

The materno-fetal interface in llama (*Lama guanicoe glama*)¹

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ABSTRACT.- Iturrizaga D.M., Verechia F.T., Santos T.C., Bombonato P.P., Teixeira D.G. & Miglino M.A. 2007. **The materno-fetal interface in llama (*Lama guanicoe glama*).** *Pesquisa Veterinária Brasileira* 27(6):221-228. Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. E-mail: miglino@usp.br

Samples from 9 llamas (28 through 36 weeks of gestation) were collected and fixed in 4% buffered paraformaldehyde (light microscopy) and in 2.5% buffered glutaraldehyde (transmission and scanning electron microscopy). The material was processed in paraplast and slides (5µm) were stained with HE, PAS, Masson-Trichrome, acid phosphatase and Perl's. The uteroferrin was immunolocalized. The results show that llama placenta is chorioallantoic, diffuse, folded and epitheliochorial, and the fetus is covered with an epidermal membrane. The trophoblast cells have variable morphology: cubic, rounded and triangular cells, with cytoplasm containing PAS-positive granules. Binucleated cells with large cytoplasm and rounded nuclei, as well as giant trophoblastic cells with multiple nuclei were also observed. Numerous blood vessels were observed beneath the cells of the uterine epithelium and around the chorionic subdivided branches. Glandular activity was shown by PAS, Perl's, and acid phosphatase positive reactions in the cytoplasm and glandular lumen, and by immunolocalization of the uteroferrin in the glandular epithelium. The uterine glands open in spaces formed by the areoles, which are filled by PAS-positive material. The llama fetus was covered by the epidermal membrane, composed of stratified epithelium, with up to seven layers of mono-, bi- or trinucleated cells. The high level of maternal and fetal vascularization surfaces indicates an intense exchange of substances across both surfaces. The metabolic activity shown in the uterine glands suggests an adaptation of the gestation to the high altitudes of the natural *habitat* of this species.

INDEX TERMS: Placenta, llama, trophoblast, uteroferrin, epitheliochorial, morphology.

RESUMO.- [A interface materno-fetal em lhamas (*Lama guanicoe glama*).] Fragmentos da placenta de 9 animais (28-36 semanas de gestação), provenientes do *Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA)*, Cusco-Peru, e da *Universidad del Altiplano (UNA)*, Puno-Peru, foram colhidos e fixados em paraformaldeído 4% em PBS para microscopia de luz e em glutaraldeído em 2,5% PBS para microscopia eletrônica de transmissão e de varredura. O material incluído em paraplast

cortado com 5 mm foi processado para HE, PAS, Tricrômio de Masson, fosfatase ácida e Perl's e para imuno-histoquímica da uteroferrina. Os resultados mostraram que a placenta da lhama é corialantóide, difusa, pregueada e epiteliocorial e o feto está recoberto pela membrana epidermal. O trofoblasto possui morfologia variada: células cúbicas, arredondadas ou triangulares, com citoplasma contendo grânulos PAS+. Células binucleadas com citoplasma aumentado e núcleo arredondado e células trofoblásticas aumentados com múltiplos núcleos, também foram observadas. Grande quantidade de vasos sanguíneos foi observada entre as células do epitélio uterino e ao redor das projeções coriônicas, as quais estavam subdivididas. A atividade glandular foi demonstrada pelas reações de PAS, Perl's e fosfatase ácida positivas e pela imunolocalização da uteroferrina na luz e epitélio glandular. As glândulas uterinas abrem-se nos espaços formados pelas aréolas, as quais estavam preenchidas por material PAS+. Os fetos das lhamas esta-

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vam recobertos pela membrana epidermal, constituída por um epitélio estratificado composto por mais de 7 camadas de células mono, bi ou trinucleadas. A alta vascularização das superfícies materna e fetais indica intensa capacidade de trocas de substâncias entre as duas superfícies, e a atividade metabólica mostrada pelas glândulas uterinas sugere adaptação da gestação às altitudes elevadas do habitat natural desta espécie.

TERMOS DE INDEXAÇÃO: Placenta, lhama, trofoblasto, uteroferrina, epiteliocorial, morfologia.

INTRODUCTION

South American camelids belong to the order Artiodactyla, sharing some characteristics with the ruminants. In Peru there are four species of the South American camelids known worldwide as alpacas, llamas, guanacos and vicunas, the first two being wild animals.

