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Microvascularization and angiogenic activity of equine corpora lutea throughout the estrous cycle

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Abstract

Corpus luteum growth and endocrine function are closely dependent on the formation of new capillaries. The objectives of this study were to evaluate (i) tissue growth and microvascular development in the equine cyclic luteal structures; (ii) in vitro angiogenic activity of luteal tissues in response to luteotrophic (LH, PGE_2) and luteolytic ($PGF_{2\alpha}$) hormones and (iii) to relate data to luteal endocrinological function. Our results show that microvascular density was increased in the early and mid luteal phase, followed by a fall in the late luteal phase and a further decrease in the corpus albicans. Hyperplasia of luteal tissue increased until the mid luteal phase and it was followed by tissue regression. Luteal explants were cultured with no hormone added, or with $PGF_{2\alpha}$, LH, PGE_2, LH + PGE_2 or LH + $PGF_{2\alpha}$. Media conditioned by equine luteal tissue from different stages of the luteal phase were able to stimulate mitogenesis of bovine aortic endothelial cells (BAEC), suggesting the presence of angiogenic activity. No difference was observed among luteal structures on their mitogenic capacity, for any treatment used. Nevertheless, Late-CL conditioned-media with $PGF_{2\alpha}$ showed a significant decrease in BAEC proliferation ($p < 0.05$) and LH + $PGF_{2\alpha}$ a tendency to reduce mitogenesis. Thus, prostaglandin $F_{2\alpha}$ may play a role on vascular regression of the CL during the late luteal phase in the

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mare. These data suggest that luteal angiogenesis and vascular regression in the mare are coordinated with the development of non-vascular tissue and might be regulated by many different factors.
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Keywords: Corpus luteum; Mare; Angiogenesis; Vascularization; Mitogenic factors

1. Introduction

The formation of luteal structures is characterized by repeating patterns of cellular proliferation [1], remodeling of extracellular matrix [2,3] and changes in luteal vascularity in each ovarian cycle [4]. Many aspects of cyclic changes in the reproductive tract during ovarian cyclicity, implantation, as well as placental function are dependent on physiologic angiogenesis [5–8], which consists of new vascular growth from pre-existing vasculature [9]. This neovasculature is essential for the blood-borne delivery of substrates for steroidogenic cells within the ovary, and enables progesterone (P_4) to be released into the blood stream [10–12]. Tissue growth and regression that occurs in the female reproductive tract appears to be controlled by stimulating or angiogenic/mitogenic substances and by inhibitory or anti-angiogenic/anti-mitogenic growth factors [13–17].

The understanding of the vital, angiogenic processes that occur in the mare's reproductive tract is lacking. Only in the recent past, studies on microvascularization of the mare's reproductive tract, such as the endometrium, follicles and corpus luteum have been carried out [18–21]. As the corpus luteum (CL) growth and its endocrine function are closely dependent on the formation of new capillaries, their dysfunction might be related to a deficient vascularization. In the future, knowledge of this process might help understanding infertility in the mare due to primary luteal function impairment.

Hormonal regulation of the production of angiogenic factors by the CL has been evaluated in vitro [22,23]. It is well documented that luteininzing hormone (LH) and prostaglandin (PG) E_2 are the main luteotrophic hormones, while prostaglandin $F_{2\alpha}$, is luteolytic in nature [24]. Lately, there is growing evidence that besides the main endocrine hormone LH, a number of locally produced regulators, such as growth factors, peptides, steroids and prostaglandins, modulate the response of the CL to these endocrine signals [25].

The objectives of this study were to evaluate (i) luteal tissue growth and microvascular development in the equine cyclic luteal structures; (ii) in vitro angiogenic activity of luteal tissues in response to luteotrophic (LH, PGE_2) and luteolytic ($PGF_{2\alpha}$) hormones and (iii) to relate data to luteal endocrinological function.

2. Materials and methods

2.1. Animals

During the equine breeding season, from the Spring equinox until the end of August, luteal tissue and blood were collected post-mortem at a slaughter facility from randomly designated cycling mares. Mares' reproductive and clinical histories were unknown, but

they were in good physical condition as determined by veterinary inspection.

2.2. Collection and classification of luteal tissues

Luteal tissue was obtained from the ovaries of 37 mares immediately after slaughter. Ovaries and ovarian structures were measured. Luteal structures were classified based on their macroscopic aspect as: early structures (corpora hemorrhagica, CH); mid-luteal phase (corpora lutea associated with follicles 15–20 mm in diameter; Mid-CL); late-luteal phase (corpora lutea associated with follicles 30–35 mm in diameter, Late-CL) and corpora albicans (CA).

Since luteal tissue was collected from mares of unknown reproductive history, their estrous cycle determination was based on post-mortem observation of the internal genitalia and further P_4 analysis. Blood samples (10 ml) were collected from the jugular vein into heparinized tubes (monovettes® Sarstedt, Numbrecht, Germany) and transported on ice to the laboratory. Plasma obtained by blood centrifugation was kept frozen (−20 °C) until progesterone assays were performed. Mares were considered to be in the luteal phase when their plasma P_4 concentration was >1 ng/ml, a CL was present. Mares in the follicular phase presented follicles >35 mm in diameter, plasma P_4 was <1 ng/ml, corpora albicans were usually present and CL were absent.

