Microvascular development and growth of uterine tissue during the estrous cycle in mares

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Objective—To document uterine growth and microvascular development in the endometrium of uteri with differing degrees of fibrosis as well as uterine growth throughout the estrous cycle of mares.

Animals—30 mares.

Procedure—Uterine tissue was obtained during the breeding season from a slaughter facility. Stage of estrous cycle of the mares was assessed on the basis of ovarian structures and plasma progesterone concentrations. Endometrium was characterized by use of light microscopy, and blood vessel walls were marked by histochemical techniques. Microvascular development was evaluated by a computerized image analysis system. Growth of uterine tissue was based on cellular content of DNA and RNA, RNA:DNA, and protein:DNA.

Results—Significant differences in vascular density were not observed in the endometrium of uteri obtained from mares euthanatized during the follicular or luteal phase of the estrous cycle, regardless of whether endometrial classification of degree of fibrosis was considered. There was a 3-fold increase in amount of DNA and RNA of endometrial cells in the follicular phase when compared to myometrium. Hypertrophy of endometrial tissue during the luteal phase was reflected by a significant increase in cell protein content and protein:DNA.

Conclusions and Clinical Relevance—Endometrial growth of vascular tissues during the estrous cycle may be coordinated with development of nonvascular tissue. Estrogen and progesterone may play a role in regulation of uterine growth and angiogenesis. (*Am J Vet Res* 2001;62:526–530)

Tissue growth depends greatly on angiogenesis, which consists of formation of new blood vessels. In adult animals, the process of neovascularization is evident mainly during tissue repair or in some pathologic situations.[1-3] However, after puberty, uterine tissue has rapid periodic growth and regression associated with physiologic changes in blood flow and angiogenesis.[1,4,5] During estrus in mares, histologic structure of the endometrium is characterized by edema attributable to an increase in vascularity and congestion; this edema decreases during diestrus.[6] However, as for most mammalian species, the mechanisms behind the steroid hormone actions on uterine growth and function are not completely defined.[7-9]

Endometritis is a limiting factor in equine reproduction. This problem results in large economic loss for the horse industry.[10,11] In mares, infertility has been associated with extensive collagen fibrosis in the endometrium. A classification system for the equine endometrium, created on the basis of the magnitude of fibrotic tissue and endometrial inflammatory alterations,[12] has been used as a prognosis for the capability of maintaining pregnancy in this species.[13]

Inflammatory and degenerative alterations in larger blood vessels and lymph vessels are the main angiopathies observed during histologic examination of the endometrium of mares.[14,15] Inflammatory vascular changes in the equine uterine mucosa, characterized by vasculitis, have been reported in approximately a fifth of a large number of samples that were evaluated.[14,15] In older mares, sclerosis of the intima and adventitia layers of blood vessels are evident.[14,15] Because there is an indirect effect of endometrial angitis on decreasing fertility through a reduction in endometrial perfusion and drainage, which is related to endometritis, angitis should be considered as an additional variable when establishing a prognosis for fertility.[14,15] Thus, angiopathies may be related to the pathogenesis of endometriosis.[a]

By day 6 after ovulation, the conceptus arrives in a mare's uterus, but implantation does not begin until day 30.[6,16] The interval from fertilization until implantation of the embryo depends solely on maternal histotroph.[17,18] Results of electron microscopy suggest vascular impairment may be associated with extensive fibrosis.[19,20] Deficient angiogenic function may be associated with an inadequate uterine environment for the normal development of the conceptus, which would result in embryonic mortality and low fertility rates in this species.

Changes in endometrial vascularity associated with the estrous cycle of mares have been reported.[6] Histologic observations on uterine growth and microvascular development have been published, but quantitative data is lacking. A better understanding of angiogenesis may contribute to improvements in the control of infertility of all animals in general and in horses in particular. Even though it is important to evaluate the role of angiogenic and growth factors involved in uterine dynamics, a more complete description of patterns of uterine growth in mares should be provided. Therefore, the objective of the study reported here was to provide a quantitative description of uterine growth and microvascular development in the equine endometrium of mares with var-

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ious degrees of uterine fibrosis as well as uterine growth throughout the estrous cycle.