There are few studies to date about ultrastructural and functional aspects of llama placenta, besides some comparisons with other species of the Camelidae family or other Artiodactyles. South American camelids have a placenta which has been classified as diffuse and epitheliochorial (Latshaw 1987). The diffuse villous placenta is found in Perissodactyles such as the horse, mule and zebra and in Cetaceans such as the dolphin, as well as in Artiodactyles such as llama, camelids and deer (Amoroso 1952, Dantzer 1999). This type of placenta is also similar to that of mares and swine, although the fetal membranes of the camelids have particular characteristics (Stevens et al. 1980, Fowler & Bravo 1998). It is stated that like in ungulates, the epitheliochorial placenta is also present in large animals with a long gestation period, like in whales, and the presence of capillaries indentation in trophoblast and uterine epithelium is important to reduce the interhemal distance (Enders & Carter 2004).

In alpacas it has been observed that the uterine surface has no hollows or specific caruncular areas similar to those described in other ruminants such as cows, sheep and goats (Bustinzá 1961). The distance between the microvilli of the fetus and the maternal surface appears to be much smaller in this species than in other ungulates, and it can be supposed that it is an adaptation to great altitudes (Stevens et al. 1980). Due to this type of placenta, placental retention is rare in camelids and typically has maternal and fetal capillaries which indent the epithelium and the trophoblast, which occur along the entire maternal-fetal interface (Olivera et al. 2003b). This type of placenta is also similar in mares and sows, although the fetal membranes of the camelids have particular characteristics (Stevens et al. 1980, Zhang et al. 1991, Fowler & Bravo 1998). As stated by some authors (Abd-Elnaeim et al. 1999, Jones et al. 2002, Olivera 2002, Olivera et al. 2003a, Wooding et al. 2003) very large cells which contain large nuclei and numerous nucleoli in the trophoblastic layer of alpacas, Bactrian camels and dromedaries have been found.

Another characteristic shared by many epitheliochorial placentae is the presence of areolae on the surface of the chorium, as found in swine (Dantzer 1984), equines (Ginther 1992), peccaries (Santos et al. 2006), and camelids (Abd-Elnaeim

2003) among others. They are related to the opening of one or more (Leiser & Dantzer 1988) uterine glands responsible for the secretion of glycoproteins, including uteroferrin (Roberts et al. 1986). In swine placenta, uteroferrin was detected in the areolas and in the glandular epithelium (Renegar et al. 1982, Leiser & Dantzer 1988). Uteroferrin is a ferritin transport glycoprotein with acid phosphatase, which provides iron for the fetuses. The synthesis of uteroferrin follows the common model for the secretion of glycoproteins. The iron can be found in the glandular epithelium of the uterine glands to be in free form in the cytoplasm or accumulated in lysosomes, but also in ferritin (Dantzer & Nielsen 1984, Roberts et al. 1986).

This study aims to describe the morphological aspects of the maternal-fetal placental interface, mainly during the last third of gestation, focusing on structure and histochemistry. This description could be used in reproductive studies in order to improve the zootechnical utilization and the conservation of llamas.

MATERIALS AND METHODS

Animals

Seven adult pregnant female animals, between 28 and 36 weeks of gestation, were obtained from the Peruvian *Instituto Veterinario de Investigaciones Tropicales y de Altura (IVITA, Cusco, Peru)* of the School of Veterinary Medicine at the *Universidad Nacional Mayor de San Marcos (UNMSM, Lima, Perú)* and two were given by the School of Veterinary Medicine and Zootechnics at the *Universidad del Altiplano (UNA, Puno, Peru)* resulting a total of nine animals for the purpose of this analysis.

The animals were submitted to general anesthesia by applying sodic pentobarbital (30mg/kg IV) and sacrificed by cervical denervation after which samples of the uterus and placenta were prepared.

Light and Electron microscopy

Tissues samples for histological analyses were fixed in 4% paraformaldehyde, in phosphate buffer 0.1M, pH 7.3, and embedded in Paraplast or Historesin. The pieces were cut into 5µm thick sections and stained with HE and Masson's trichrome after which were submitted to the PAS, acid phosphatase and Perl's Prussian blue reactions and immunohistochemical analysis for uteroferrin.

Small fragments (0.3 mm) were fixed in 2.5% glutaraldehyde, phosphate buffer 0.1M, pH 7.3, and processed for electron microscopy. For transmission electron microscopy the samples were embedded in Araldite® resin (Araldite-502 Embedding, Electron Microscopy Science, Hatfield, PA, USA). Ultrathin sections 60 nm thick were post-fixed in 1% osmium tetroxide and 2% tannic acid for 1 hour, and analyzed by transmission electron microscopy (TEM) (JEOL 1010). Other samples were prepared for scanning electron microscopy (SEM) (Leo® 435 VP, USA).