2.3. Experiment I—in situ study

Luteal tissue was fixed in Carnoy solution for 6 h, dehydrated in a series of ethanol dilutions, infiltrated in xylene and embedded in paraffin for histological studies and determination of vascular areas (CH, $n = 7$; Mid-CL, $n = 17$; Late-CL $n = 8$; CA, $n = 5$). Other pieces of tissue were transported, on dry ice, to the laboratory and kept at −70 °C, until DNA, RNA and protein determinations were performed (CH, $n = 7$; Mid-CL, $n = 17$; Late-CL $n = 8$; CA, $n = 5$).

2.4. Experiment II—in vitro study

In order to evaluate the angiogenic activity of equine corpora lutea throughout the estrous cycle, small samples of corpora lutea (CH, $n = 5$; Mid-CL, $n = 7$; Late-CL, $n = 7$) were rinsed thrice in sterile PBS with penicillin and streptomycin, and transported, on ice, to the laboratory in culture medium for further incubation and endothelial cells mitogenesis assays. Medium consisted of Earle's Balanced Salts medium with Hepes (25 mM), streptomycin (100 μg/ml) and penicillin (100 U.I./ml)(all reagents from Sigma, St. Louis, MO, USA) added. Corpora albicans were not used in this part of the experiment due to lack of available tissue.

Luteal tissue was minced into small (approximately 1 mm^3, 200 mg/3 ml) explants (CH = 5; Mid-CL = 7, Late CL = 7) and pre-incubated for 1 h in polystyrene culture tubes (Sarstedt, Numbrecht, Germany), in a tissue incubator (Biosafe Eco-Integra Biosciences, Chur, Switzerland; 37 °C, 5% CO_2, 95% air), on a shaker (Titertek; Huntsville, AL, USA; 150 rpm). The culture medium consisted of DMEM and Ham's F12 (1:1, v/v), and was supplemented with BSA (4 mg/ml, Sigma), transferrin (5 μg/ml; Sigma), hidrocortisone

(40 ng/ml, Sigma), insulin (5 μg/ml, Sigma), penicillin (50 U/ml, Sigma) and streptomycin (50 μg/ml, Sigma). After the pre-incubation period, luteal explants were further incubated for 6 h with (1) no hormone, (2) LH (1 μg USDA-bs/ml, Sigma), (3) $PGF_{2\alpha}$ (1 μg/ml, Upjohn), (4) PGE_2 (1 μg/ml, Sigma) and (5) LH + PGE_2 (1 μg/ml each) or LH + $PGF_{2\alpha}$ (1 μg/ml each). These hormonal concentrations have been previously tested on in vitro bovine luteal tissue incubations for endothelial cells mitogenesis assays [23]. For control, both media with or without hormones added were incubated in the absence of tissue. Media from negative controls and conditioned by luteal tissues were stored at −70 °C, to be later tested for P_4 production and for their ability to stimulate mitogenesis of bovine aortic endothelial cells (BAEC).

2.5. Determination of vascular areas

Six-micrometer-thick luteal tissue sections were cut with a rotatory microtome (Leica® RM2125RT, Leica Instruments, Nubloch, Germany) and walls of blood vessels were marked using Periodic Acid Shiff reagent (PAS)(Sigma). This reagent strongly reacts with carbohydrates in the microvascular basement membrane and has been widely used as a marker of endothelial cells [26]. For each mare, number of vessels and microvascular areas were determined on histologic sections, on 10 randomly chosen microscope fields, using a light microscope at a total magnification of 400×, connected to a computerized cell analysis system (CAS, Becton & Dickinson, Erembodegem, Belgium). All blood vessels in luteal tissue were evaluated equally without distinguishing their nature (arterioles, venules and capillaries). Vascular area was calculated as the percentage of the area occupied by blood vessels with respect to the total area in each microscopic field on all 10 histologic sections evaluated per each mare. Vessel numbers were also counted on the same histologic sections used for microvascular area determination. Total vascular area and blood vessels number were determined as the mean values for all 10 microscopic fields of the luteal tissue evaluated for each mare.

2.6. Determination of total DNA, RNA and protein content

To assess cellular growth and regression of equine luteal tissues, concentrations of genomic DNA, RNA and protein were analyzed. Samples (100 mg) of luteal tissue were placed in 1.2 ml of digestion buffer (100 nM NaCl, 10 mM Tris–Cl, pH 8, 25 mM EDTA, pH 8, 0.5% sodium dodecyl sulfate, 0.1 mg/ml proteinase K) and ground by use of a polytron (Ultraturrax T8, IKA® Werke, Staufen, Germany) at 4 °C. Nucleic acids were extracted with phenol/chloroform/isoamyl alcohol, and DNA was purified as described elsewhere [27]. For total RNA extraction, luteal tissue (100 mg) was also ground with a polytron in 1 ml of Trizol (Sigma), then separated by using chloroform and centrifugation. RNA was precipitated from the aqueous phase with isopropyl alcohol. After a 10 min incubation at room temperature, followed by a 10 min centrifugation (12,000 × *g*, 4 °C), the pellet was washed with 70% ethanol in Trizol and redissolved in DPEC water (Sigma). Quantification of nucleic acids was achieved on the basis of optical density of the sample using a UV photometer (Ultrospec 3100 Pro, Amersham Pharmacia Biotech, Piscataway, NJ, USA)

operated at a wavelength of 260 nm.

