



Endometrial alterations, early placentation and maternal fetal interaction in carnivores *Alterações endometriais da placentação inicial e interação materno-fetal em carnívoros*

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Abstract

The canine and coati morphology of the endometrial glands during different phases of the estrous cycle and begin of pregnancy were compared; thus evaluating morphological alterations related mainly to endometrial chambers and microvessels distribution. We aimed to analyze a triad of uterine components and their morphophysiological features that were the disposal of the endometrial glands, microvascular net and relation with begin of gestational period and their morphological alterations for blastocyst/embryo reception. We can consider that canine endometrial glands follow demarcated alterations of the lumen to adjust it to the period of the estrous cycle. A bleeding process was followed among endometrial tissue. A capillary network reflects to the period of the estrous cycle, either with microvascular increases for estrous and diestrus or regression compared with proestrus highlighting the reduction of future hemorrhage that proceeds this estrous period. Endometrial glands delimit the trophoblast invasion when compared as no altered morphology related to the deeper endometrial glands. We stand out the extreme importance of the microvascular net (spirals artery) and the structural behavior of the endometrial glands since it begins to make possible the pregnancy confirmation in the type of endotheliochorial placenta and embryo implantation inside the lumen.

Keywords: canine, coati, endometrium, estrous cycle, morphology, pregnancy.

Resumo

A morfologia endometrial de caninos e quatis durante diferentes fases do ciclo estral e começo de gestação foi comparada e suas alterações e evoluções morfológicas foram descritas principalmente quanto às câmaras endometriais e à distribuição de microvasos. Foram estudadas uma tríade de componentes uterinos e suas características morfofisiológicas, as quais são a disposição das glândulas endometriais, sua rede microvascular e as relações com o início da prenhez e suas alterações quanto à recepção do blastocisto/embrião. Pode-se considerar que as glândulas endometriais seguem alterações evidentes do lúmen em ajuste a cada ciclo estral. O processo de sangramento foi acompanhado no tecido endometrial. A rede capilar reflete a cada período também, tanto com aumento microvascular no estro e diestro quanto para regressão destes no proestro, tornando evidente a redução do sangramento que se segue após a fase do estro. As glândulas endometriais superficiais delimitam a invasão do trofoblasto quando comparadas às profundas, que são inalteradas morfológicamente. Enfatizou-se a importância da rede microvascular (artérias espirais) e o comportamento estrutural destas glândulas, as quais podem tornar possível a confirmação de gestação em relação a este tipo de placenta, endoteliocorial, e a implantação do embrião no lúmen uterino.

Palavras-chave: caninos, ciclo estral, endométrio, gestação, morfologia, quati.

Introduction

The canine reproductive aspects possess some characteristics that cannot be seen in other mammals; for example: monogamy, pseudopregnacy and delayed ovulation. In the canine female, the manifestation of the endocrines events during the estrous cycle follows a particular standard. Specific aspects until now partially elucidated, as the expression or inhibition of hormones in the phases of the cycle and during moments of transition between these phases are poorly commented in literature. The main function of the uterus is gestational, to guarantee the complete development of new individuals (concepts) until the birth (gestation the term). In accordance with the phase of the estrous cycle, or gestation, some substances as the prostaglandins and secretions of the endometrial glands are produced by this organ, allowing its correct physiological activity. For Rodrigues and Rodrigues (2002), the mechanisms of regulation, secretion and hormonal interactions responsible

for changes which trigger or occur during the estrous cycle in dogs has been the target of a significant number of experiments. Many specific aspects of the estrous cycle of the bitch has not been revealed, and old concepts are being reviewed because the universe of canine reproduction should be better studied. A century ago, Turner (1876) noted that the openings of uterine glands were located in areas of the endometrium, with a similar pattern of blood vessels. Studies of Tsutsumi (1962) produced confirmatory evidences. Davies (1950) examined eight uteri containing embryos seal with a diameter less than 6mm and a length of 180mm and found that in the early stage there is marked increase in activity of the uterine glands, coinciding with the formation of crypts on the surface of the endometrium comparable to bitch. At this stage, no trace of the obliterated umbilical vein or calf-intestinal bag was verified. MacDonald (1976) studied the uterine vascularization of pregnant sows by electron microscopy. Through catheters, injected colored latex into the arteries and veins of sixteen pregnant uteri obtained from sows at various stages of pregnancy. The study of molds obtained indicated that in early pregnancy, the capillary network that serves the preliminary transversal fold of the membrane of the uterine mucosa was composed of parallel layers of low sulci and vascular gutters. Ambrósio and Miglino (2001) and Miglino et al., 2006 studied the exchange systems of maternal-fetal placenta at term of mongrel bitches about their vascular aspects through anatomy macro, microscopic and scanning electron microscopy. The vascularization of this organ is performed by funicular vessels, arteries and veins, which are distributed in the middle third of the umbilical cord emitting two branches to each placental side, a placental central branch and another branch as placental chorionic one.

Materials and Methods

Animals

For this research were used uteri of 34 bitches that were divided into the following phases of the estrous cycle: 7 uterus in proestrus phase, 7 uterus in diestrus phase, 12 uterus in anestrus phase, 4 uterus in estrus phase and 4 uteri of bitches in early gestation. All uteri were obtained by ovariohysterectomy. One pregnant uterus at begin of pregnancy from a coati was collected by the same surgical procedure at CECRIMPAS (Center of Raised and Preservation of Wild Animals) at São João da Boa Vista, UNIFEOP.

Cycle determination

Vaginal cytology was performed on each bitch, moments before ovariohysterectomy, to check their estrous cycle phase. The vaginal samples were collected according to the techniques indicated by others authors (Evans and Cole, 1977; Jöchle and Andersen, 1977; Feldman and Nelson, 1997).

Light microscopy

The uterus had their vessels perfused with an aqueous solution of 4% paraformaldehyde and subsequently sectioned. Preparations were fixed with the same solution with a pH of 7.3. The blocks were dehydrated with series of alcohols with increasing concentrations (70-100%) and diaphanized in xylol followed by inclusion in Paraplast (Paraplast Embedding Media-Paraplast Plus, Oxford Lab, USA) and sectioned in a Polyartmicrotomo (Leica). The stainings performed for evaluation of uterine histology were hematoxylin eosin and Sirius Red photomicrographs were taken (Nikon Eclipse E-400) for processing of the results.

