

Characteristics of the Equine Placenta at First Trimester

Características de la Placenta Equina en el Primer Trimestre

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SUMMARY: The equine placenta is a simple apposition of fetal and maternal tissues, becoming more complex with the formation of microcotyledons around days 75 and 100 of gestation. The present study aimed to describe the gross and microscopic morphology of early equine placenta. Embryonic/fetal membranes from thirty-seven mares were submitted to macroscopic description, light, scanning and transmission microscopy. Overall the gross characteristics of membranes were similar with already described for older stages. However, transmission electron microscopy evidenced high metabolic rate in chorion and allantois, and high secretion profile in amnion and even higher in yolk sac. Gene ontologies enrichment, using published data, pointed several common ontologies in allantoic and amniotic fluids, related to oxygen and iron transport, extracellular space and high-density lipoprotein receptor binding. Overall, the morphological and ontology enrichment could indicate allantois and amnion crosstalk.

KEY WORDS: Horse; Placental development; Pregnancy.

INTRODUCTION

The extra-embryonic membranes represent the communication link between the mother and the fetus, established by the placenta, a vital organ for fetal development and growth (Wolf *et al.*, 2003). The anatomy of the placenta has been studied in several ungulate species at macroscopic and ultrastructural levels (swine, sheep, bovine and equine) (Amoroso, 1954; Mossman, 1987), however, detailed morphological aspects of early fetal membranes in horses remains poorly covered in the literature (Ginther, 1992; Dantzer & Leiser, 1993; Wodding & Flint, 1994).

Equine pregnancy has some particularities, such as, the early conceptus remains spherical and moves freely within the uterine lumen until day 16 after ovulation, when it becomes fixed at the base of the uterine horn (Ginther; Stout & Allen, 2001; Walter *et al.*, 2010). The yolk sac begins to regress from around day 22, while the allantois enlarges rapidly and fuses with the overlying chorion to form the allantochorion. In the embryonic pedicle at the period of regressing yolk sac and growing allantois, the trophoblast cells start to differentiate to form the chorionic girdle (Allen

& Wilsher, 2009) and by day 35 the trophoblast has fully differentiated into its minor invasive (chorionic girdle) and major non-invasive (allantochorion) components. Between days 35 to 38 the chorionic girdle cells become binucleated and invade the maternal endometrium to form endometrial cups (Gerstenberg *et al.*, 1999). Until day 150 of pregnancy, the placenta develops to full maturity (Ginther).

The importance of studying placenta development and functions rely upon achieving knowledge as to improve the maintenance of the fetus (Wells *et al.*, 1999). Understanding placenta morphophysiology depends upon a detailed characterization of the normal process of early placentation. Information concerning horse conceptus development and regarding materno-fetal contacts, are scarce in the literature, and many aspects remain poorly understood. Also knowledge about membrane development is important, because estrogen precursors, produced by hyperplastic fetal gonads, are aromatized by the equine placenta (Allen & Stewart; Barreto *et al.*, 2018). Thus, the objective of the present study was to provide a morphological description,

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at gross, fine and ultrastructural levels, using tools as scanning and transmission electron microscopy (SEM and TEM, respectively), of the horse extra-embryonic membranes from days 15 and 107 of gestational age.

MATERIAL AND METHOD

Sample collection. The project was approved by Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science of University of São Paulo (Protocol number 1475/2008). Uteri from 37 pregnant mix breed mares were collected at an abattoir located in Pelotas, Rio Grande do Sul, Brazil. Pieces of membranes nearby the embryo/fetus were fixed in 4 % buffered paraformaldehyde or 2.5 % buffered glutaraldehyde. Gestational age were estimated according to crown-rump length and characteristics described by Evans & Sack (1973), and samples were divided by age intervals, 15-35 days of pregnancy (n=19), 36-55 (n=8), 56-75 (n=3), 76-95 (n=3) and 96-107 (n=4).

Light microscopy. After 4 % paraformaldehyde fixation, fragments were routinely processed for basic histology and stained with hematoxylin-eosin (HE), periodic acid-Schiff (PAS) and picosirius [13] before histological examination and documentation under light microscope (Nikon Eclipse E-800).

Scanning electron microscopy. After 24 hours of 2.5 % glutaraldehyde fixation the membrane fragments were rinsed three times of 15 min each in phosphate buffer solution (PBS). Then, they were post-fixed in 1 % osmium tetroxide, rinsed again before, and immersed in 1 % tannic acid in water at 4 °C for 1 h. The samples were thrice rinsed in distilled water and dehydrated in a crescent ethanol gradient for 10 minutes each. Finally, samples were completely dried using a Balzers vacuum system (CPD 020), placed on metallic support slides, and coated with a layer of gold (Emitech K550). A LEO 435 VO scanning electron microscope was used to examine the samples.