Protein determination was performed on sample homogenates by using the Coomassie Plus protein assay (Pierce Chemical Co., Rockford, IL, USA). Bovine serum albumin was used as a standard. Luteal tissue hyperplasia was indicated by cell content of DNA [6,28]. Ratios of RNA:DNA and protein:DNA were considered to be an index of tissue hypertrophy [6,28].

2.7. *Endothelial cells mitogenesis assay*

The ability of equine luteal tissue conditioned media to stimulate mitogenesis of bovine aortic endothelial cells was studied as described by Redmer et al. [23]. Vascular endothelial growth factor (VEGF; Sigma) was used as a positive control, at different concentrations (10, 20, 25 and 50 ng/ml). The positive control was used to evaluate the ability of an angiogenic factor (VEGF) to stimulate BAEC proliferation under the same experimental conditions. Briefly, BAEC (2×10^4 cells/ml) were allowed to adhere to the bottom of a 24-well culture plate (Nucleon-Nunc, Ballerup, Denmark), and incubated for 24 h in a tissue incubator (Biosafe Eco Integra Biosciences, Chur, Switzerland; 37 °C, 5% CO_2, 95% air). Afterwards, the conditioned media samples were added in triplicate wells at a final concentration of 30%, and incubated for another 72 h. Proliferative response of BAEC to samples was evaluated under the light micoscope (Olympus CK40, Wetzlar-Nauborn, Germany), by determining the number of cells in each well, using a neubauer chamber and further compared to controls. For each mare, treatment effects were assessed by comparing BAEC proliferation in luteal tissue conditioned media with media from negative controls (with no luteal tissue). Therefore, the percentage of BAEC proliferation in equine luteal tissue conditioned media was calculated with respect to negative controls, which were considered 100% of cell mitogenesis.

2.8. *Progesterone assays*

Progesterone concentration in both plasma and luteal tissue conditioned media was evaluated using a solid-phase radioimmunoassay (Coat-a-Count Progesterone, Diagnostic Product Corp., Los Angeles, CA, USA). Intra and inter assay coefficients were, respectively, 6.4% and 18.8% for the level of 3.2 nmol/l (1 ng/ml) and 4.2% and 8.0% for the level of 15.9 nmol/l (5 ng/ml).

2.9. *Statistical analysis*

Since values regarding microvascular areas on different luteal structures were not normally distributed, data were subjected to arcsine transformation and analyzed by use of a one-way ANOVA. Data on blood vessels count per luteal strucuture were also analyzed by one-way ANOVA. Significance was defined as values of $p < 0.05$.

Data for DNA, RNA and protein content, RNA:DNA and protein:DNA in 100 mg of luteal tissue were processed by use of a one-way ANOVA. The same statistical procedure was used for endothelial cells mitogenesis data and P_4 concentration in both plasma and luteal tissue conditioned media. Significance was defined as values of $p < 0.05$. Whenever

a significant difference was detected, post hoc comparison tests (Scheffé *F*-test or Fisher least-significant difference test) were performed (Statistic for Windows, Statsoft Inc., 1995, Tulsa, OK, USA).

3. Results

3.1. Determination of vascular areas

Vascular areas were greater during the early luteal phase (CH) and the mid luteal phase (Mid-CL) than in the Late-CL and CA ($p < 0.05$; Fig. 1A). Regarding the mean number of vessels in luteal tissue there was a significant increase during Mid- and Late-CL compared to CH and CA ($p < 0.05$; Fig. 1B). Even though the vascular area in the regressed and afunctional luteal structure (CA) was lower than in the early luteal structure (CH), the number of blood vessels between both structures did not differ ($p > 0.05$; Fig. 1B).

3.2. Determination of DNA, RNA and protein

The DNA concentration (Table 1) of early luteal phase structures was much lower than Mid-CL ($p < 0.001$)(Table 1). Even though late luteal structures DNA concentration was not different from early structures, it was lower than observed in the mid luteal phase CL ($p < 0.001$). The afunctional luteal structure (CA) showed a reduction in DNA when

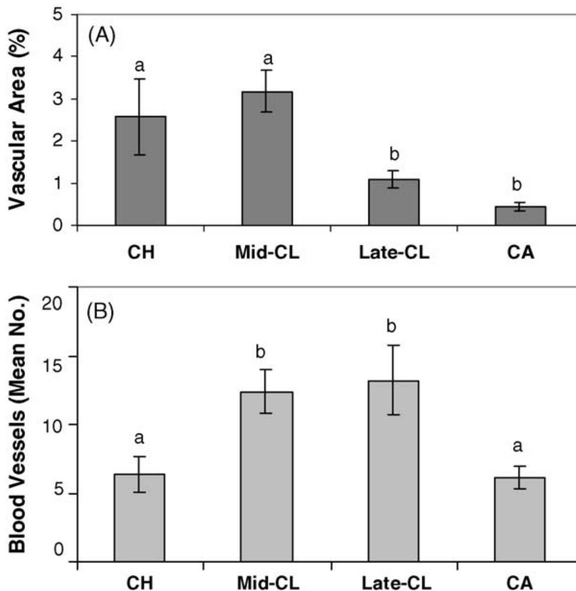

Fig. 1. Vascular areas (A) and mean blood vessel number (B) in luteal tissue obtained from mares during the estrous cycle. Values reported are means ± S.E.M. (a and b) Values differ significantly ($p < 0.05$, Fisher least significant test). CH, corpus hemorrhagicum; Mid-CL, corpus luteum from mid luteal phase; Late-CL, corpus luteum from late luteal phase; CA, corpus albicans.