Scanning electron microscopy

The vessels of fresh uteri were injected with Mercor (methyl methacrylate). The molds were immersed in water for about 24 hours to complete the hardening of Mercor. Subsequently they were submerged in a solution of 15% NaOH and distilled water at a temperature of 60° C to complete the technique of corrosion. These molds were washed for several days in distilled water and liquid soap (neutral Extram) and dried in an incubator. After this procedure, the molds were fragmented under stereoscopic lenses and mounted on aluminum base (stubs) with carbon cement. They were covered with gold (3 nm) and examined by scanning electron microscope Zeiss - LEO located at ICB - Institute of Biological Sciences at USP. To visualize the lumen of pregnant uterus between 0-20 days of gestation, the protocol was different, without injection of methyl methacrylate, therefore was followed by the fragmentation of the uterine horns and then submerged in 2.5% glutaraldehyde in phosphate buffer for fixation. After 48 hours fixation, the material was washed three times in 0.1 molar phosphate buffer (pH 7.4) for 10 minutes, totaling 30 minutes of immersion. The material was post fixed in osmium tetroxide for 2 hours and again washed three times in 0.1 molar phosphate buffer (pH 7.4) for 10 minutes. Dehydration occurred in batteries of 15 minutes with increasing concentrations of alcohol (50, 70, 80, 90 and 95%) passing subsequently in absolute alcohol for 15 minutes four times. The critical point was obtained by the equipment (CPD 020 - Balzers) UNION, where all the alcohol was removed from the material leaving it completely dry for the metallization with gold. As a final stage

of processing, the samples were fixed on stubs of aluminum with carbon glue, and left in incubator for 12 hours for drying and subjected to bathing gold in sputter (Emitech - K 550). The analyses of electron micrographs were performed in a scanning electron microscope (LEO - 435 PV).

Results

Light microscopy: uterine histology

In early anestrus, crypt regions still exist, but these were composed only of epithelial cells and its stroma was rare or absent. Columnar epithelium cells were still hypertrophic with picnotic nucleus in several cells, while the cytoplasm was, in many cells, replaced by foamy vacuoles. At the beginning this estrous phase was possible to find endometrial glands with different characteristics: most of these glands presented rectilinear shape, but there were several with curvilinear appearance suggesting the end of progesterone phase (Fig. 1A and B).

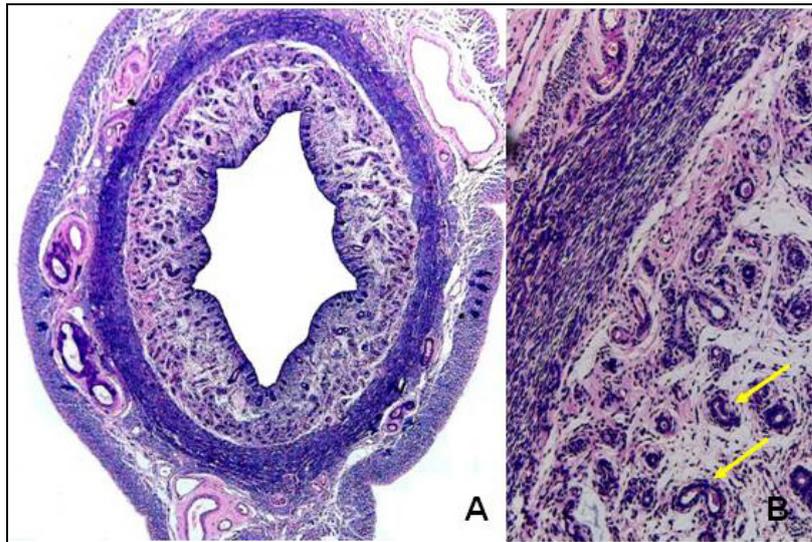


Figure 1. Photomicrograph of a canine uterus in anoestrus. The endometrium has actually regressed, it is fine, but many glands are still very curvy and have secretions (arrows). Increases: A - 4x B - 20x. H.E. Stain - Hematoxylin and Eosin.

The endometrium of animals in the middle of this stage was covered with a layer of simple cubic surface epithelium. In late anoestrus, epithelial surface was formed by simple cuboidal cells with large and round nuclei (Fig. 2A and 2B).

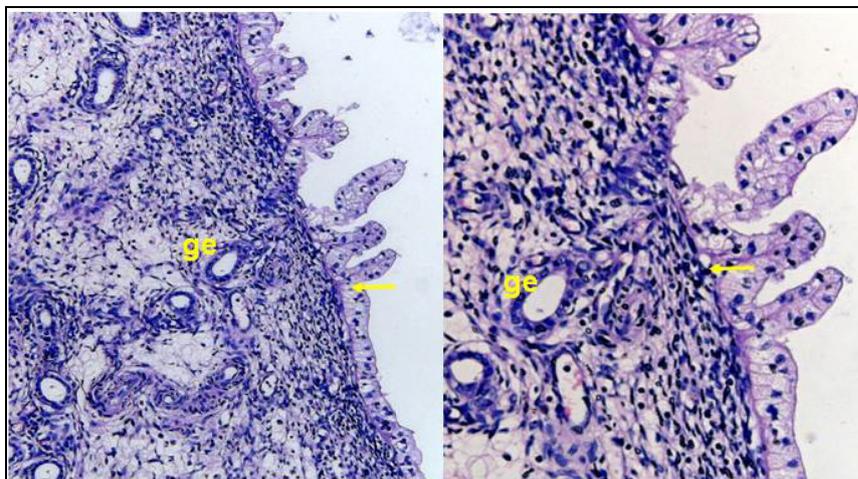


Figure 2. Photomicrograph of the uterus of a bitch during anoestrus, two months postpartum. We observed some glands without secretion (g) and the cuboid luminal epithelium is altered, showing cellular disorganization (arrow). Increases: A - 10x B - 20x. H.E. Stain - Hematoxylin and Eosin.