Immunohistochemistry

Sections 5µm were submitted to immunohistochemical analysis for uteroferrin detection. The sections were desparafinized, and the endogenous peroxidase activity was blocked with 2% H₂O₂ in methanol for 30min. Soon afterwards the hydrated sections were treated with buffered citrate pH 6.0 in the microwave oven for 15min, washed in PBS and incubated overnight with rabbit polyclonal antibody anti-uteroferrin IGG in PBS (from Prof. Fuller W. Bazer) (1:1000). The sections were washed in PBS and incubated with biotinylated anti-rabbit secondary antibody from Dako (KIT LSAB®, Dako, Washington, DC, USA) for 50 min, followed by incubation with streptavidin-biotin-per-

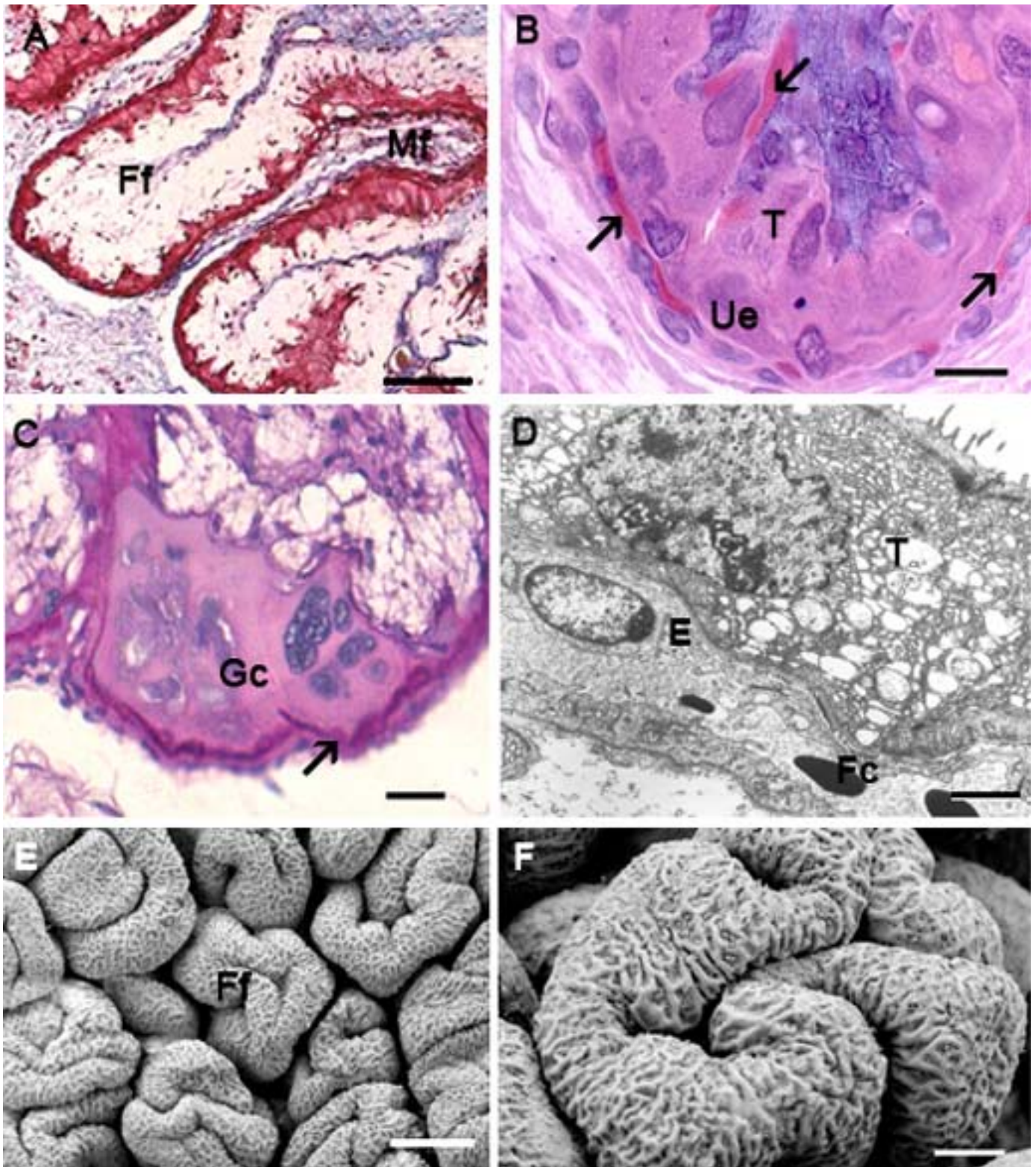


Fig.1. Llama placenta morphology of the placental barrier. A) The fetal (Ff) and maternal (Mf) folds interdigitating each other. B) Detail of placental barrier composed by trophoblast (T) and by uterine epithelium (Ue); note the capillary (arrow) indentation in both epithelium. C) PAS positive reaction on maternal-fetal barrier (arrow) and the giant cells with various nuclei (Gc) on the top of the fetal fold. D) Ultrastructural aspect of the trophoblastic cell (T) and its relation with fetal capillary (Fc). E Endothelial cell. E-F) SEM from fetal folds (Ff), after manual separation from the maternal membranes. (A) Masson, (B) HE, (C) PAS. Scale bar: (A) 100 μ m, (B-C) 20 μ m, (D) 5 μ m, (E) 10 μ m e (F) 100 μ m.

