receptors during the mid-luteal stage compared to the early stage (Wiles *et al.*, 2014). Also, the relaxing effect of leptin on blood vessels was partially mediated by the NO-dependent pathway and phenylephrine-contracted arterial rings were relaxed by leptin that mediated through endothelial NO production (Kimura *et al.*, 2002).

In several species, IGF-1 enhances ovarian activity by stimulating granulosa and thecal cell proliferation and mitogene-

sis (Deichsel *et al.*, 2006). The spreading of capillaries appears to be induced by growth factors such as IGF-1 for supporting the synthesis of progesterone (Khanghah & kor, 2013). The increased levels of IGF-1 during early luteal growth phase could be referred to their actions in enhancing ovarian activity through stimulating thecal cell proliferation and mitogenesis (Deichsel *et al.*, 2006). Additionally, products of luteal origin such as E₂ and insulin like growth factor-1 (IGF-1) play a role in the regulation of these cells from apoptosis (Devoto *et al.*, 2009). The positive correlation previously recorded between E₂ and leptin during foal heat in lactating Arabian mares (Abo El-Maaty & Gabr, 2010) was also observed during the luteal phase of non-lactating mares but even higher and explain the synergistic actions of both hormones in regulating ovulation and corpus luteum development.

It could be concluded that power and colour blood flow vascularisation areas were greatly affected by the day after ovulation, luteal phase and circulating ovarian hormones, IGF-1, NO and leptin levels indicating their important role during early CL development.

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