The proestrus is characterized by endometrial growth, so the endometrium at this stage of estrous was thicker and the luminal epithelial cells became columnar. The endometrial glands increased in size, became more linear (Fig. 3), indicating that they were under the influence of estrogen (arrows). Glandular secretions were absent or rare and in tiny quantities.

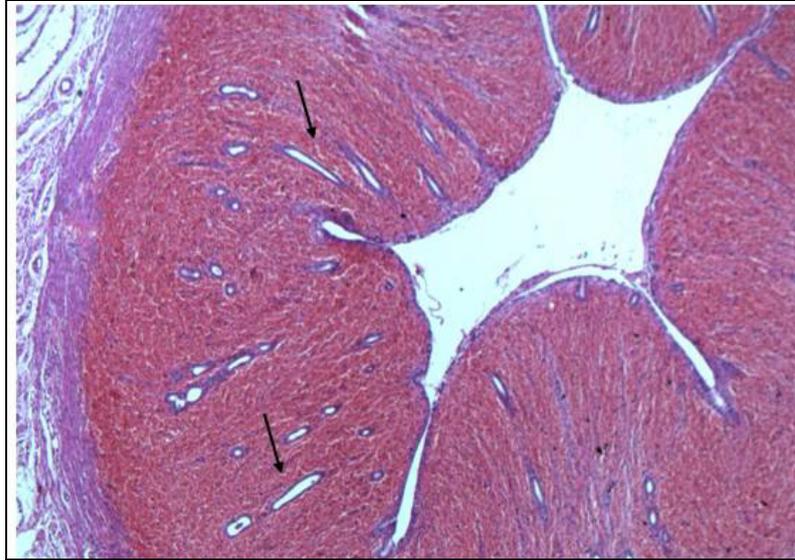


Figure 3. Photomicrograph of transversal cross section of canine uterus at proestrus. A very thick endometrium and uterine endometrial glands straight, featuring a stage of the influence of estrogen (arrows). Magnification: 4x. H.E. Stain - Hematoxylin and Eosin.

Mitotic figures were observed around the glands or within them, indicating a phase of glandular proliferation (Fig. 4).

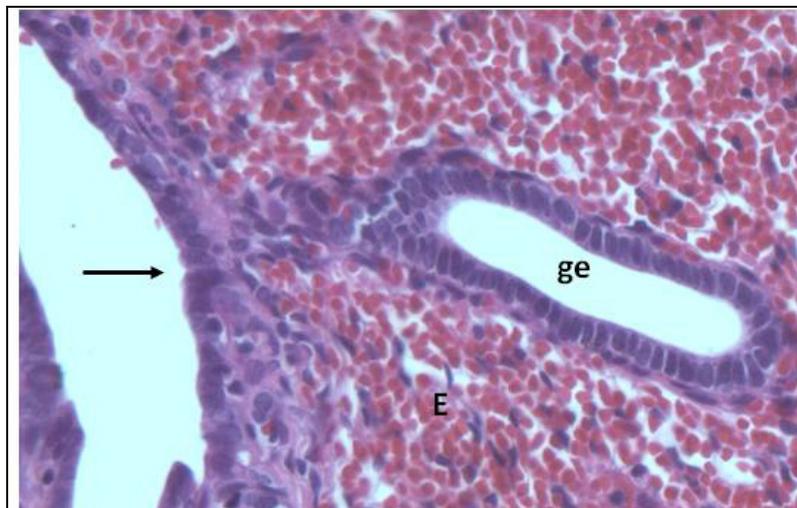


Figure 4. Photomicrograph of the same animal from the previous figure, showing the rectilinear shape of the endometrial glands (g) and the presence of mitotic figures. Columnar cells (arrow), erythrocytes (E). Magnification: 40x. H.E. Stain - Hematoxylin and Eosin

Histological examination of the uterus of bitches in estrus showed a general increase in the size of the uterus, the endometrium with a very thick, and large glandular proliferation with highly developed glands (Fig. 5 and 6); The epithelium at this stage presents a cylindrical top. The uterine lumen it is sufficiently diminished in this phase. The connective tissue is characterized by congestion, edema and bleeding.

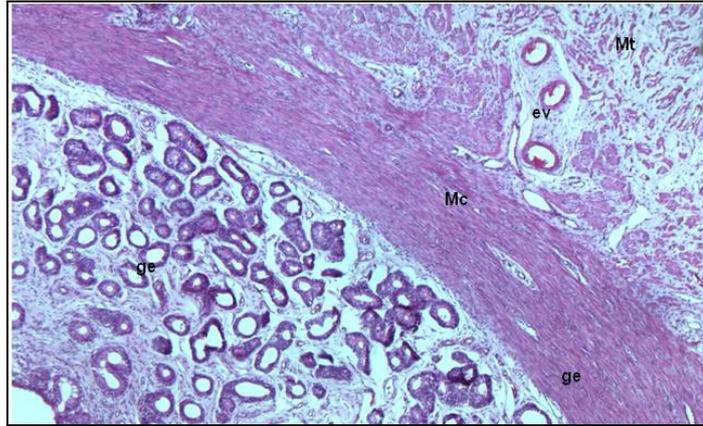


Figure 5. Photomicrograph of transversal cross of canine uterus in estrus. Note the glandular proliferation and general increase of the uterus with thick endometrium. Endometrial glands (g) vascular layer (v), circular myometrium (Mc), myometrium transversal cross (Mt). Magnification: 4x. H.E. Stain - Hematoxylin and Eosin.

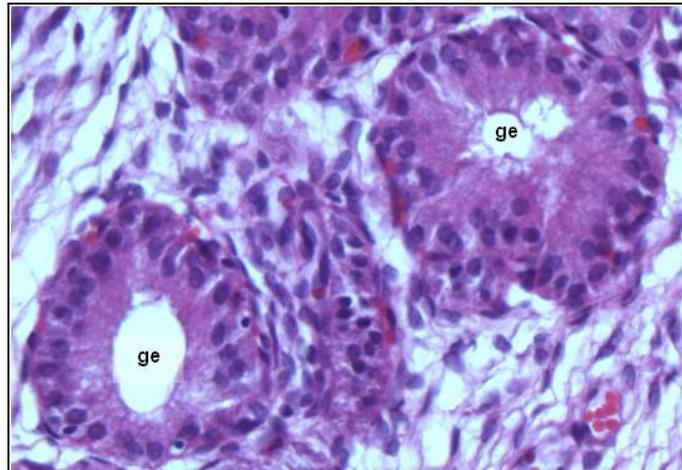


Figure 6. Photomicrograph of canine uterus in estrus, indicating the high degree of development of endometrial glands (g). Magnification: 10x. Stain: H.E. - Hematoxylin and Eosin.