Transmission electron microscopy. Samples were fixed, rinsed, post-fixed and alcohol dehydrated as scanning electron microscopy samples. After, were kept overnight under slow agitation in a solution of propylene oxide (Polysciences, Inc., EUA) and Spurr resine (Sigma Chemical Co.) in a 1:1 proportion. Then, the solution was replaced by straight resine and polymerized at 69 °C for 72 h. Ultrathin sections (60 nm) were sliced in an automatic ultramicrotome (Ultracut, Leica Microsystems, Nussloch, German), transferred to cooper grids, counterstained with 2 % uranyl

acetate for 5 min and 0.5 % plumb acetate for 10 min. Finally, sections were observed in a transmission electron microscope (JEOL CX-II-100, Peabody, MA, EUA).

Protein expression from amniotic and allantoic fluids.

To evaluate the similarities between amniotic and allantoic fluids we used the database generated by Loux & Ball (2018), where both liquids was submitted to proteomic analysis. Firstly, we separated 3 groups of proteins by origin: amniotic, allantoic and common. Those proteins was converted to gene ID on David Bioinformatics Resource 6.8 (<https://david.ncifcrf.gov>) and then submitted to gene ontology enrichment analysis on Panther software (<http://www.geneontology.org/page/go-enrichment-analysis>) using default parameters.

RESULTS

Chorion. Early on, the chorion was a smooth membrane that make direct contacted with the uterine epithelium (Fig. 1). In the period of 56-75 days of pregnancy is possible to observe the early microvilli development (Fig. 1 A-C). After this, the microvilli are formed and give a brownish color to the chorion (Fig. 1D) Those microvilli make delicate association between chorion and endometrium, characterizing the epitheliochorial placenta. Even in early stages, is possible to observe the chorionic vascularization as a ramification from umbilical funiculus to allow maternal-fetal exchanges (Fig. 1).

Microscopically, even from early development of the microvilli, the chorionic epithelium, trophoblast, was a monolayer of columnar mononuclear cells intercalated (Fig. 2A) by some bi- or multinuclear cells (Fig. 2A). The trophoblast cells have a round format (Fig. 2B) and presented intense PAS stain (Fig. 2A). Ultrastructurally, trophoblast has large nucleus with areas of dense and loose chromatin (Fig. 2C), and, in apical pole, presented several mitochondria (Fig. 2C) and electrodense vesicles (Fig. 2D), and the Golgi apparatus was dispersed throughout cytoplasm. Underneath the trophoblast, mesenchyme was highly vascularized and collagen-rich (Fig. 2A). No differences of cellular morphology were observed trough the analyzed periods.

Allantois. The allantois was richly vascularized even in early stages, displaying thick vessels (Fig. 1). Those vessels become more complex and fused with chorionic vessels after 36-55 days of pregnancy, forming the chorioallantois membrane (Fig. 1B-D). Histologically, along the analyzed period, the allantois epithelium presented a smooth aspect, whose cells had pentagonal shape, with microvilli in the