Table 1
Concentration of DNA, RNA, protein, RNA:DNA and protein:DNA of luteal tissue obtained from mares during the estrous cycle

Luteal tissue				
Variable	CH	Mid-CL	Late-CL	CA
DNA (mg/g of tissue)	29.9 ± 4.8^{a}	56.8 ± 5.5^{b}	29.3 ± 3.5^{a}	17.5 ± 2.5^{c}
RNA (mg/g of tissue)	7.5 ± 4.0^{d}	25.8 ± 5.6^{e}	10.7 ± 3.6^{d}	5.7 ± 0.8^{d}
Protein (mg/g of tissue)	308.2 ± 29.2^{f}	313.2 ± 22.6^{f}	210.1 ± 38.6^{g}	101.6 ± 25.7^{h}
RNA:DNA	0.41 ± 0.2	0.65 ± 0.2	0.34 ± 0.12	0.34 ± 0.07
Protein:DNA	14.1 ± 2.1^{f}	7.4 ± 0.99^{g}	$9.0 \pm 3.0^{f,g}$	5.5 ± 0.9^{g}

Values reported are means ± S.E.M. (a–c) Values differ significantly ($p < 0.001$). (d and e) Values differ significantly ($p < 0.01$). (f–h) Values differ significantly ($p < 0.05$, Fisher least significant test). CH, corpus hemorrhagicum; Mid-CL, corpus luteum from mid luteal phase; Late-CL, corpus luteum from late luteal phase; CA, corpus albicans.

compared to all the other luteal structures evaluated ($p < 0.0001$). Even though RNA concentration was much higher in the Mid-CL when compared to CH ($p < 0.01$), protein concentration did not differ. In addition, RNA concentration was similar between early, late CL and CA ($p > 0.05$), but it was lower than the Mid-CL (Table 1). Furthermore, protein concentrations were also increased in the early and Mid-CL, and were followed by a decrease in late luteal structures ($p < 0.05$). Another fall in protein concentration was observed in the afunctional luteal structure ($p < 0.05$; Table 1). Even though protein-DNA ratio was the highest in the CH when compared to all the other luteal structures ($p < 0.05$), no difference was noted on RNA:DNA for any of the other tissue samples evaluated (Table 1).

3.3. Endothelial mitogenesis assays

Equine luteal tissue alone showed the capability to increase BAEC proliferation (Fig. 2), when compared to negative controls ($p < 0.05$). However, no difference was observed among luteal structures on their mitogenic capacity, for any treatment used. Nevertheless, Late-

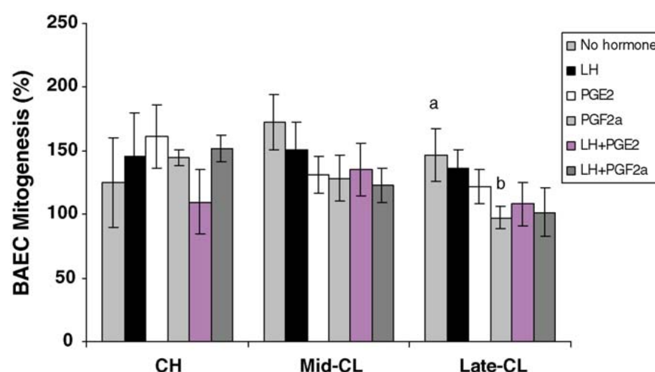

Fig. 2. Mitogenic capacity of different luteal structures on bovine aortic endotelial cells (BAEC) in the presence or absence (control) of different hormones. Values reported are means ± S.E.M. (a and b) Values differ significantly ($p < 0.05$, Fisher least significant test). CH, corpus hemorrhagicum; Mid-CL, corpus luteum from mid luteal phase; Late-CL, corpus luteum from late luteal phase.

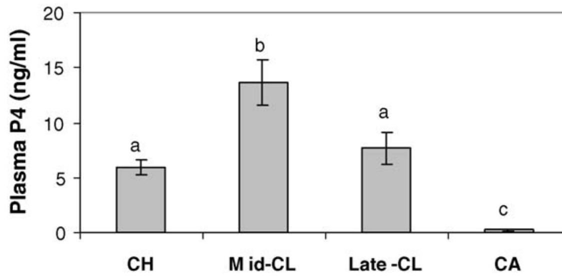

Fig. 3. Plasma progesterone (P4) concentrations from mares during different stages of the estrous cycle. Values reported are means ± S.E.M. (a–c) Values differ significantly ($p < 0.05$, Fisher least significant test). CH, corpus hemorrhagicum; Mid-CL, corpus luteum from mid luteal phase; Late-CL, corpus luteum from late luteal phase; CA, corpus albicans.

CL conditioned media with $PGF_{2\alpha}$ showed a significant decrease in BAEC proliferation ($p < 0.05$) and LH + $PGF_{2\alpha}$ a tendency to reduce mitogenesis but did not reach statistical significance ($p = 0.07$).

Positive controls showed the ability of the angiogenic factor VEGF to stimulate BAEC proliferation under the experimental conditions, at different concentrations (10, 20, 25 and 50 ng/ml). Even though there was a 25% increase in BAEC proliferation from 10 ng/ml (164.4% ± 6) to 20 ng/ml (189.21% ± 5) no further increase was observed for higher doses.