oxidase complex from the same kit for 50min. Visualization was performed with 0.5mg/mL 3,3-diaminobenzidine (DAB) (Sigma Chemical Co., Sta Luis, CA, USA) diluted in PBS (with hydrogen peroxide addition at a final concentration of 0.075% for 5min). Between each step the samples were washed in PBS for 15min and stained with Harris's haematoxylin.

RESULTS

The macroscopic characteristics of the uterus and placental membranes in all the specimens used in the present study were similar. The pregnant uterine horn, in all cases, was the left one and the corium exhibited chorionic projections towards the endometrium.

Histological results from transversal sections showed folds composed by four or five ramifications (Fig.1A), covered by a trophoblast layer. The trophoblastic cells had a variable morphology: cubic, round and almost triangular. Uninucleated cells, few bi- and scarce multinucleated trophoblastic giant cells were observed (Fig.1B-C). In most of the cells a round shaped nucleus, with basophilic nucleoli, were present. Scarce nucleated giant trophoblastic cells are located on the top of the chorionic projections and were easily distinguishable from other cells of the trophoblastic layer by their basophilic cytoplasm, containing 5, 6 or even more nuclei (Fig.1C). Ultrastructural, the presence of an electron dense amorphous material inside the nuclei, microvilli on the apical surface and desmosomes among the membranes of neighboring cells were observed (Fig.1D). The folded aspect of the materno-fetal interface was confirmed by SEM of the fetal surface after disconnection (Fig.1E-F).

The trophoblast showed PAS-positive reactivity in the cytoplasmic granules and in the basal layer and positive reaction to acid phosphatase enzyme, visualized as dark irregular accumulations in the cytoplasm. The maternal-fetal interface had strong PAS-positive reaction (Fig.1C) and weak positive reaction to the acid phosphatase enzyme.

Blood vessels of different diameters, proximal to the trophoblastic layer, were found intermixed with the trophoblastic cells, forming a subtrophoblastic network (Fig.1B,D). Collagen fibers of the mesenchyme were oriented toward the internal part of the chorionic folds and their ramifications.

The allantoic membrane of llamas was formed by a unique layer of columnar cells of different heights (Fig.2A). The face directed toward the interior part of the chorioallantoic cavity showed an irregular appearance with some adhered areas. The apical surface of these cells had a different shape without a clearly defined limit. A number of cells possess on their surface a concentration of microvilli, while another was characterized by a depression on the surface of the epithelium (Fig.2B).

In the maternal side, the uterine epithelium was composed by a layer of irregular cubic cells with spherical nucleus and eosinophilic cytoplasm (Fig.1C). The apical surface of uterine epithelium had microvilli that interdigitated with the corresponding microvilli of the trophoblast. This epithelium was anchored over a loose connective tissue layer, rich in blood vessels and uterine glands. The capillaries formed a subepi-