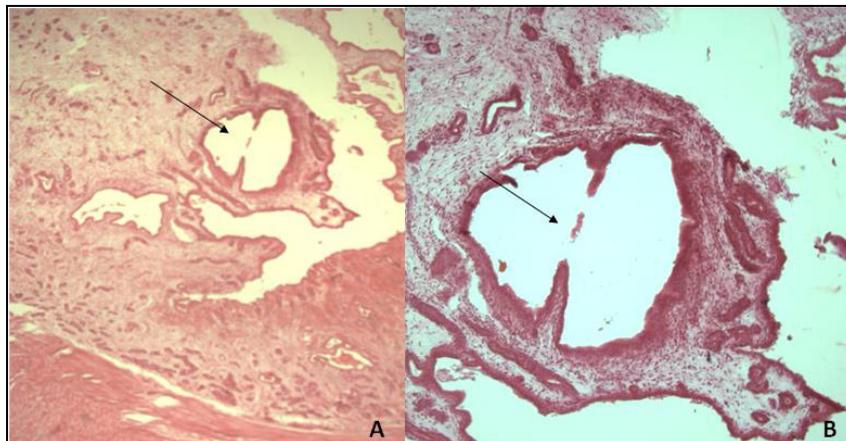


Figure 7. Photomicrograph of a cross section of canine uterus with less of 20 days of pregnancy. Possible area of implantation of the embryo (arrows). Magnification: A - 4x. B - 10x. Stain H.E. - Hematoxylin and Eosin.

Until 17 days of pregnancy, is possible to visualize the implantation site and embryonic body inside the endometrial glands rearrangement (Fig. 7). The endometrial glands become filled with narrow openings to the lumen. The citotrophoblast invasion started following via lumen to myometrium.

The endometrium of animals that were in early to mid-diestrus presented with hyperplastic and dilated, with edematous stroma and blood vessels redness. The endometrial glands were very convoluted with high amount of secretion (Fig. 8).

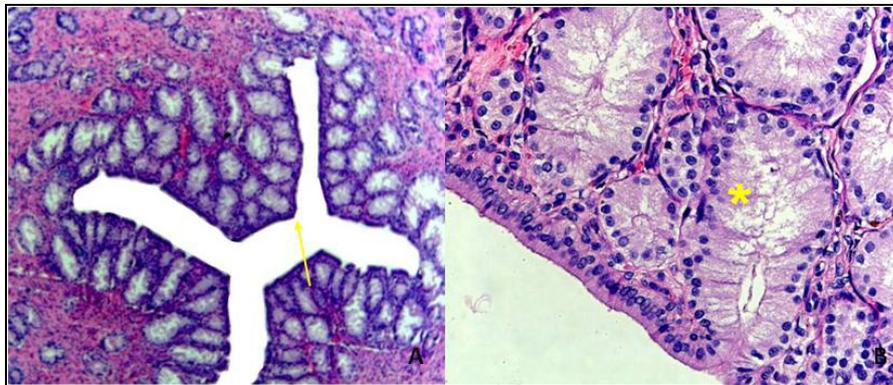


Figure 8. Photomicrograph of canine uterus in diestrus. There is clearly the degree of maximum development of endometrial glands (*), which are full of secretion, indicating the dominance of progesterone in this phase. The lumen it is very narrow (arrow). Increases: A - B and 4x - 20x. H.E. Stain - Hematoxylin and Eosin.

The endometrial crypts, present since the onset of estrus, were better delineated at the end of this stage, when some cells with picnotic nucleus were already observed. In this phase the endometrium and its glands were fully developed (Fig. 9).

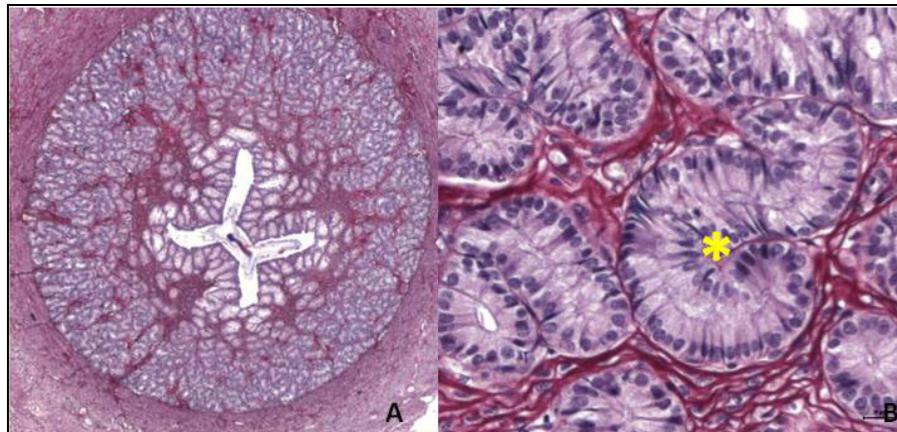


Figure 9. Photomicrograph of the same animal to the previous figure. In "A" overview of the endometrium, totally taken by developed glands (arrows). In "B" glands (*) overdeveloped and filled with secretions. Increases: A - 2.5x and B - 40x. Stain Sirius Red with H.E. base.

Scanning electron microscopy

The microvascular structure of the canine uterus shows accentuated during anestrus, and the envelopment of the arteries of the perimeter is visible as nourishing base for this period of uterine rest. The uterine lumen is uniform and the endometrial glands are placed in sustentation of the endometrium. The microvascular network originates from the spiral arteries that leave the myometrium and penetrate the endometrium. When studying the ultra structure of vascular molds, we distinguish between the arterial and the venous vessels, through the endothelial negative impressions on the same ones. For the description, we consider for the arteries, is: longitudinal impressions and parallel bars, and for the veins, impressions to perhaps; circular and random. The spirals arteries are convoluted and going in direction to the uterine lumen, originating the capillary network. The capillary network during anestrus reveals a reduced bore and a circular and uniform, disposal, which originates from arterioles (Fig. 10A and B).