3.4. Progesterone analyses

In this study, the analysis of plasma P_4 (Fig. 3) revealed differences in the synthesis of this hormone by several luteal structures, depending on their nature (i.e. CH, Mid-CL and Late-CL). There was a significant rise in plasma P_4 from CH stage to mid luteal phase CL ($p < 0.01$), followed by a fall in the Late-CL ($p < 0.05$; Fig. 3). Plasma P_4 synthesized by

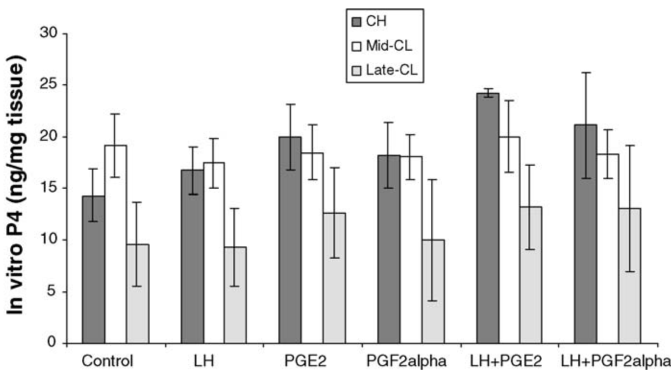

Fig. 4. In vitro progesterone (P4) concentrations in culture medium conditioned by cycling mares' luteal tissue in the presence or absence (control) of different hormones. Values reported are means ± S.E.M. CH, corpus hemorrhagicum; Mid-CL, corpus luteum from mid luteal phase; Late-CL, corpus luteum from late luteal phase.

early luteal phase CL was not different from the levels produced by late luteal structures (Fig. 3). In the follicular phase, when the afunctional CA was present in the ovary, plasma P_4 concentration was much lower than in the presence of any other luteal structures in the luteal phase ($p < 0.001$).

The release of P_4 in culture medium without exogenous hormones, showed a tendency ($p = 0.07$) to rise from CH (14.5 ± 2.5 ng/mg of luteal tissue) to Mid-CL (19.1 ± 3.1 ng/mg of tissue), and to decrease in Late-CL (9.6 ± 3.8 ng/mg of tissue), similar to plasma P_4. Early luteal phase structure showed a 1.7-fold increase (69%) in in vitro P_4 secretion, when in the presence of both luteotrophic hormones tested (LH + PGE_2) with respect to control, even though no significant difference was observed (Fig. 4).

4. Discussion

Changes in microvascularization on equine cyclic luteal structures were present throughout the luteal phase, suggesting that numerous but not yet defined angiogenic factors are produced by the equine CL, during luteal development. Mitogenic factors, such as vascular endothelial growth factor, fibroblast growth factor (FGF), angiogenin, angiopoietins, nitric oxide and monocyte chemottractant protein-1 (MCP-1 factor), among others, promote endothelial cell proliferation and migration and therefore vascular growth in ovarian tissues from different species [8,10,11,29–34]. In this study, a marked increase in microvascular area in the early and mid-luteal phases was observed, even though vessel number was the highest during mid and late luteal phases. Similar results have been previously reported in the cow and in the mare [35–37]. In the present study, the high number of blood vessels in the late CL, associated with a decrease in vascular areas, might be explained by a decrease in blood vessel lumen and by vessel contraction giving the appearance of concentric rings [36]. This decrease in capillary diameter may lead to a fall in blood flow and could initiate and/or accelerate CL regression [38]. The further fall in blood vessel density and number observed in the mare's CA, might be ascribed to the occlusion of blood vessels that disappear as the residual luteal structure organizes into the corpus albicans [36].

A rise in DNA content in the mid luteal phase suggests luteal tissue hyperplasia in the mare. This might be due not only to luteal cells proliferation [39], but also to endothelial cells proliferation (1). Also, in the cow, ewe and sow, DNA content and rate of DNA synthesis increased linearly, from early CL until the mature CL was formed [1,40,41]. The decrease in both microvascular areas and DNA content in Late-CL and regressed luteal structure in the present study suggests that blood vessel regression is associated with tissue involution that occurs during regression of the luteal structures [35,42]. In addition, the highest protein:DNA ratio in the early luteal structure suggests hypertrophy of equine luteal tissue after ovulation. Thus, this could be ascribed to hypertrophy of fibroblasts and other stromal cells or to the presence of a large amount of red blood cells that lack DNA [43]. Protein content drop in late luteal phase tissue and in the afunctional luteal structure suggests luteal regression, which is accompanied by vascular regression.

Plasma P_4 concentration was the highest in mares presenting a mature CL, when vascular areas and blood vessels count were also elevated. Increase in blood supply in the mature CL as well as P_4 secretion was also reported for other species [1,16]. The fall in plasma

P_4 concentration in late CL in the present study confirmed the occurrence of functional luteolysis, followed by structural luteolysis in the CA, when this hormone concentration falls below basal levels [44]. Actually, in the present study, vascular areas were the highest during early and mid-luteal stages, and fell afterwards, when P_4 secretion also dropped. A decrease in plasma P_4 concentration coinciding with a fall in the density of blood vessels and endothelial cell proliferation during early luteolysis has been reported in primates [8].