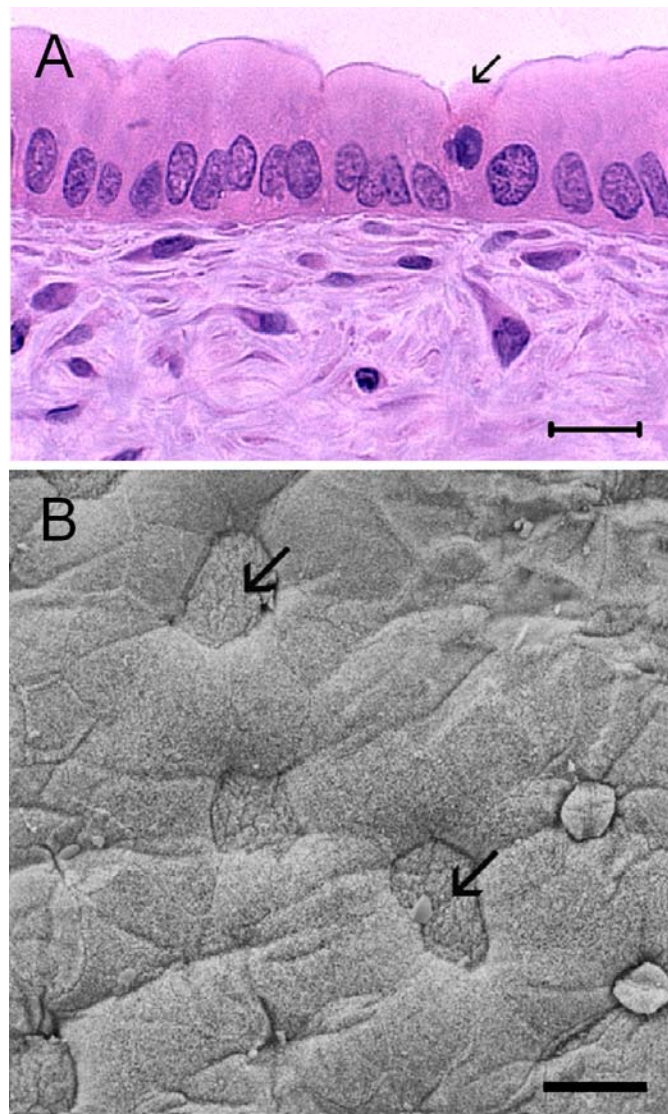


Fig.2. The allantois morphology from llama placenta at term. A) The allantoic epithelium was composed by a simple layer of columnar cells with some cells characterized by a picnotic nucleus and eosinophilic cytoplasm (arrow). B) Overview of the apical surface of the allantois (fetal side) evidencing some cells with differentiated morphology (arrows). (A) HE. Scale bar: (A) 20 μ m, (B) 30 μ m.

thelial capillarie network with indentation among uterine epithelial cells.

The uterine glands were simple and with a large lumen full of eosinophilic secretion. The glandular epithelial cells were basophilic and possessed clear vesicles, usually in the apical pole (Fig.3A). Ultrastructural, the glandular cells contained Golgi complex and a great amount of rough endoplasmatic reticulum, especially around the nucleus (Fig.3B). Numerous mitochondria were also observed in the supranuclear position and close to the apical portion of the cells. In the glandular lumen it was possible to observe irregular bodies of different sizes and electron densities. Microvilosities were observed on the apical surface of the cells. Less fre-