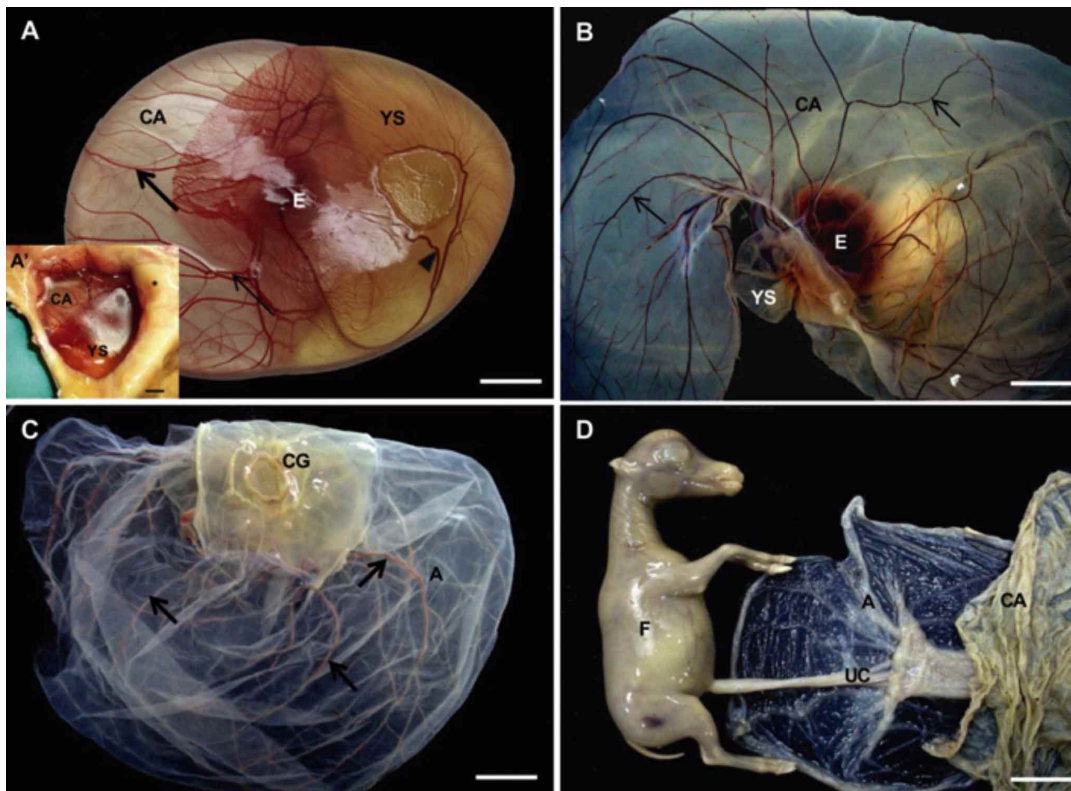


Fig. 1. Photographs of equine early membrane development. In A. 15-35 days of pregnancy. Embryo (E) in the center, chorioallantoic membrane (CA) supplied by allantoic vessels (arrows) and by vitelline vein (arrowhead) from yolk sac (YS). In A', detail of A, showing the close relationship of the chorioallantoic membrane (CA) and yolk sac (YS) with the uterine mucosa (*). In B. 36-55 days of pregnancy. Same structures as observed in A. In C. 36-55 days of pregnancy. Chorionic girdle (CG), the allantoic vessels (arrows) and amnion (A). In D. 76-95 days of pregnancy. Developed fetus (F) with umbilical cord (UC), amnion (A) and chorioallantoic (CA). Bar = 1 cm.

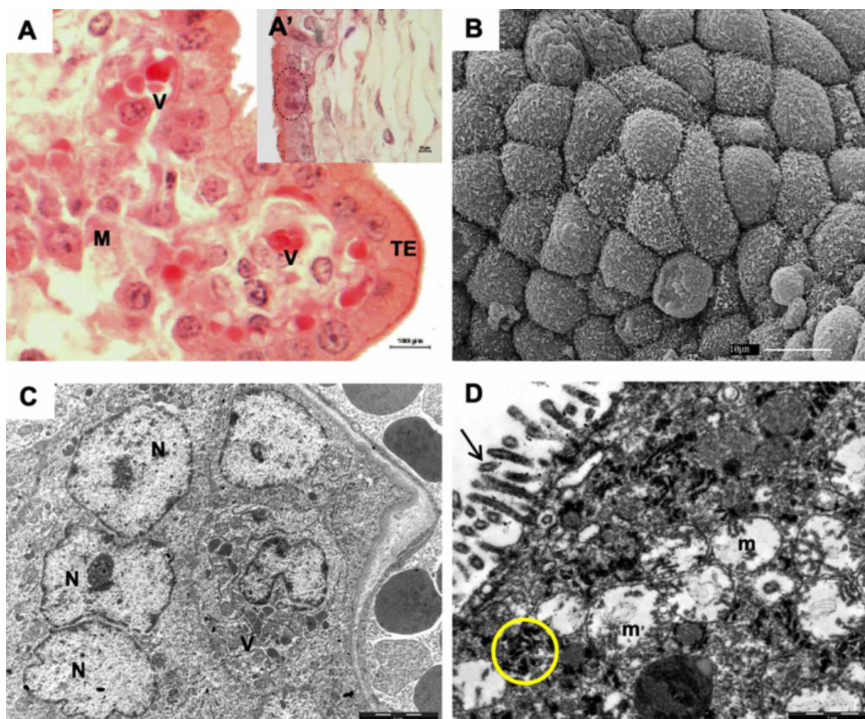


Fig. 2. Images of structure equine chorion membrane under light microscopy, scanning (SEM) and transmission electron microscopy (TEM). In A, 36-55 days of pregnancy, periodic acid-Schiff stain. Epithelium composed by trophoblastic cells and apposed in a richly vascularized (V) mesenchyme (M). In A', detail of A. Presence of binucleate cells (circle). In B, 36-55 days of pregnancy, SEM. Chorionic surface covered with numerous microvilli. In C, 76-95 days of pregnancy, TEM. Trinucleate trophoblastic cell. In D, 76-95 days of pregnancy, TEM. Trophoblastic cell apex with microvilli (arrow), mitochondria (m) and some electron dense vesicles (circle) dispersed in the cytoplasm.