In vitro P_4 production by equine luteal tissues in this study shows the incubation adequacy for the maintenance of normal luteal function [23]. In several animal species, luteal cells in vitro were able to secrete P_4 in response to LH [45,46]. Luteinizing hormone, alone or in combination with $PGF_{2\alpha}$, was capable of stimulating P_4 secretion from bovine luteal cells in vitro in mid and late luteal phases, but not from early CL [46,47]. Nevertheless, other studies showed that bovine and equine CL were not responsive to LH in vitro [48,49], which is in agreement with our observations.

All equine luteal structures from different stages of the luteal phase showed in vitro angiogenic activity by stimulating the proliferation of endothelial cells, as previously described for the cow [23]. As a matter of fact, endothelial cells used here were from bovine aorta and not from equine specific endothelial cells. However, a number of physiologic functions, such as the secretion of PGI_2 and ET-1 are shared by all endothelial cells [50,51]. As reported for the cow [22], these data show that LH did not cause a significant increase in angiogenic activity by any equine luteal structure evaluated. However, there is substantial evidence that luteal angiogenesis is hormonally regulated by luteinizing hormone (LH) as most of the major potential regulatory factors are produced by lutein cells, which respond to changing LH stimulation [52]. Neither any stimulatory effect on BAEC was observed for any luteal structures when in the presence of PGE_2 (a luteotrophic hormone in other species). However, when the regressing luteal structure was in the presence of a luteolytic hormone ($PGF_{2\alpha}$), a decrease in mitogenic activity was observed. Also, when $PGF_{2\alpha}$ was combined to LH, there was a tendency to reduce BAEC mitogenesis, when compared to controls. On the contrary, conditioned media from Late-CL in the cow exerted the greatest angiogenic response when compared to media conditioned by mid-cycle or corpora hemorrhagica [23]. In another study, the expression of the angiogenic factor FGF-2 was increased during functional luteolysis and decreased afterwards in the bovine corpus luteum [44]. The decrease in endothelial cell mitogenesis observed in mare's late CL when in the presence of $PGF_{2\alpha}$, could be due not only to a lack of synthesis of angiogenic factors, but also to a rise in anti-angiogenic factors, such as angiostatin, endostatin [53,54], thrombospondins [55] and platelet factor 4 [56], which may be modulating angiogenesis and luteal regression. Some anti-angiogenic factors might be involved in luteal formation to avoid excessive vascular development and to mediate endothelial cells apoptosis during regression [9]. Thus, according to our results, $PGF_{2\alpha}$ may play a role in vascular regression of the CL during the late luteal phase in the mare. However, in the cow, a paracrine role for $PGF_{2\alpha}$ on proliferation and survival of bovine derived endothelial cells has been recently reported [57]. Morphological alterations of both steroidogenic cells and blood vessels are essential for regression of cyclic CL [8], with microvascular endothelial cells being the first ones to undergo apoptosis [58]. Since in the mare, overall apoptosis also increases in the late luteal phase CL [59,60], it makes sense that in this study vascular regression was also increased in this stage of the luteal phase, as shown by a decrease in both microvascular areas and endothelial cell

mitogenesis under the influence of $PGF_{2\alpha}$. In conclusion, vascular growth and regression appear to be coordinated with development of non vascular tissue. Also, $PGF_{2\alpha}$ may play a role on vascular regression, acting on endothelial cells during the late luteal phase. These data suggest that luteal angiogenesis and vascular regression in the mare is a very complex process that might be regulated by many different factors and needs further investigation.

Acknowledgments

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References

[1] Jablonka-Shariff A, Grazul-Bilska AT, Redmer DA, Reynolds LP. Growth and cellular proliferatin of ovine corpora lutea throughout the estrous cycle. Endocrinology 1993;133:1871–9.
[2] Goldberg M, Moses MA, Tsang PCW. Identification of matrix metalloproteinases and metalloproteinases inhibitors in bovine corpora lutea and their variation during the estrous cycle. J Anim Sci 1996;74:849–57.
[3] Curry TE, Osteen KG. Cyclic changes in the matrix metalloproteinase system in the ovary and uterus. Biol Reprod 2001;64:1285–96.
[4] Redmer DA, Reynolds LP. Angiogenesis in the ovary. Rev Reprod 1996;1:182–92.
[5] Reynolds LP, Millaway DS, Kirsch JD, Infeld JE, Redmer DA. Growth and in-vitro metabolism of placental tissues of cows from Day 100 to Day 250 of gestation. J Reprod Fertil 1990;89:213–22.
[6] Reynolds LP, Killilea SD, Redmer DA. Angiogenesis in the female reproductive tract. FASEB J 1992;6:886–92.
[7] Augustin HG, Braun K, Telemenakis I, Modlich U, Kuhn W. Phenotypic characterization of endothelial cells in a physiological model of blood vessel growth and regression. Am J Pathol 1995;147:339–51.
[8] Stouffer RL, Martinez-Chequer JC, Molsknee TA, Xu F, Hazzard TM. Regulation and action of angiogenic factors in primate ovary. Arch Med Res 2001;32:567–75.
[9] Tamanini C, De Ambrogi M. Angiogenesis in developing follicle and corpus luteum. Reprod Domest Anim 2004;39:206–16.
[10] Phillips HS, Hains J, Leung DW, Ferrara N. Vascular endothelial growth factor is expressed in rat corpus luteum. Endocrinology 1990;172(2):965–7.
[11] Yan Z, Weich HA, Bernart W, Breckwoldt M, Neulen J. Vascular endothelial growth factor (VEGF) messenger ribonucleic acid (mRNA) expression in luteinized human granulosa cells in vitro. J Clin Endocrinol Metab 1993;77(6):1723–5.
[12] Grasselli F, Basini G, Bussolati S, Tamanini C. Effects of VEGF and bFGF on proliferation and production of steroids and nitric oxide in porcine granulosa cells. Reprod Domest Anim 2002;37:362–8.
[13] Hudlicka O. Development of microcirculation: capillary growth and adaptation. In: Renkin EM, Michael CC, editors. Handbook of physiology, vol. IV, Part 1. Washington, DC: American Physiological Society; 1984. p. 165–216.
[14] Folkman J, Klagsburn M. Angiogenic factors. Science 1987;235:442–7.
[15] Redmer DA, Kirsch JD, Reynolds LP. Production of mitogenic factors by cell types of bovine large estrogen-active and estrogen-inactive follicles. J Anim Sci 1991;69:237–45.
[16] Reynolds LP, Grazul-Bilska AT, Killilea SD, Redmer DA. Mitogenic factors of corpora lutea. Prog Growth Factor Res 1994;5:159–75.