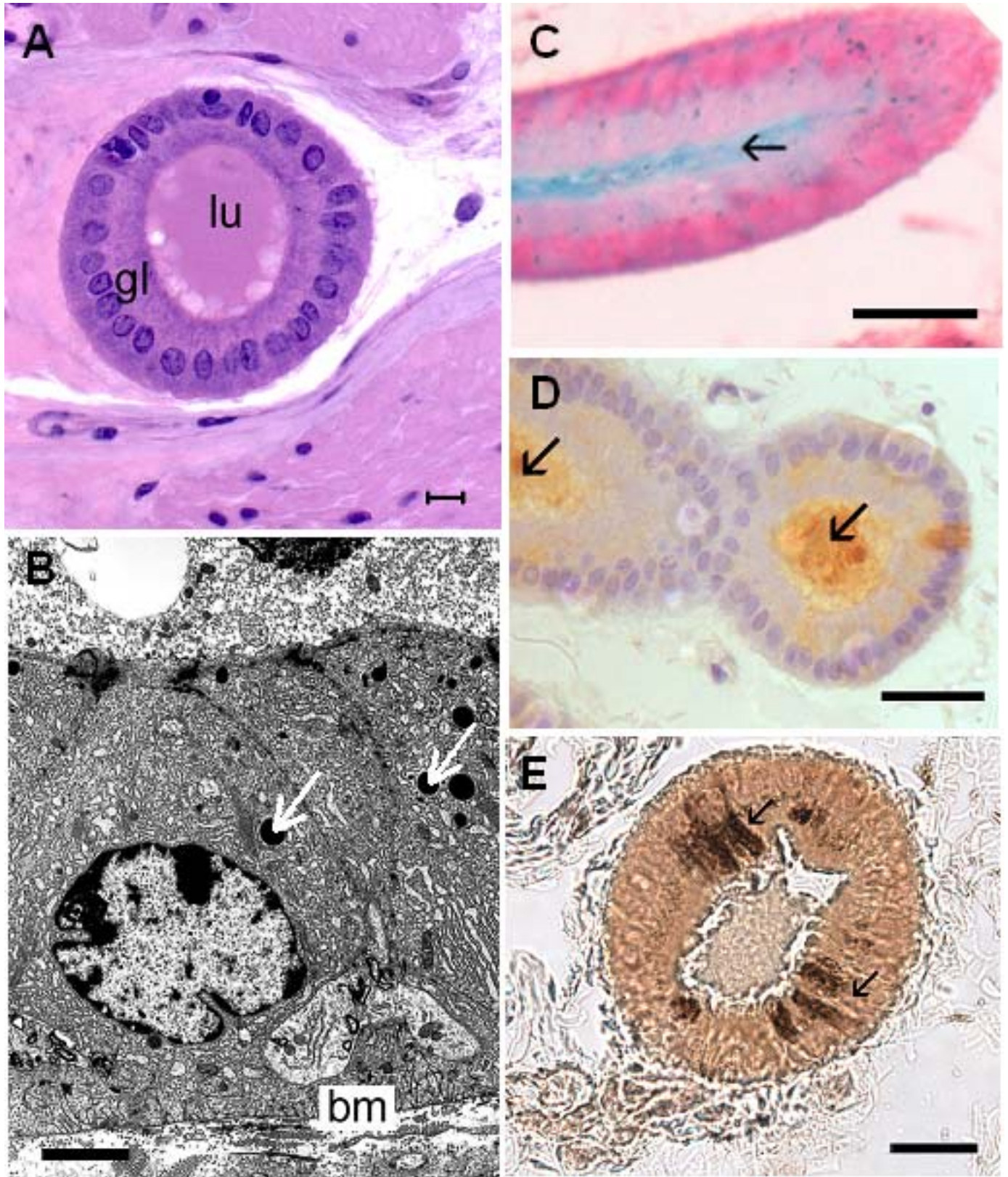


Fig.3. Morphological aspects of uterine glands from llama placenta at term. A) The epithelium is composed by columnar, basophilic glandular cells (gl) with the lumen (lu) filled by secretion. B) Ultrastructurally the secretory granules (shadowed arrow) and the basal membrane (bm) are noticed. C-D) The glandular activity (black arrows) is demonstrated by Perl's+ reaction (C), immunolocalization of uteroferrin+ (D) and reaction to acid phosphatase+ (E). A) HE, C) Perl's, D) DAB, E) Acid phosphatase. Scale bar: A) 10 μ m, B) 2 μ m, C) 25 μ m, D,E) 20 μ m.

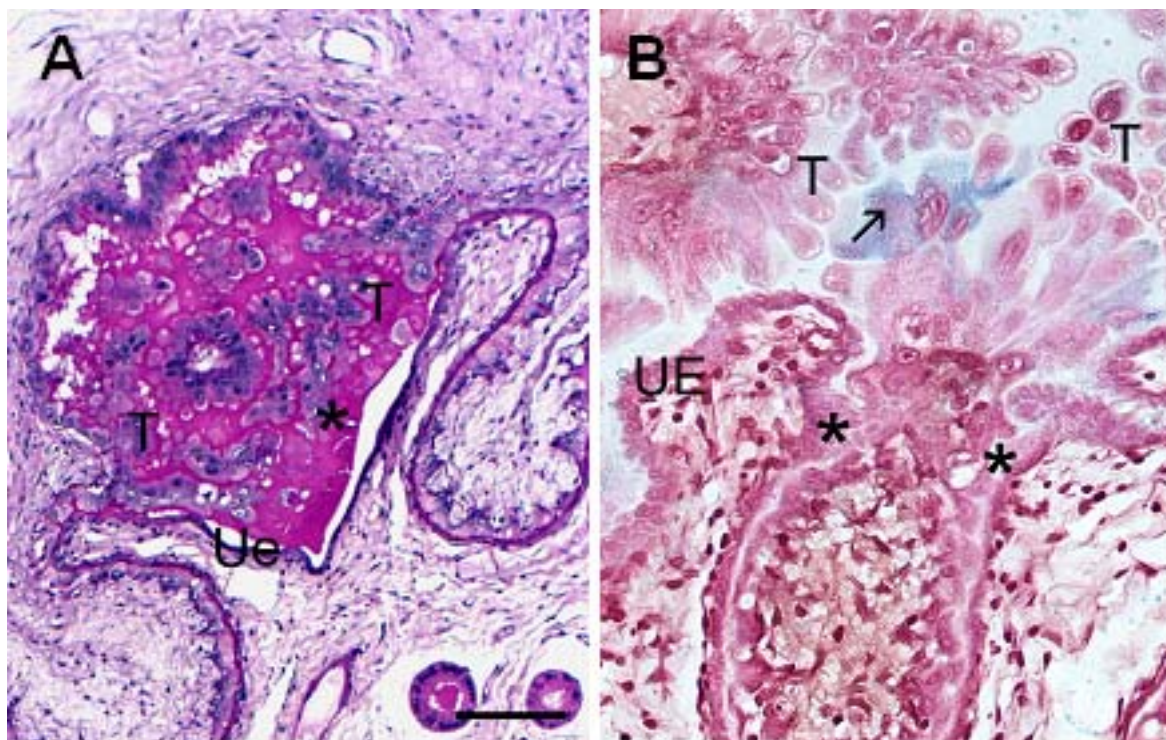


Fig.4. Llama placenta areola. A) Into the areolar cavity the trophoblast (T) forms elaborated folds distant of the uterine epithelium (Ue). Here, the secretive areolar content is PAS+ (*). B) Positive Perl's reaction in the areolar cavity (arrow) disperses nearby trophoblastic cells (T). Two mouths (*) bordered by uterine epithelium (Ue). A) PAS, B) Perl's. Scale bar: A) 100 μ m, B) 40 μ m.