apical surface (Fig 3B). Some button-like structures formed by cell shedding were present in the surface (Fig. 3C). Ultrastructurally, the allantois epithelium was single columnar with large globous nuclei. The cytoplasm is polarized, where the basal pole was rich in rough

endoplasmic reticulum whereas the apical pole had a dense mitochondria concentration (Fig. 3D). Under epithelium, the mesenchyme with low cell density have an abundant extracellular matrix containing blood vessels with erythroblasts in the lumen (Fig. 3A).

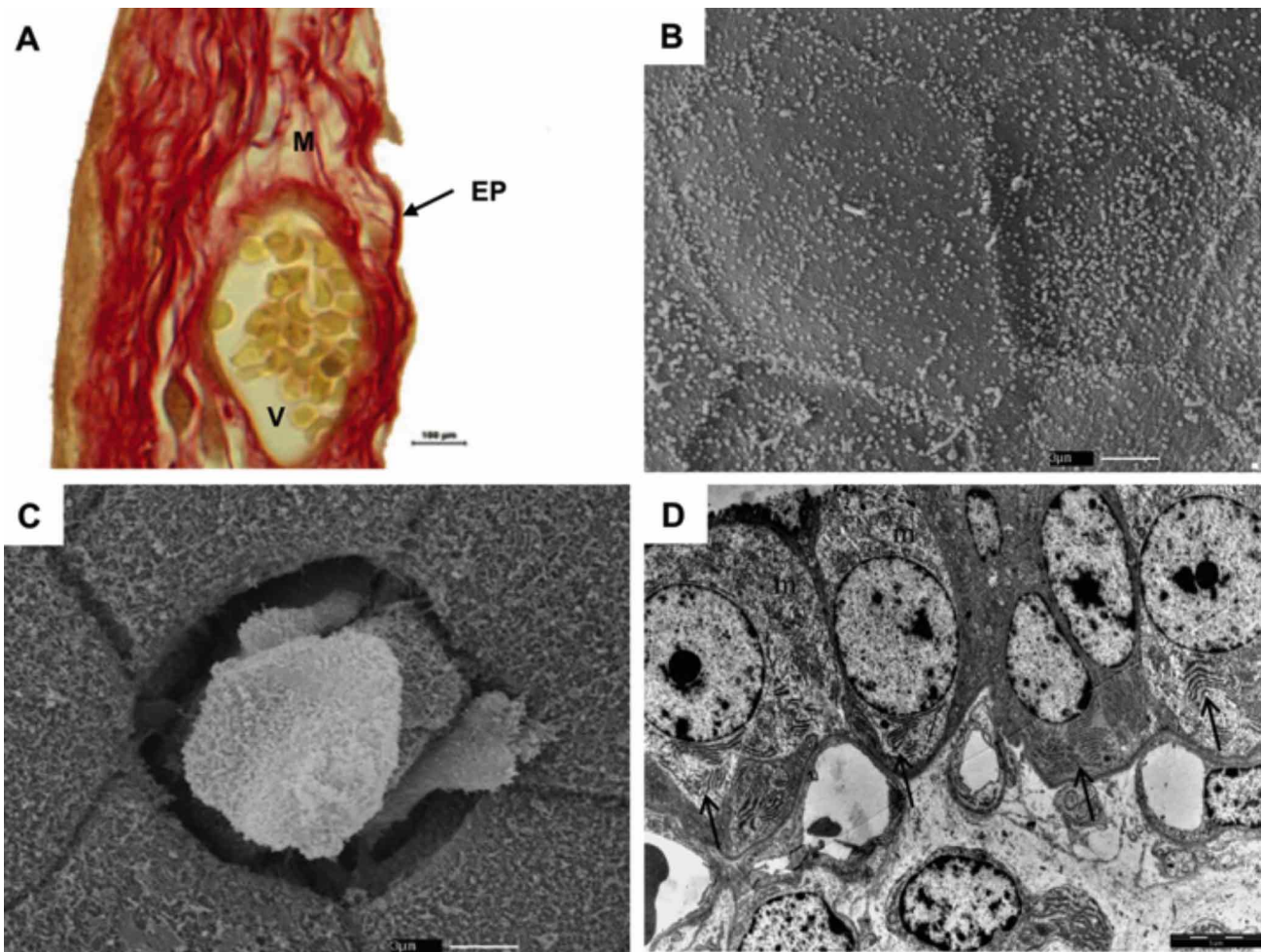


Fig. 3. Images of structure equine allantois membrane under light microscopy, scanning (SEM) and transmission electron microscopy (TEM). In A, 15-35 days of pregnancy, picosirius stain. Simple squamous epithelium (Ep) apposed at the mesenchyme (M) that is filled with collagen fibers and blood vessels (V). In B, 96-107 days of pregnancy, SEM. Apical surface of epithelial cells is covered by microvilli. In C, 96-107 days of pregnancy, SEM. Observe cell shedding in the surface. In D, 96-107 days of pregnancy, TEM. Simple columnar epithelium and the large number of mitochondria (M) in the apical cell and endoplasmic reticulum in the cell base (arrows).

Amnion. The amnion was the innermost membrane and was directly related to the embryos/fetus (Fig. 1D). In early stages (until 36-55 days of pregnancy) make contact with the chorion (Fig. 4A), however after is in close contact with the allantois (Fig. 1D). Is an avascular membrane that contain a small amount of clear and slightly viscous liquid. Overall, in similarity to allantois, the surface presented cells with pentagonal shape, covered by microvilli (Fig. 4B), cell shedding observed as button shape structures (as in the allantois) were also present (Fig. 4C). The epithelium was composed by a monolayer of pavement cells laying over

basement membrane and mesenchyme (Fig. 4A, D). The epithelium contains several vesicles with eletrodense intensities in the apical pole, just above the microvilli (Fig. 4D).

Yolk sac. The yolk sac had a yellowish color and was highly vascularized and regressed around 40 days of pregnancy (Fig. 1A, B), disappearing at 56-75 days of pregnancy. The surface of the yolk sac was similar to allantois and amnion. Independent of age, the yolk sac epithelium presented a single layer of cells varying from cuboidal to columnar shape and mono- or binuclear cells (Fig. 5A).