[17] Grazul-Bilska AT, Redmer DA, Killilea SD, Kraft KC, Reynolds LP. Production of mitogenic factor(s) by ovine corpora lutea throughout the estrous cycle. Endocrinology 1992;130(6):3625–32.
[18] Ferreira-Dias G, Serrão P, Costa Durão J, Robalo Silva J, Mateus L, Correia J, et al. Vascular density in the equine endometrium throughout the estrous cycle. In: Second Conference of the European Society for Domestic Animal Reproduction. 1998. p. 80–1.
[19] AL-ziĭabi MO, Fraser HM, Hinojosa A, Watson ED. Changes in the vasculature of the corpus luteum throughout the oestrous cycle and after $PGF_{2\alpha}$ induced luteolysis. In: 14th International Congress on Animal Reproduction, vol. I. 2000. p. 60 [Abstracts].
[20] Ferreira-Dias G, Serrão PM, Costa Durão J, Robalo Silva J. Microvascular development and growth of the uterus during the estrous cycle in the mare. Am J Vet Res 2001;62(4):526–30.
[21] Watson E, Ai-zi'abi M. Characterization of morphology and angiogenesis in follicles of mares during spring transition and the breeding season. Reproduction 2002;124:227–34.
[22] Redmer DA, Rone JD, Goodman AL. Evidence for a non-steroidal angiotropic factor from the primate corpus luteum: stimulation of endothelial cell migration in vitro. In: Proceedings of the Society for Exp. Biology and Medicine, vol. 179. 1985. p. 136–40.
[23] Redmer DA, Grazul AT, Kirsch DJ, Reynolds LP. Angiogenic activity of bovine corpora lutea at several stages of luteal development. J Reprod Fertil 1988;82:627–34.
[24] Juengel JL, Niswender GD. Molecular regulation of luteal progesterone synthesis in domestic animals. J Reprod Fertil Suppl 1999;54:193–205.
[25] Shams D, Berisha B. Regulation of corpus luteum function in cattle—an overview. Reprod Domest Anim 2004;39:241–51.
[26] Luna, LG. Polysaccharides and mucopolysaccharides. Histopathologic methods and color atlas of special stains and tissue artifacts, Gaithersburg, MD: American Histolabs Inc.; 1992. p. 364–98.
[27] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JD, Smith JA, et al. Preparation and analysis of DNA. Short protocols in molecular biology. 2nd ed. New York, USA: John Wiley & Sons Harvard Medical School; 1992. p. 2-8–2-9.
[28] Baserga R. In: Baserga R, editor. Tissue growth. The biology of cell reproduction. Cambridge: Harvard University Press; 1985. p. 46–58.
[29] Redmer DA, Dai Y, Li J, Charnock-Jones DS, Smith SK, Reynolds LP. Moor RM characterization and expression of vascular endothelial growth factor (VEGF) in the ovine corpus luteum. J Reprod Fertil 1996;108:157–65.
[30] Goede V, Brogelli L, Ziche M, Augustin HG. Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1. Int J Cancer 1999;82(5):765–70.
[31] Lee HS, Lee IS, Kang TC, Jeong GB, Chang SI. Angiogenin is involved in morphological changes and angiogenesis in the ovary. Biochem Biophys Res Commun 1999;257(1):182–6.
[32] Berisha B, Schams D, Kosmann M, Amselruber, Einspanier R. Expression and tissue concentration of vascular endothelial growth factor, its receptors, and localization in the bovine corpus luteum during the estrous cycle and pregnancy. Biol Reprod 2000;63:1106–14.
[33] Reynolds LP, Grazul-Bilska AT, Redmer DA. Angiogenesis in the corpus luteum. Endocrine 2000;12:1–9.
[34] Tamanini C, Basini G, Grasselli F, Tirelli M. Nitric oxide and the ovary. J Anim Sci 2003;81:E1–7.
[35] Zheng J, Redmer DA, Reynolds LP. Vascular development and heparin-binding growth factors in the bovine corpus luteum at several stages of the estrous cycle. Biol Reprod 1993;49:1177–89.
[36] Modlich U, Kau F-J, Augustin H. Cyclic angiogenesis and blood vessel regression in the ovary: blood vessel regression during luteolysis involved endothelial cell detachment and vessel occlusion. Lab Invest 1996;74(4):771–80.
[37] AL-zi'abi MO, Watson ED, Fraser HM. Angiogenesis and vascular endothelial growth factor expression in the equine corpus luteum. Reproduction 2003;125:259–70.
[38] Gaytán F, Morales C, García-Pardo L, Reymundo C, Bellido C, Sánchez-Criado JE. A quantitative study of changes in the human corpus luteum microvasculature during the menstrual cycle. Biol Reprod 1999;60:914–9.
[39] Roberto da Costa RP, Branco V, Pessa P, Robalo Silva J, Ferreira-Dias G. Progesterone receptors and proliferating cell nuclear antigen expression in the equine luteal tissue. Reprod Fertil Dev; 2005.