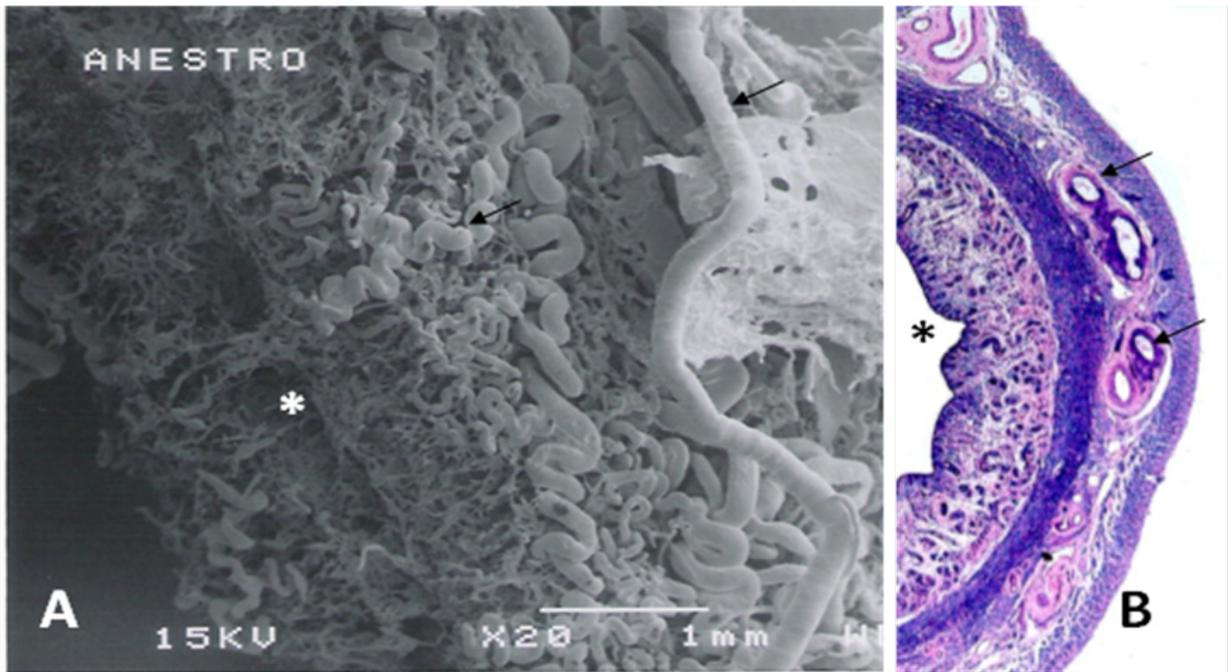


Figure 10. Scanning electron micrograph of perimytrium vessels and capillary network branching to lumen direction (*), 20X. B: Photomicrography of uterine components in contrast of microvascular ultrastructure with analogy with the microvessels (arrows).

The capillary network during anestrus reveals a reduced bore and obeys a circular and uniform, disposal, which originates from arterioles (Fig. 11).

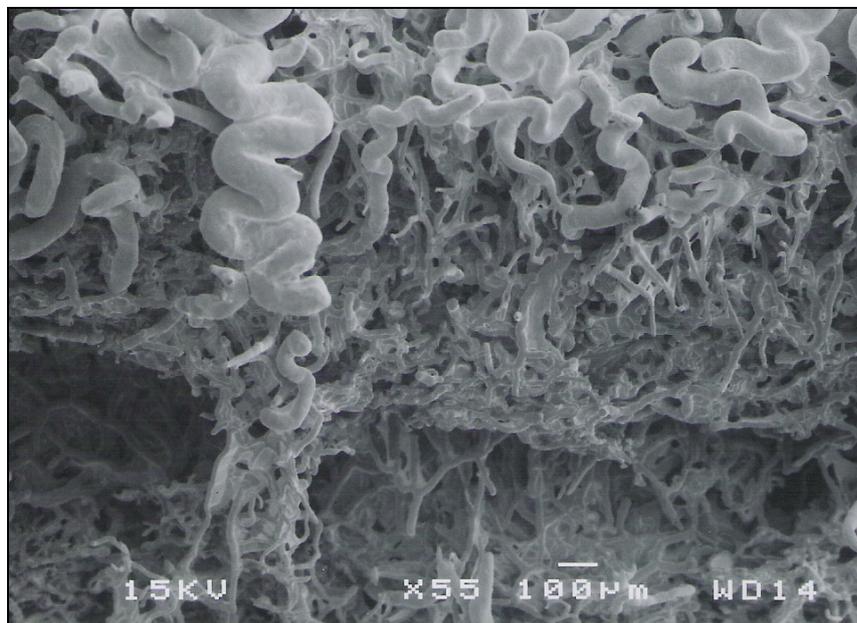


Figure 11. Scanning electron micrograph of the spiral artery and its disposition to the uterine lumen. Magnification: 55X.

In the phase of proestrus, the conformation of the vascular network of the perimeter remains similar to anestrus; however we can see a reduction of bore of the arteries spirals (Fig. 12) and a bigger support of venous return.

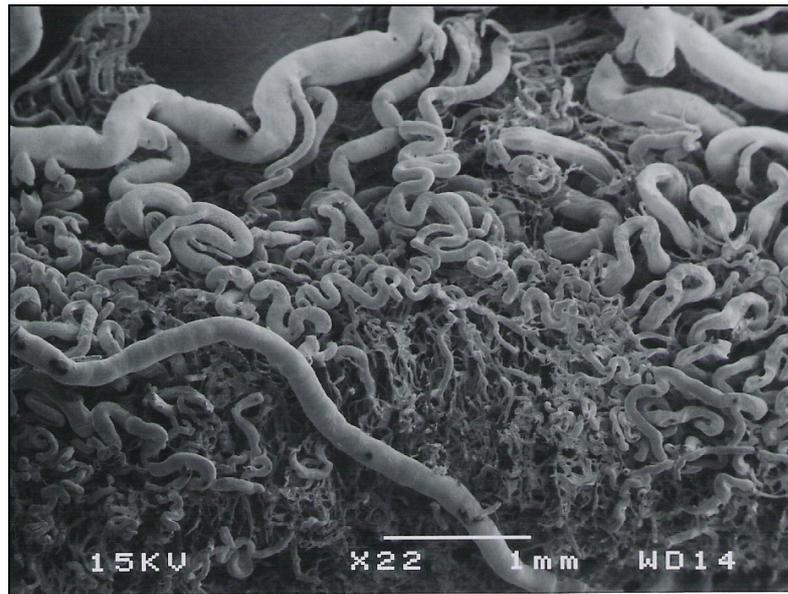


Figure 12. Scanning electron micrograph of pro-estrous uterus showing the disposition of perimytrium vessels and spiral arteries origin.