Materials and Methods

Animals——Mares with unknown reproductive and clinical histories were used in the study. Tissue samples were collected at a slaughter facility from randomly designated mares in good physical condition, as determined during veterinary inspection. Blood samples were obtained, and ovarian structures were measured and recorded.

Collection and preparation of uterine tissues—Tissue was obtained from 14 mares for evaluation of endometrial vascular density. Immediately after slaughter, 3 samples (0.5 × 0.5 cm) of uterine mucosa were collected from each mare. Endometrial samples for histologic examination were fixed in Carnoy solution for 6 hours, dehydrated in a series of ethanol solutions, and embedded in paraffin. Six-micron-thick sections were cut. Sections were stained with H&E and evaluated, using light microscopy (400× magnification). Sections were classified in accordance with the criteria of Kenney.[12] Healthy endometrial tissue (ie, without fibrosis or inflammation) was considered to be a category-I endometrium, whereas severe histologic lesions characterized category-III endometrium. The intermediate situation between these 2 extremes was considered to be a feature of category-II endometrium.

Samples of endometrium and myometrium were obtained from 16 mares for the evaluation of uterine growth. Samples were analyzed for concentrations of DNA, RNA, and protein. Immediately after slaughter, uterine tissues were carefully dissected, and samples of endometrium and myometrium were transported to the laboratory on dry ice and stored at –70 C until analyses were performed.

Determination of the stage of the estrous cycle—Because uterine samples were obtained from mares with an unknown reproductive history, the stage of the estrous cycle was determined on the basis of endocrine analysis and results of postmortem examination of the reproductive tract. Immediately after slaughter, a blood sample (10 ml) was collected from a jugular vein into heparinized syringes[b] and transported on ice to our laboratory. After centrifugation, aliquots of plasma were stored frozen (–20 C) until analyzed for progesterone concentration, using solid-phase radioimmunoassay.[c]

Ovaries and ovarian structures such as follicles, corpora hemorrhagica, corpora lutea, and corpora albicans were measured. Cyclic activity was determined on the basis of detection of a preovulatory follicle with a recent corpus albicans, corpus hemorrhagicum, or corpus luteum.[21] Previous ovulation was indicated by detection of active luteal structures.[21] The follicular phase was characterized by detection of a follicle ≥ 35 mm in diameter and plasma progesterone concentration < 1 ng/ml.[22,23] In contrast, mares were considered to be in the luteal phase when a corpus luteum was detected, none of the follicles were ≥ 35 mm in diameter, and plasma concentration of progesterone was > 1 ng/ml. Phase of the estrous cycle also was confirmed by histologic examination of the endometrium, using light microscopy.[12]

Microvascular density in the endometrium—Walls of blood vessels in the endometrium were marked, using periodic acid-Schiff stain, which strongly reacts with carbohydrates in the microvascular basement membrane[24] and has been widely used as a marker of endothelial cells. For each mare, 10 randomly chosen endometrial fields were photographed, using light microscopy at 400× magnification. Photomicrographs were printed and scanned, and vascular areas then were measured, using a computerized image analysis system.[d] All vessels were evaluated equally without distinguishing among arterioles, venules, and capillaries.[25] Vascular density was assessed as the percentage of the area occupied by blood vessels with respect to the entire area of each micrograph. Total vascular area was calculated as the mean value for all 10 micrographs of the endometrial samples of each mare.