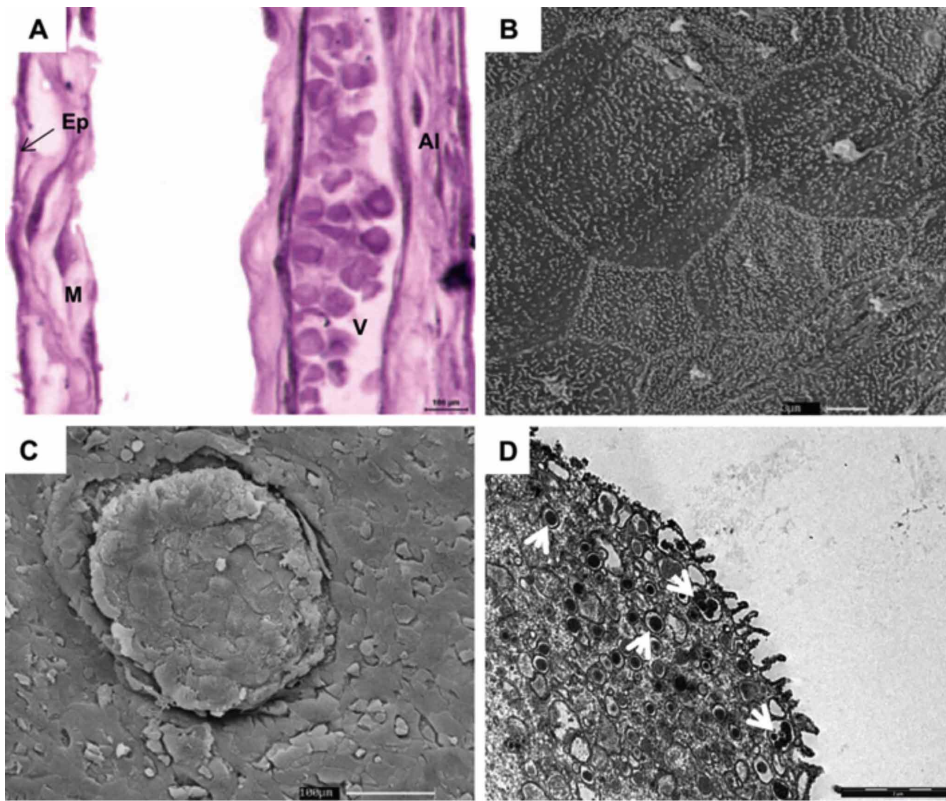


Fig. 4. Images of structure equine amniotic membrane under light microscopy, scanning (SEM) and transmission electron microscopy (TEM). In A, 36-55 days of pregnancy, HE. Simple squamous epithelium (arrow) apposing the mesenchyme (M) and its relationship with the vessels (V) of allantois membrane (Al). In B, 56-75 days of pregnancy, SEM. As in the chorioallantois, the apical surface of amniotic epithelium is covered by microvilli. In C, 56-75 days of pregnancy, SEM. Cell shedding found on the surface of the amnion. In D, 76-95 days of pregnancy, TEM. Amniotic cell apex showing the large number of electron-glycogen granules (arrows).

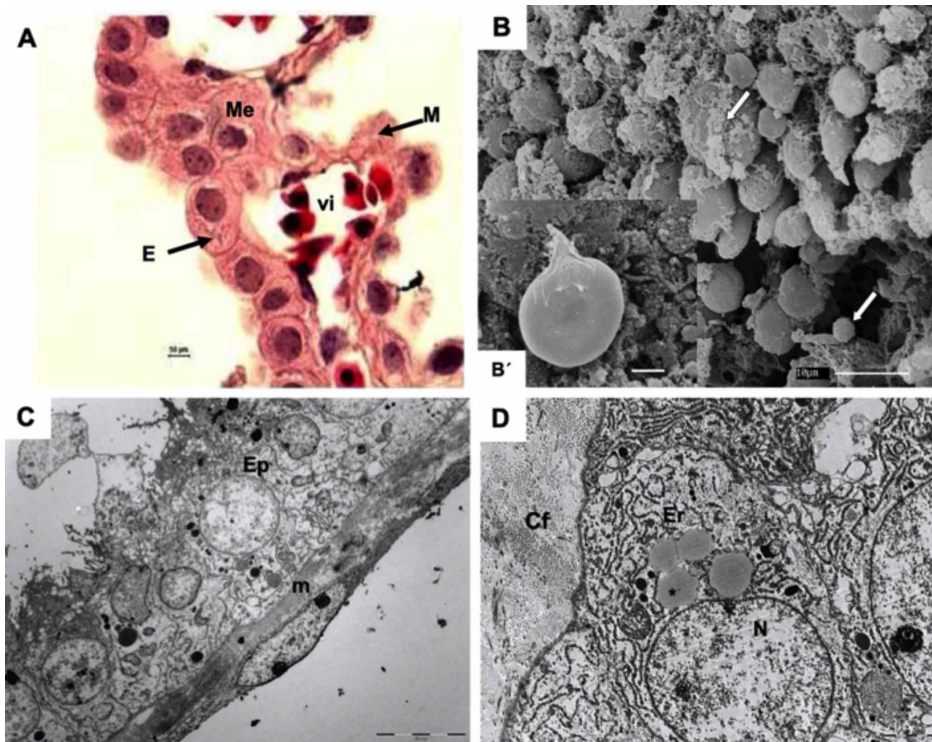


Fig. 5. Images of structure equine yolk sac membrane under light microscopy, scanning (SEM) and transmission electron microscopy (TEM). In A, 36-55 days of pregnancy, HE. The yolk sac histological structure consists of three layers: the mesothelium (M) composed by goblet cells (E) with secretory apex, vascular islands (vi) with still nucleated blood cells, and mesenchyme (Me). In B, 15-35 days of pregnancy, SEM. The surface of mesothelial cells is covered by droplets of secretion (arrows). In B', detail of B. droplet in a moment of exocytosis. In C, 15-35 days of pregnancy, TEM. The simple epithelium (Ep) of globular mesothelial cells apposed on mesenchyme (m). In D, 15-35 days of pregnancy, TEM. Globular mesothelial cells are full of rough endoplasmic reticulum (Er) and some electron dense vesicles (asterisk) surrounding the nucleus (N).

The internal epithelial layer that make contact with the embryo, the mesothelium, presented cuboidal shape with

secretory aspect due to vesicles in the apical surface (exocytosis) (Fig. 5A-C). Whereas the external layer

monolayer faced the coelom, the hemangioblasts, presented cuboidal to columnar shape being mono or binucleated. In general, their cytoplasm had dispersed and abundant rough endoplasmic reticulum and few vesicles, whereas the apical pole was abundant in mitochondria (Fig. 5A, C, D). Those two epithelial layers were intercalated by a highly vascularized mesenchyme that contained numerous vascular islets with blood precursor cells in the lumen (Fig. 5A). Vascular islets were evident since early times of development, characterizing an early hematopoiesis in horses.