However, when we look at the disposal of the endometrial capillaries during the period of estrous, it is evident the reduction of the bore of the capillaries, in an attempt to prevent any kind of disruption. In this period. The endometrial glands are immersed in a great lake of overflowed blood, watering down the hormone's alterations for the beginning of estrous and its consequent hemorrhage. During the phase of estrous we had some problems with the methylmethacrylate injection, probably due to occurred hemorrhagic region in this phase. We had vast extravasations of the solution that when making solid, presented an amorphous mass after the corrosion of soft fabrics. Different protocols had been made to fulfill the vascular net with the solution, however we had exaggerated by emptying points, we did not have definition of the capillaries. In the period of diestrus the enlargement of the endometrial glands with consequent envelopment of the net was clear. In this phase, the endometrial glands look like a tissue reorganization and renewal vascular or better, revitalization of the endometrium (Fig. 13).

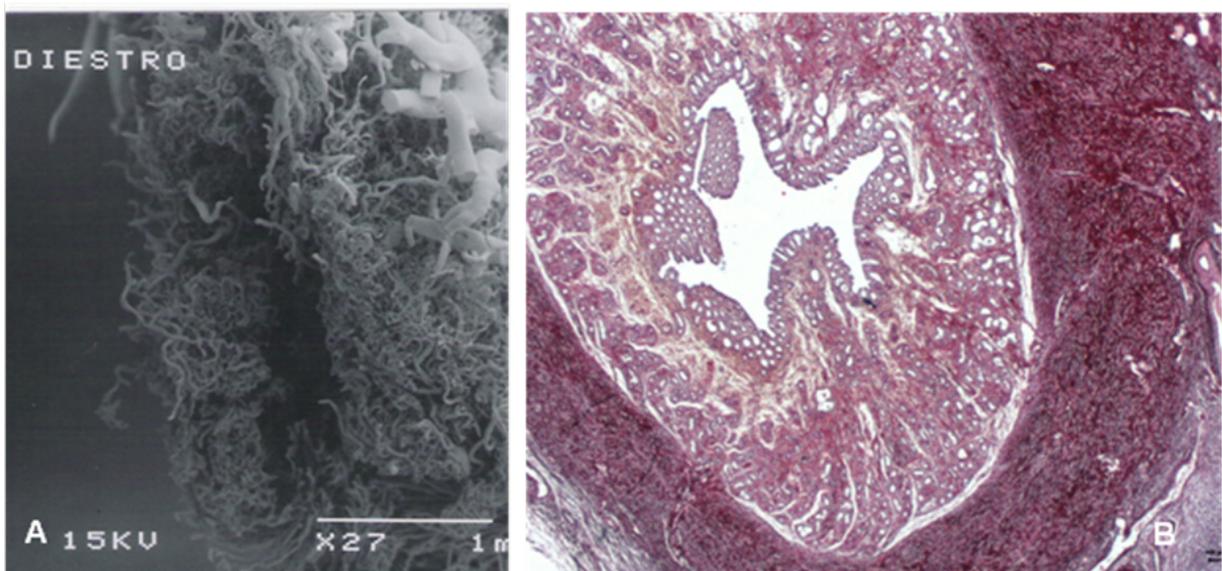


Figure 13. A: Scanning electron micrograph of uterine horn at diestrus with microvascular net disorganized as effect of endometrial glands enlargement (B).

For pregnant, dogs with up to 25 days of gestation, the endometrial glands in analysis of the uterine lumen for scanning electron microscopy (SEM) surface, showing a parallelism between itself (Fig. 14A) and, moreover, they activate secretions; as we can see in the proven histology and in the SEM (Fig. 14B).

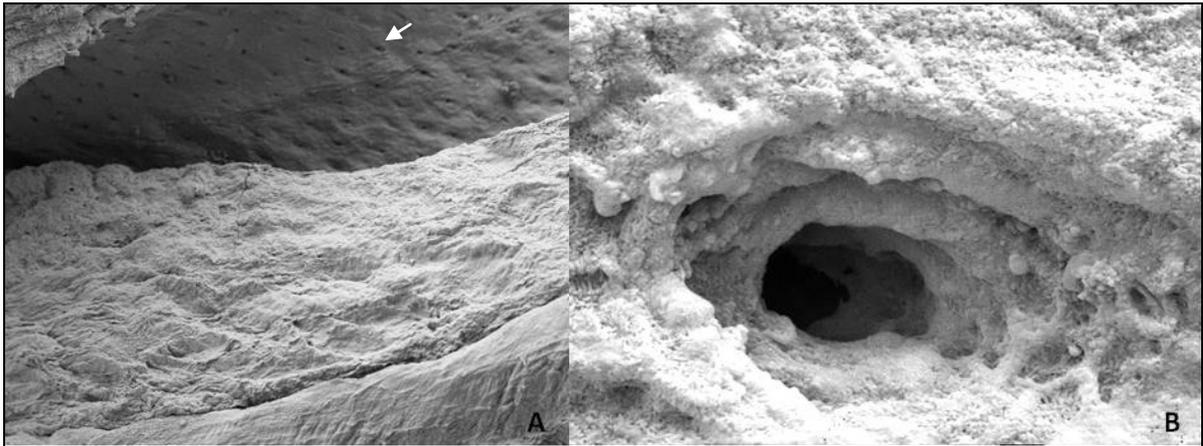


Figure 14. A: Scanning electron micrograph of pregnant uterus at begins showing the parallelism among endometrial glands opening (arrow). B: overview of the opened endometrial gland.