Determination of DNA, RNA, and protein content—To assess cellular growth of equine uterine tissues, concentrations of DNA, RNA, and protein were analyzed in endometrium and myometrium.[25,26] Samples (800 mg) of endometrium or myometrium were placed in 10 ml of buffer solution (50 m*M* tris-acetate, pH 7.8) and then ground by use of a polytron[e] at 4 C. The DNA and RNA were extracted from uterine tissue homogenates, using a commercial kit[f] in accordance with the manufacturer's instructions. Briefly, a 250-µl sample of homogenate was diluted (1:1) in buffer solution (50 m*M* tris-acetate, 4*M* guanidine thiocyanate, pH 7.8). Nucleic acids were precipitated with isopropanol. After adsorption, RNA was eluted from the cartridge by use of another buffer solution (100 m*M* tris-acetate, 15% ethanol, 400 m*M* KCl, pH 6.3). The sample containing DNA then was poured into a cartridge, and DNA was eluted by use of a buffer solution (100 m*M* tris-H_3PO_4, 15% ethanol, 1,000 m*M* KCl, pH 8.5). Quantification of nucleic acids was determined on the basis of optical density of the sample, using a UV photometer[g] operated at a wavelength of 260 nm.

Protein determination was performed on sample homogenates, using a method for a protein assay.[h] Bovine serum albumin was used as a standard. Cell content of DNA was considered to be an index of tissue hyperplasia.[25,26] Hypertrophy was indicated by analysis of the ratios of RNA:DNA and protein:DNA.[25,26]

Statistical analysis—Most horses sold to the slaughter facility used in our study are infertile. Thus, it was extremely difficult to obtain a sufficient number of mares with category-I endometrium. Therefore, analysis of vascular density during the estrous cycle (follicular vs luteal) was accomplished by evaluating data for the following groups: mares with category-I or -II classification, mares with category-III classification, and all mares. It appears that the ultrastructure does not differ substantially between endometrium from mares with category-I or -II classification, but it does differ substantially between those mares and mares with category-III classification.[19,20] Because values were not normally distributed, data were subjected to arcsine transformation and analyzed by use of a 1-way ANOVA. Significance was defined as values of $P < 0.05$.[i]

Data for DNA, RNA, and protein content, RNA:DNA, and protein:DNA in 1 g of endometrial or myometrial tissue as well as RNA:DNA and protein:DNA were analyzed by use of a 1-way ANOVA. 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.[i]

Results

Of the original 14 mares, 6 had a uterine classification of category-I or -II (3 in follicular phase, 3 in luteal phase), and 8 had a uterine classification of category-III (3 in follicular phase, 5 in luteal phase). We did not detect significant differences in vascular density of the endometrium for either phase of the estrous cycle, regardless of uterine endometrial classification (**Fig 1**).

Of the 16 mares used for analysis of uterine growth, 7 were in the follicular phase and 9 were in the

luteal phase at the time of slaughter. Although there was a 3-fold increase in the amount of DNA and RNA in the endometrium during the follicular phase, the values did not differ significantly in samples between the follicular and luteal phase (**Table 1**). Protein concentration, RNA:DNA, and protein:DNA were identical in endometrial samples obtained from mares in the follicular and luteal phases.

Values for amount of DNA, RNA, or protein, as well as RNA:DNA, or protein:DNA were not significantly different among samples of myometrium collected at either phase of the estrous cycle. These results were in contrast to those for endometrial tissue. However, when comparing endometrium and myometrium during the luteal phase, increases in protein content ($P < 0.001$) and protein:DNA ($P = 0.01$) were significantly higher in the endometrium than in the myometrium (Table 1). In contrast, significant differences were not observed for DNA or RNA content or RNA:DNA between the 2 uterine tissues in the luteal phase of the cycle. In the follicular phase, we did not detect differences for any of the variables evaluated.