Protein expression from amniotic and allantoic fluids. Using Loux & Ball proteomic data, from 129 proteins, 35 (27.1 %) were unique on amniotic, 17 (13.2 %) on allantoic fluids, and 77 (59.7 %) were common in both (Fig. 6A). From those data, the amniotic fluid enriched only extracellular space ontology, that is inside cellular component domain (Fig. 6B). In the allantoic fluid only peroxiredoxin activity was enriched, that is inside molecular function domain (Fig. 6C). Unexpectedly, the common proteins for both fluids enriched more ontology for the three domains: cellular component, biological function and molecular function (Fig. 6D).

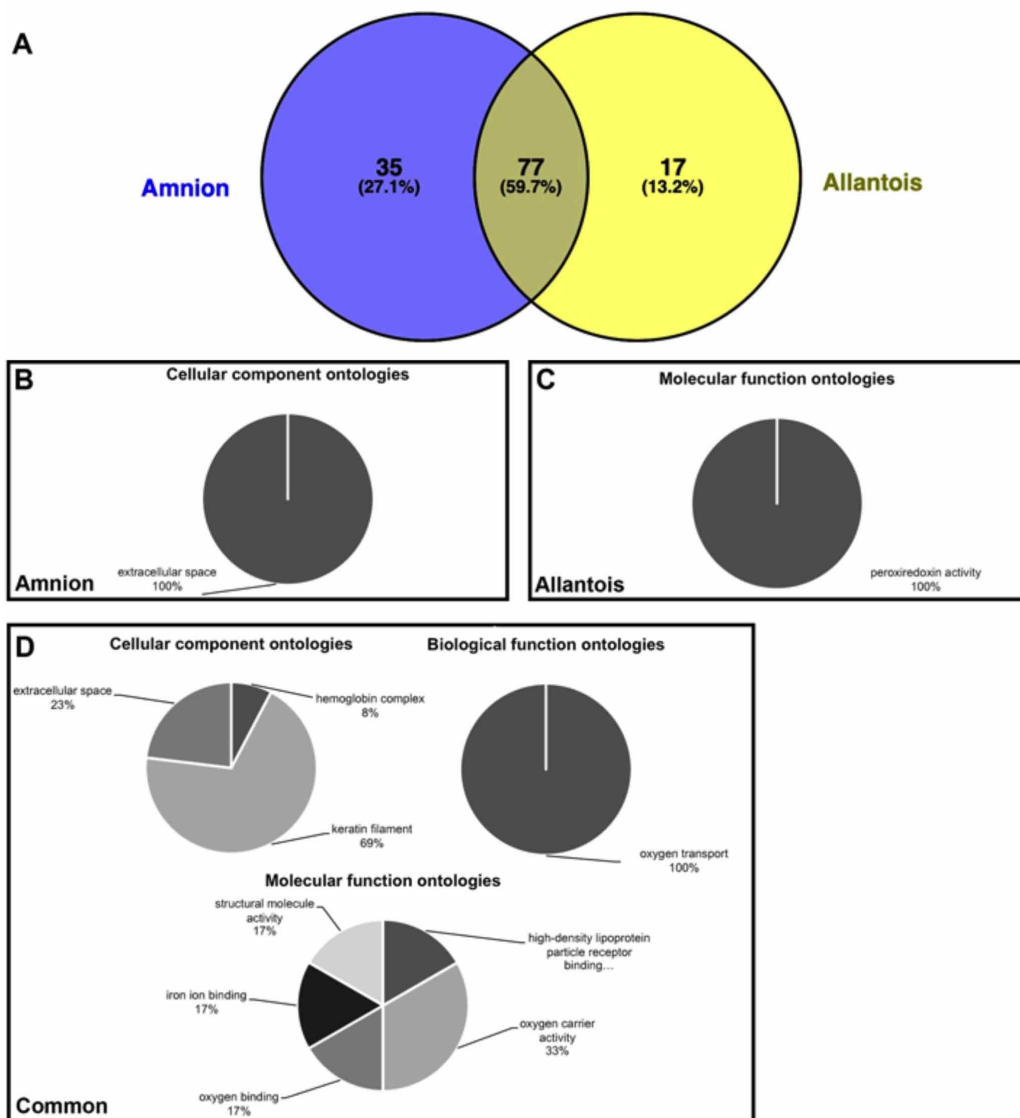


Fig. 6. Gene ontology enrichment from protein of amniotic and allantoic fluid. In A, unique proteins expressed in each fluids and proteins common in both fluids. In B, only gene ontologies inside cellular component domain were enriched for amniotic fluid. In C, only gene ontology inside molecular function domain was enriched for allantoic fluid. In D, gene ontologies for all three domains were enriched for protein commonly present in both fluids.