[40] Fricke PM, Zheng J, Redmer DA. Compositional and histochemical characterization of luteal growth and development throughout the estros cycle in cows. Biol Reprod 1985;48(Suppl 1):156.
[41] Ricke WA, Redmer DA, Reynolds LP. Growth and cellular proliferation of pig corpora lutea throughout the oestrous cycle. J Reprod Fertil 1999;117:369–77.
[42] Augustin HG, Braun K, Telemenakis I, Modlich U, Kuhn W. Ovarian angiogenesis: phenotypic characterization of endothelial cells in a physiological model of blood vessels growth and regression. Am J Pathol 1995;147:339–51.
[43] Ginther OJ. Reproductive efficiency. In: Reproductive biology of the mare—basic and applied aspects. 2nd ed. Equiservices, Cross Plains, Wisc; 1992. p. 199–202.
[44] Neuvians TP, Schams D, Berisha B, Pfaffl MW. Involvement of pro-inflammatory cytokines, mediators of inflammation, and basic fibroblast growth factor in prostaglandin $F_{2\alpha}$-induced luteolysis in bovine corpus luteum. Biol Reprod 2004;70:473–80.
[45] Campbell BK, Baird DT, Webb R. Effects of dose of LH on androgen production and luteinization of ovine theca cells cultured in a serum-free medium. J Reprod Fertil 1998;112:69–77.
[46] Grazul AT, Kirsch JD, Slanger WD, Marchello MJ, Redmer DA. Prostaglandin-F2α, oxytocin and progesterone secretion by bovine luteal cells at several stages of luteal development: effects of oxytocin, luteinizing hormone, prostaglandin-F2α and estradiol 17 beta. Prostaglandin 1989;38:307–18.
[47] Girsh E, Greber Y, Meidan R. Luteotrophic and luteolytic interaction between bovine small and large luteal-like cells and endothelial cells. Biol Reprod 1995;52:954–62.
[48] Redmer DA, Kirsch JD, Grazul AT. In vitro production of angiotropic factor by bovine corpus luteum: partial characterization of activities that are chemotactic and mitogenic for endothelial cells. Adv Exp Med Biol 1987;219:683–8.
[49] Broadley C, Menzies GS, Bramley TA, Watson ED. Isolation of cell populations from the mare corpus luteum: comparison of mechanical and collagenase dissociation. J Reprod Fertil 1994;102:7–15.
[50] MacIntyre DE, Pearson JD, Gordon JL. Localization and stimulation of prostacyclin production in vascular cells. Nature 1978;271:549–51.
[51] Yanagisawa M, Kurihara H, Kimura S, Tomobo Y, Koboyashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988;332:411–5.
[52] Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E. Patterns of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J Clin Invest 1993;91:2235–43.
[53] Espinosa Cervantes MC, Rosado Garcia A. Angiogenesis in reproductive physiology. Follicular development, formation and maintenance of the corpus luteum. Gynecol Obstet Mex 2002;70:17–27.
[54] Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997;277:55–60.
[55] Hazzard TM, Rohan RM, Molskness TA, Fanton JW, Dámato RJ, Stouffer RL. Injection of anti-angiogenic agents into the macaque preovulatory follicle: disruption of corpus luteum development and function. Endocrine 2002;17:199–206.
[56] Perollet C, Han ZC, Savona C, Caen JP, Bikfalvi A. Platelet factor 4 modulates fibroblast growth factor 2 (FGF-2) activity and inhibits FGF-2 dimerization. Blood 1998;91:3289–99.
[57] Meidan R, Podlovni H, Kisliouk T, Spanel-Borowski K, Rosiansku M. Prostaglandin F2 alpha induces proliferation and survival of bovine luteal endothelial cells. Biol Reprod 2004 [Sp. Issue, 37th Annual Meeting for the Study of Reproduction, Abstract 21, p. 97].
[58] Davis JS, Rueda BR, Spanel-Borowski K. Microvascular endothelial cells of the corpus luteum. Reprod Biol Endocrinol 2003;1:89.
[59] AL-zi'abi MO, Fraser HM, Watson ED. Cell death during natural and induced luteal regression in mares. Reproduction 2002;123:67–77.
[60] Ferreira-Dias G, Mateus L, Solá S, Ramalho RM, Castro RE. Rodrigues CMP apoptosis in the equine cyclic corpus luteum. Reprod Domest Anim 2004;39(4):284.