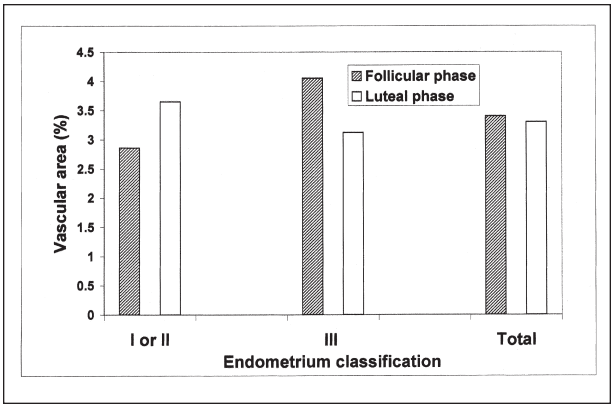

Figure 1—Vascular density in samples of equine endometrium during the follicular and luteal phases of the estrous cycle for mares with a histologic classification of category I or II, category III, or all mares regardless of histologic classification. Values did not differ significantly ($P \geq 0.05$).

Table 1—Concentrations of DNA, RNA, and protein RNA:DNA, and protein:DNA of endometrial and myometrial tissues obtained from mares during the follicular and luteal phases of the estrous cycle

Variable	Uterine tissue	Follicular phase (n = 7)	Luteal phase (n = 9)
DNA (mg/g of tissue)			
	Endo	0.603 ± 0.434	0.162 ± 0.006
	Myo	0.223 ± 0.031	0.170 ± 0.012
RNA (mg/g of tissue)			
	Endo	0.489 ± 0.346	0.148 ± 0.014
	Myo	0.154 ± 0.024	0.167 ± 0.007
Protein (mg/g of tissue)			
	Endo	52.46 ± 3.28	54.96 ± 2.37[a]
	Myo	45.87 ± 4.69	37.85 ± 3.07[b]
RNA:DNA			
	Endo	0.856 ± 0.134	0.939 ± 0.097
	Myo	0.752 ± 0.140	0.996 ± 0.060
Protein:DNA			
	Endo	243.95 ± 56.04	340.64 ± 14.60[c]
	Myo	221.34 ± 35.96	227.31 ± 24.97[d]

Values reported are mean ± SEM.
[a,b]Values differ significantly ($P < 0.001$; Fisher least-significant difference test or Scheffe F test). [c,d]Values differ significantly ($P = 0.01$; Fisher least-significant difference test or Scheffe F test).Endo = Endometrium. Myo = Myometrium.

Discussion

The influence of ovarian steroid hormones on endometrial growth and regression as well as vascularization has been repoerted.[2] During the estrous cycle, estrogen effects predominate during the follicular phase, whereas progesterone is the main ovarian steroid that influences uterine dynamics during the luteal phase.[27] Cyclic patterns of histologic changes in the endometrium throughout the estrous cycle have been attributed to the effects of ovarian steroids.[12,27-29] It is during the period immediately preceding ovulation that uterine blood flow is the greatest, and it reaches a minimum during the luteal phase.[30] These systematic changes in uterine blood flow appear to be associated with the ratio of concentrations of estrogen-to-progesterone in the blood.[30] The existence of estrogen receptors in the uterus is essential for angiogenesis.[31]

Analysis of results of the study reported here revealed that microvascular area in the endometrium did not differ between the follicular and luteal phase. Our data are in agreement with those obtained by Reynolds et al[29] and suggest that endometrial growth of vascular tissues is coordinated with development of nonvascular tissue. However, because the reproductive status of our mares was determined solely on the basis of observations of ovarian structures and systemic concentrations of progesterone, it was not possible to predict the exact day of the estrous cycle. In a study of ewes,[29] weight of the uterine horns was approximately 40% greater during estrus than on day 8 after estrus. Tissue weight remained constant from day 8 to 15 after estrus. Results for total microvascular volume revealed a pattern similar to that for uterine growth.[29] However, when considering microvascular volume as a percentage of endometrial tissue volume, it did not differ among days of the estrous cycle.[25] Contradictory observations have been reported for other species.[32,33] In cows, an increase in endometrial vasculature has been observed during the early luteal phase but not during estrus.[32] In a study on angiogenesis in humans, investigators did not find significant differences in endothelial cell proliferation between the luteal and follicular phases of the menstrual cycle.[33] Women with menorrhagia had an increase in endothelial cell proliferation in the endometrium, compared with women with a normal endometrium.[33]