quently there were well developed cilia and scarce secretory vesicles were found. In these cells the ferric ferritin detection was especially evident as small granulations in the lumen and cytoplasm and the uteroferrin was immuno-localized in the cytoplasm and lumens (Fig.3C-D). The reaction of

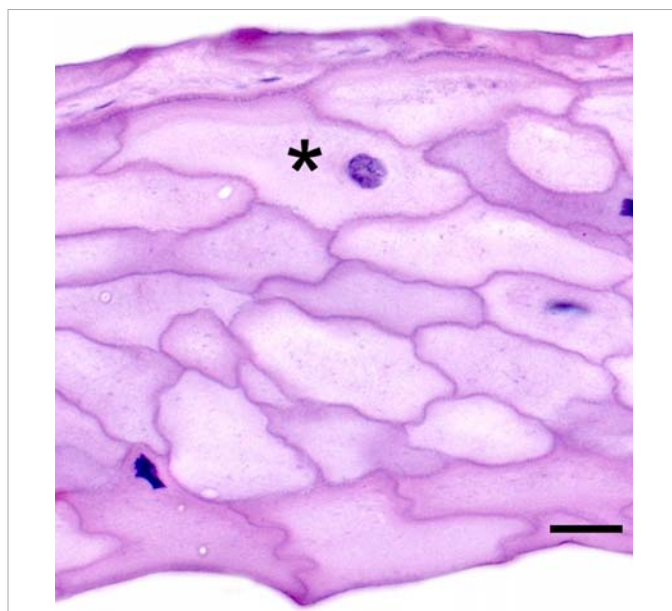


Fig.5. The epidermal membrane is composed by layers of eosinophilic cells eventually nucleated (*). HE. Scale bar: 10 μ m.

the acid phosphatase is weak inside the granular cells (Fig.3E).

The areolae were scarcely dispersed among the chorionic projections as spaces separating the fetal and maternal faces. The spaces are filled with an amorphous material strongly PAS-positive and Perl's-positive (Fig.4A-B), and in their interior cellular debris and the uterine gland outlets were found. The areolar trophoblast exhibited a cylindrical-like shape and covers the elaborated corium folds. The whole fetal surface was covered by the epidermal membrane. This membrane was formed by elongated and irregular cells, overlapping each other, forming a stratified epithelium of seven or more layers. Some cells presented two or three nuclei and in others mitosis is observed and the cytoplasm was eosinophilic (Fig.5).

DISCUSSION

The localization of the fetuses in the left horn of llamas confirms the hypothesis that implantation of embryos in the left horn offers better conditions for their development (Sumar & Leyva 1979).

The cells of the trophoblastic layer have an active participation in the uptake and metabolism of important molecules for the nutrition and maintenance of the fetus. The presence of giant trophoblastic cells suggests that they have special functions in the synthesis of metabolic products, differing from or more complex than those produced in other trophoblastic cells. The morphological variability of the trophoblastic cells

has also been described in other species of camelids, such as in dromedaries (Gorokhovskii et al. 1975, Abd-Elnaeim et al. 1999, Jones et al. 2000, Wooding et al. 2003) and in alpacas (Bustanza 1961, Jones et al. 2000). Both uni- and binucleated cells of the trophoblastic layer exhibited PAS-positive reactivity similar to that observed in dromedaries and alpacas (Jones et al. 2000, Olivera et al. 2003a). Acid phosphatase enzyme reactivity was observed in some trophoblastic cells localized, mainly localized at the top of the chorionic projections, differing from that found in the alpacas where this reactivity was only noted in the trophoblastic cells related to the areolae (Olivera et al. 2003a).

The PAS and acid phosphatase reactions showed that the trophoblastic cells actively participate in the uptake of products coming from the maternal surface, as well as in their metabolism and catalysis. The greater reactivity in llamas, compared to alpacas and dromedaries, can suggest a higher and more efficient metabolic activity of the trophoblastic cells.