Already the microvascular study it shows differentiated of the glands, that present a disposal in “honeycomb” in the extremities of the microvascular arrangements in the region of the uterine lumen. (Fig. 15A). A prolongation of the vessels exists to give support mainly to the beginning of the gestation and to the placenta. (Fig. 15B).

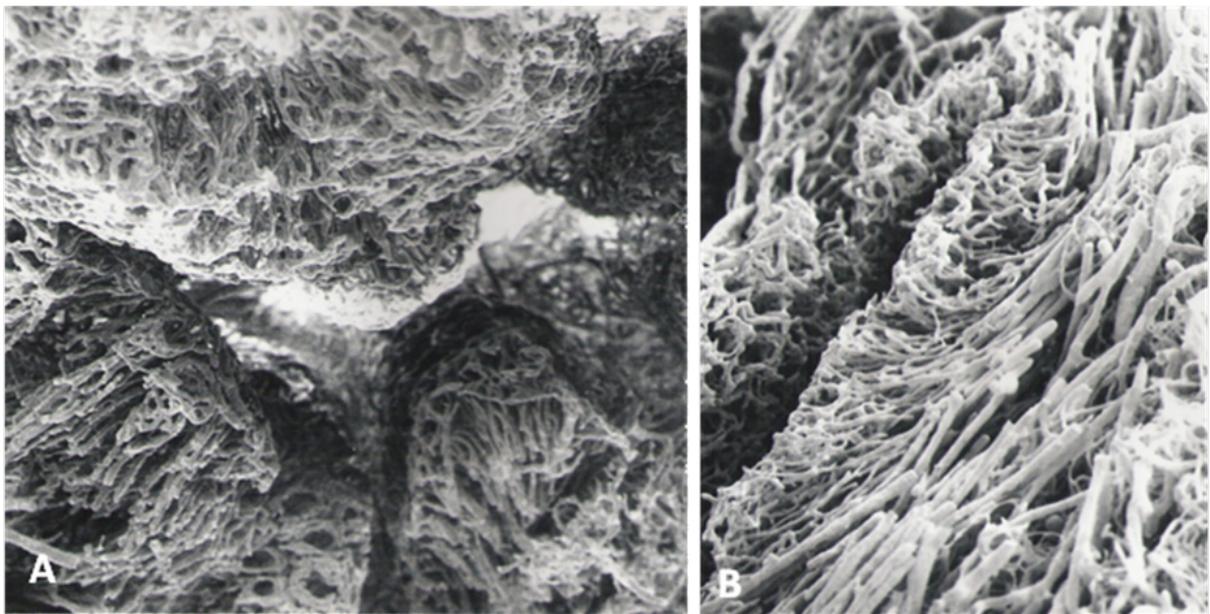


Figure 15. Microvessels disposition at begin of pregnancy in dogs. A: Uterine lumen overview and in B, disposition of the vessels branching to perimetrium.

Wild carnivore's (Coati, *Nasua nasua*) uterine considerations

The possible pregnancy was considered because of a less increase of one of the horns and also the presence of corpus luteum at the ovary of the same side. Tissues were fixed in 10% formaldehyde and prepared for light microscopy and immunohistochemistry by standard procedures. The bicornuate uterus was composed by perimetrium, myometrium, and endometrium (Fig. 16A).

At the myometrium were found a large number of vessels strongly stained by vimentin. Two distinct arrangement of muscle cells were observed at this layer, one circular and other longitudinal separated by vessels. The endometrium consists in a simple cubic epithelium with numerous glands with high activity of secretion. Those glands and the endometrium layer of the lumen present cytokeatin positive stain at its cytoplasm. As we know, cytokeatin was expressed at the cytoskeleton of trophoblast cells, but it was found at the lumen of endometrial gland cells, however, a disorganized area was found related to embryo site of implantation. The embryo cannot be found, and because of it we cannot confirm the gestation, but the high activity of the uterine glands its rearrangement more deep and the positive stain of cytokeatin gave us evidence that the maternal environment was prepared for the embryo attachment as know in domestic carnivores (Fig. 16B-E).