Effects of estradiol that result in an increase in uterine size and cell proliferation have been reported elsewhere[7-9,29,31] and are not in complete agreement with results obtained in our study, because the 3-fold increase in DNA content of endometrial cells in our mares during the follicular phase was not significantly different from values for the luteal phase. However, the increase in DNA and RNA content in the follicular phase suggests a stimulatory influence of estradiol on hyperplasia of the equine endometrium. In a study of sheep,[27] DNA content of the endometrium did not differ significantly between samples collected on the day of estrus and on day 10 during mid-diestrus. Similar results were observed for ovariectomized sheep that

received estradiol, progesterone, or estradiol in combination with progesterone.[27]

In our study, it appears that there was hypertrophy of endometrial tissue during the luteal phase, compared with values for myometrial tissue, as reflected by protein content and protein:DNA. This significant increase in protein content and protein:DNA in the endometrium during diestrus may be explained by the normal increase in protein synthesis that is evident in the uterus of a mare under the influence of progesterone.[34] In addition, it is the endometrium, not the myometrium, that contains uterine glands.[12] Those glands are the site where histotroph, which consists of proteins, is synthesized under the influence of progesterone.[35] An increase in uterine size observed in sheep under the influence of a combination of estradiol and progesterone was attributed to hypertrophy and can be related to RNA:DNA and protein:DNA.[27]

Glandular and luminal epithelial cells in the endometrium are constantly replaced by new cells. In equine endometrium under the physiologic influence of estrogen during estrus, stromal cells in the stratum compactum of the endometrium proliferate faster, whereas few cells are mitotically active in the stratum spongiosum.[36] This pattern of cellular proliferation continues for 3 days after the end of estrus and extends into the early luteal phase. Later, between days 3 and 7 after ovulation, the rate of cellular multiplication increases in the epithelial cells of the secretory portions of the endometrial glands.[36] This may be ascribed to a delayed response to the decrease in plasma estrogen concentrations.[36] After day 7 of diestrus, mitotic activity in equine endometrium is depressed for all cell types as long as progesterone concentrations remain high.[36] The fact that we did not find a significant difference in DNA content in the endometrium between follicular and luteal phases may be explained by the fact that the exact day of the estrous cycle on which we collected uterine tissues was not known. Because early and mid-diestrus samples were included in the same experimental group, and because they behave mitotically in a similar manner to endometrial cells during the follicular phase,[36] we may have been comparing cell populations that did not differ. Therefore, these observations need to be confirmed in endometral biopsy specimens obtained from mares during various phases of the estrous cycle. It would be necessary to strictly monitor the estrous cycles of such mares. However, because endometrial biopsy instruments only collect a small amount of endometrial tissue in mares, it is not practical to procure samples of myometrium for study by use of this method.

[a]Schoon D, Gruninger B, Wrede S, et al. Vascular lesions in the equine endometrium (abstr), in *Proceedings*. Int Conf Endometritis/Endometrose beim pferd. *Pferdeheilkunde* 1997; 5:546.

[b]Monovette, Sarstedt, Numbrecht, Germany.

[c]Coat-a-Count Progesterone, Diagnostic Product Corp, Los Angeles, Calif.

[d]Scion Image, National Institutes of Health, Bethesda, Md.

[e]Ultra-Turrax T25, IKA-Labortechnik, Janke & Kunkel GMBH & Co KG, Staufen, Germany.

[f]Nucleobond AXR/80, Macherey-Nagel, Duren, Germany.

[g]UV 1101, Biotech Photometer, Cambridge, UK.

[h]Coomassie Plus protein assay, No. 1856210, Pierce Chemical Co, Rockford, Ill. [i]Statview program for Macintosh, Adept Scientific, Letchworth, UK.

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