DISCUSSION

Herein were provided more ultrastructural details of the equine membranes from the first trimester of pregnancy. Overall the gross characteristics corroborate with previous descriptions about the equine placenta (Allen & Wilsher), that is characterized as chorioallantoic (formed by fusion of the allantoic and chorionic mesoderm), epitheliochorial (chorionic and uterine epithelia in contact), diffuse (chorionic villi distributed diffusely), indecidual (without loss of maternal tissue during parturition) and villous (chorionic projections) (Allen, 2001).

The chorion has the same morphology as presented for others and in other species (Samuel *et al.*, 1975; Steven, 1982; Mossman, 1987; Allen & Stewart). The trophoblast, chorionic epithelium, even in early or late first trimester, presented as monolayer, with intense PAS stain for glycoproteins, well established cytoplasmic polarity, loose chromatin, dispersed Golgi apparatus and microvilli in their apical surface. Altogether those cellular characteristics indicates a high metabolic activity, necessary for fetal signaling to endometrium in the materno-fetal interface that is established by chorion during early first trimester (Wilsher & Allen, 2012).

The allantois and chorionic vasculature fuses around day 40, establishing the chorioallantoic membrane. Even before fusion, the allantois presented well-structured vessels, that become more complex after fusion, to vascularize the villi projections in the chorionic surface (Allen & Stewart; Wilsher & Allen). The allantois visceral epithelium is also polarized as in trophoblast, having loose chromatin, high concentrations of endoplasmic reticulum and Golgi apparatus, suggesting a high metabolic rate and secretion. In addition, the most enriched ontology in allantois fluid is the peroxidoxin activity. Peroxidoxin in an antioxidant enzyme related to genome stability (West *et al.*, 2018). Then, we can hypothesize that the high metabolic allantois epithelium could produce peroxidoxin to inhibit possible oxidants eliminated in the allantois fluid by fetal metabolism.

The amniotic membrane epithelium has numerous apical pole vesicles, also the most enriched ontology on amniotic fluid was the extracellular space with several proteins related to secretion (Loux & Ball), suggesting a secretion activity. The protein groups commonly present in allantoic and amniotic fluids enriched several ontologies, but majorly related to oxygen and iron transport, extracellular space and high-density lipoprotein receptor binding. Then, together, those two membranes could have combined function, and maybe a crosstalk, in the transport of some molecules.

The involution of equine yolk sac overlays the period of chorionic and allantoic vascular fusion, as was previous described (Wilsher & Allen; Wooding & Burton, 2008) and indicates the transition from vitelline to chorioallantoic placentation, as is observed in ruminants (Wilsher & Allen). From all membranes, the yolk sac has the most secretory profile, due presence of several and large vesicles in the mesothelial surface to embryonic/fetal nutrition during vitelline placentation (Murphy & Martinuk, 1991; Rüsse *et al.*, 1992; Hoppen, 1994).

Overall, the morphological and ontology enrichment indicating crosstalk and combined function. And involution of yolk sac overlays the chorioallantois development to replacement of vitelline to chorioallantoic placentation.

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RESUMEN: La placenta equina es una simple aposición de tejidos fetales y maternos, que se vuelve más compleja con la formación de microcotiledones alrededor de los días 75 y 100 de gestación. El presente estudio tuvo como objetivo describir la morfología macroscópica y microscópica de la placenta equina temprana. Las membranas embrionarias / fetales de treinta y siete yeguas fueron sometidas a descripción macroscópica, luz, escaneo y microscopía de transmisión. En general, las características generales de las membranas fueron similares a las ya descritas para las etapas más antiguas. Sin embargo, la microscopía electrónica de transmisión mostró una alta tasa metabólica en corion y alantoides, y un alto perfil de secreción en amnios e incluso mayor en el saco vitelino. El enriquecimiento de ontologías génicas, utilizando datos publicados, señaló varias ontologías comunes en fluidos alantoideos y amnióticos, relacionados con el transporte de oxígeno y hierro, espacio extracelular y unión a receptores de lipoproteínas de alta densidad. En general, el enriquecimiento morfológico y ontológico podría indicar alantoides y diafonía de amnios.

PALABRAS CLAVE: Equino; Desarrollo placentario; Preñez.

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