Giant trophoblastic cells were less frequently observed than in other species of camelids, such as in alpacas, and even less than in those seen in dromedaries (Jones et al. 2000, Olivera et al. 2003a). Remains of intercellular membranes observed in the cytoplasm of some giant cells fit the proposition that the source of these cells in dromedaries results from the fusion of contiguous mononucleated cells (Skidmore et al. 1996). We noted that after their formation these cells do not maintain the histochemical properties of the mononucleated cells, which leads us to believe that the giant trophoblastic cells have different or specialized functions.

The mesenchyma on the inside of the fetal folds and their subdivisions is filled by collagenous fibers, as was observed in alpacas (Jones et al. 2004). This fact indicates that the collagenous fibers are reinforcements in the chorionic projections, acting as a framework support.

The characteristics of the allantois are similar to those in swine (Tiedemann 1979), but, according to our observations, the allantois cells appear to have a secreting function in llamas, denoted by the villous surface. We believe that the differentiated cells found on the surface reveal a functional specialization of the allantois.

In alpaca placentae, the maternal-fetal interface is a strongly stained with PAS. This material was described as glycoproteins of great diversity in the differing species and different stages of gestation (Jones et al. 1995, Jones et al. 1997). In sow placenta, this material is attributed with having functions of adhesion, control and inhibition of the invasive process of the trophoblast, as well as being related to the presence of signaling molecules of hormones, enzymes and growth factors (Dantzer 1985, Dantzer & Leiser 1993).

The presence of microvilli and cilia in the lumen of the uterine glands in our observations allows us to state that the flow of secretions is regulated and directed by the movement of these structures, so that the contents are taken to the excretory duct of the glands in the areolar cavity.

The results suggest an active passage of glycoproteins between both the maternal and the fetal surfaces while the

areolae constitute a source of reservoir of substances for the fetus, as well as allowing the trophoblast to use them continually. Generally speaking, the morphological and histochemical characteristics of the areolae in our study were very similar to those described in peccaries (Santos 2006), sows (Dantzer 1984) and alpacas (Olivera 2002, Olivera et al. 2003a). The classification of regular and irregular areolae as observed in sows was not found in the llama (Abd-Elnaeim et al. 2003). In sows there is a system of specific vessels for the areolae, suggesting that this system is important for maintaining the transfer of substances in the areolae, as well as their secretion, metabolism and absorption (Dantzer 1984, Dantzer & Leiser 1993, Santos 2006).

By analyzing the llama placenta, the uteroferrin was immunolocalized to the glandular lumen and in the cytoplasm of the glandular cells in the form of granulations. These findings largely match the studies made in sows' placentae (Enegar et al. 1982, Dantzer & Nielsen 1984, Raub et al. 1985, Roberts et al. 1986, Bazer et al. 1991), and in studies which show that the secretion of this protein is a specific genetic expression of the glandular cells (Reed et al. 1996).

Uteroferrin in llamas can also be characterized as a transport glycoprotein, with acid phosphatase activity, with the lumen and the glandular epithelial cells having an intense PAS-positive and acid phosphatase-positive activity. Studies performed in swine showed similar results for placentae (Buih et al. 1979, Dantzer & Nielsen 1984) and that the uteroferrin is produced and secreted by the glandular epithelium. The uteroferrin also acts as a protein which function is transportation of iron, going to the fetal surface in different forms of ferritin or ferric ferrocyanide. The uteroferrin is returned to the maternal circulation having left the iron at the receptors of the trophoblastic cells. The morphological similarities between both species lead us to suppose that this phenomenon may also occur in llama placentae. Determining the mechanisms for the transport and the molecular forms in which the iron is transferred to the fetal receptors deserves further studies using this species for confirmation.

As regards the epidermal membrane of llamas, the morphological characteristics match the descriptions already made for llamas (Fowler & Olander 1990, Olivera 2002) and also for other members of the Camelidae family (Musa 1977, Merkt et al. 1988). Based on the structural results it can be stated that this membrane has a mechanical role of protecting the internal surface of the fetal membranes as well as facilitating llamas' parturition.

Epitheliochorial placentae in general have large sub-epithelial capillary networks to compensate for the thickness of the placental barrier between the maternal and fetal blood (Fowler & Olander 1990). Another factor which contributes to this characteristic, particularly in llamas, is the high altitude at which these animals are bred, giving them a dense sub-epithelial capillary network on both the maternal and the fetal side.

After these considerations, we can conclude that the llama placenta is chorioallantoic, epitheliochorial, and diffuse, as well as having areas specialized in absorbing substances

coming from the uterine glands, the areolae. These morphological characteristics are common to other mammals with an epitheliochorial placenta, such as in swine, tayassu and other members of the Camelidae family (alpaca), suggesting that in llamas the placental physiology obeys a similar pattern as is seen among the various related species.

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