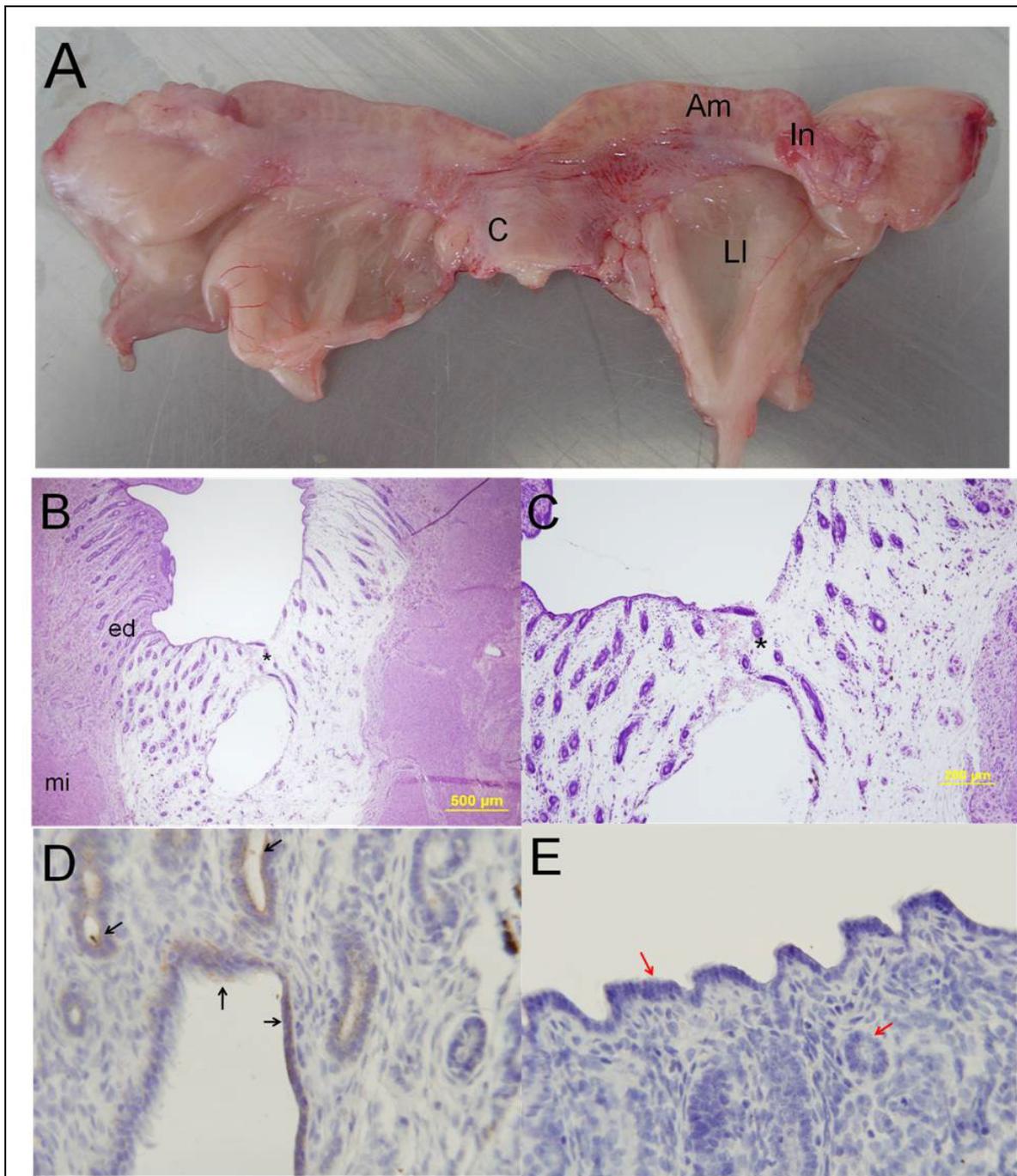


Figure 16. Figure A represent the uterus in Coati (*Nasua nasua*), this uterus consists of two horns, a body (C), infundibulum (In), funnel (Am) and broad ligament (LI). Observed in B endometrium (ed), myometrium (mi) and area of implantation (*). Figure C shows the region of implantation in the uterus enlarged to better visualization. Figures D show histological slides stained with vimentin where the simple cubic trophoblastic (Ec) epithelium and marked on the cellular skeleton (Black arrow). In E show the negative control of the D the same area and not marked (red arrow).

Discussion

In this study, endometrial morphology and microvasculature of dogs at different stages of the estrous cycle and first third of pregnancy were evaluated by histological and scanning electron microscopy. Vaginal cytology were performed in all dogs, moments before the animals were submitted to ovariohysterectomy. Vaginal cytology provides a simple and rapid diagnosis, which occasionally supplies the need for histological examination. The vaginal cytology aimed to assist in confirming the period of the estrous cycle for each female was moments before the surgery. Although many of the consulted authors (Onclin e Vertstegen, 1997; Cain,

2001; De Bosscher et al., 2002) also exalter the importance and exactness of the serum dosage of P4, using this examination as the main way to classify and to divide the animals used in the experiments in groups according the estrous cycle phase; in our study no sanguine sample of the animals was evaluated, therefore our objective was to evaluate and to classify the animals considering only the alterations and morphologic characteristics that were found in the evaluated samples. This objective was reached; therefore the results obtained in the microscopic evaluations of the canine endometrium had been very similar to those ones described by authors who had used hormones dosages in their methodology of research. The morphologic changes of the canine endometrium in estrous and end of diestrus, for example, imply functional mechanisms; the increase in the length and number of the microvillus in the end of estrous can be important for the blastocyst-uterine interaction in the beginning of gestation. In proliferative phase, endometrial cells human beings are differentiated and expressed molecules of adhesion for blastocysts. According to Van Cruchten et al. (2002); if these cells are also present in dogs, they should be important not only for deployment, but also have a role in the pathogenesis of cystic endometrial hyperplasia complex. We also consider that morphologic alterations in the canine endometrium during the phases of the estrous cycle are very important in pathogenesis of pyometra. Our conclusions about the disposal of the endometrial glands and its structure had been similar to the discoveries of Elnaeim-Abd et al., 2001 for studies in uteri of asinine during estrous phase. Concerning that we may confirm the real function of these glands for reception of conceptus. They must secrete histotrofo, therefore it is will be the first phase of nutrition to the embryo, the call imbibitions nutrition. When we look at the beginning of the gestation, the conformation of the uterine glands appears as the apposition of citotrofoblast and the blastocist; and its reaction to trofoblastic invasion. Mainly in accordance with the form of the glands, therefore the same ones are the support of origin of the trofoblastic infiltrated one stop in direction to the myometrium. However, Davies, 1950 studying placentas in the beginning of gestation in seals show crypts uterine delimiting the area of placentation and its form, the same salient in our research. However we must take into account the type of placentation in each species and its organization of endometrial tissue for reception of concept, therefore MacDonald and Bosma (1985), by studying pregnant uterus of pigs shows formation of distinct transversal ridges in the uterine light, in reply to its epitheliocorial placentation, although it differs that ones that possess endoteliocorial placenta and with form to lamellar (Ambrósio and Miglino, 2001), however its endometrial glands determine the trofoblastic invasion. No literature in carnivores was found telling the disposal of the endometrial glands, its net to microvasculature and relation with the gestational period, and we can only infer that the study delimited clearly this triad and its morphologic alterations for reception of conceptus.

Conclusions

The canine endometrial glands follow demarcated alterations of via lumen to adjust it into the estrous cycle that if just find the in dogs. A capillary network reflects the period of the estrous cycle, either with microvascular increase for estrous and diestrus, or poor for proestrus, with sights of the reduction of future hemorrhage that proceeds this estrous period. The endometrial glands delimit the trofoblastic invasion. We highlighted the extreme importance of the microvascular network (spiral arterial) and the structural organization of the endometrial glands in the beginning at pregnancy to make possible the confirmation of pregnancy in the canine species of the gestation and uterine alterations. We also show for the first time uterine structure in a wild south America carnivore, Coati, with emphasis to endometrial glands organization and really begin of pregnancy in this specie, compared as similar with dogs, but philogenetically little different.

Acknowledgments

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