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Morphological study of equine amniotic compartment

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Abstract

Exfoliative cytology of human amniotic fluid (AF) has been extensively studied since 1940s, but no data exist in equine species. The AF compartment represents the environment in which the foetus grows and matures, and its composition changes, reflecting foetal well-being and development. The aim of this study was to describe for the first time the morphology of equine AF cells and amniotic membrane (AM) with light microscopy (LM) and transmission electron microscopy (TEM). AF was collected at parturition within 5 min after the appearance of the AM with a 60 mL syringe from 34 mares and samples of AM were collected from a subset of 7 mares with normal pregnancy hospitalized for attended parturition. For LM observation, a sample of cytocentrifuged fresh AF was stained with May-Grünwald Giemsa and AM sections were stained with H-E. For TEM observation, AF and AM were fixed, embedded in epoxy resins, then sectioned and stained with uranyl acetate and lead citrate solutions. Nucleated and anucleated squamous cells with basophilic cytoplasm, intensely basophilic cornified cells, polymorphonuclear cells, and clusters of eosinophilic amorphous substance were observed. Cells presumably derived from tracheal epithelium and small round nucleated cells with eosinophilic cytoplasm presumably derived from amniotic or urinary epithelium were occasionally found. Lamellar body-like structures (LBs) were present in some epithelial cells. In AM, epithelial, basal and mesenchymal layers were clearly visible with both techniques as previously described. Epithelial cells had several cytoplasmic vacuolization and microvilli were present on apical surface. The connective tissue presented fibroblasts, mesenchymal and rare polymorphonuclear cells, surrounded by abundant extracellular matrix, with distribution of collagen fibres. This is the first report about equine amniotic compartment description by LM and TEM. As recently reported in human medicine, the AM could be a second potential source of pulmonary surfactant, given the finding of LBs inside the cells which could have the same function as in humans. Further studies in samples collected at different gestational ages could increase the knowledge of AF

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cells and their modification during pregnancy, as well as a better comprehension of the role of AM as a secondary source of pulmonary surfactant in the horse. The diagnostic evaluation of AF cellular composition in high-risk pregnancies may also be investigated.

Keywords: mares, amniotic fluid, cytology, amniotic membrane, electron microscopy, lamellar bodies

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1. Introduction

In mammals, many anatomical and histological studies have conducted about foetal membrane and in particular about the amnion, the inner foetal membrane, which is an elastic, opaque to translucent and thin membrane [1,2]. It surrounds the foetus, delimiting the amniotic cavity that contains the amniotic fluid (AF) and it was contiguous to umbilical cord and foetal skin [1]. The amnion consists of a cuboid epithelial layer deriving from epiblast (ectodermal). Outside the amniotic ectoderm, there is a thin layer of mesoderm, adjacent to the mesodermal lining of chorion [1,3].

Despite the extensive research conducted on the amniotic membrane (AM), there is still much to be understood about the fundamental aspects of its structure, functions, evolution and development [4]. Histologically, the amnion is composed by three layers: epithelial monolayer, acellular compact layer, and fibroblast layer. The first layer consists of cuboidal cells that come into direct contact with AF. The cells of the first layer secrete glycoproteins and collagen fibres, which constitute the basal membrane [3]. The acellular compact layer consists of a stromal matrix forming fibrous skeleton of AM. Interstitial collagens of type I and III predominate and are secreted by mesenchymal cells situated in the fibroblast layer, that is the thickest layer of the amnion, composed of a loose fibroblast network embedded in a reticular matrix [1-3].

Furthermore, it is known that AM is a source of mesenchymal stem cells (MSCs). Some studies, in fact, reported the successful isolation of MSCs derived from AM in the horse [5,6].

Many studies have been conducted on biochemical composition of equine AF [7-13], but little is known about the cellular composition. Only a research group [14,15] investigated the presence of polymorphonuclear leucocytes (PMNs) in equine AF, performing cytological evaluation on samples collected at parturition with direct puncture of amniotic vesicle during stage II. In the mare, unlike in the women [16,17], PMNs have also been found in AF in normal pregnancy, identifying their origin

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in the foetus [14,15]. Furthermore, it was established the presence of squamous cells, keratin scales and cellular debris [14,15].

Conversely, exfoliative cytology of human AF has been studied extensively since the early last century [18,19]. In human AF, studies published in the '60s described cell types according to their morphology, maturity, and staining characteristics, despite the low quality of the images in relation to the recent technologies. Different classifications of those cells have been made, based on authors' experience, cellular staining affinities, cellular maturity and morphology. Several studies [20-23] identified a cell classification including five different cellular types: anucleated and nucleated squamous cells, eosinophilic polygonal cells, small round cells, and tall columnar cells. A different classification [24-25] identified only two cell types: large cells with irregular contours, which includes the first three types described above, and round cells. Moreover, PMNs were identified, especially neutrophils, and several studies evaluated their increase as an indicator of bacterial amniotic infection [16,17]. These studies demonstrated that during intramniotic infection there is a foetal inflammatory response and that the PMNs found in the AF are of foetal origin supposing their passage through the alveoli, the umbilical cord and the chorionic vessels [16,17].\

As regards transmission electron microscopy (TEM) observation, numerous studies about amniotic compartment are present in women [26-30], few studies in sheep [31] and cattle [32], but there are no studies in the equine species. In women, AF analysis by TEM has identified two cell types, which can be referred to the second classification described above [26,28]. In sheep and cattle, no studies about AF analysis by TEM are present and it was reported that AM cells had size and number of apical microvilli which, respectively, decrease and increase with advancing pregnancy [31,32].

In domestic animals, amniotic cavity is formed at very early stages and is present throughout foetal development until parturition [1,3]. It represents the environment in which the foetus grows and

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matures, and its composition changes as the pregnancy progresses, reflecting foetal well-being and development [9,15,17,23,33].

The aim of the present study was to describe the morphology of amniotic compartment, in particular to describe equine AF cells and AM in normal pregnancy. To better investigate the morphological aspect of amniotic compartment, light microscopy (LM) was combined with high resolution TEM.

2. Materials and Methods

2.1 Animals

Thirty-four healthy mares with normal pregnancy hospitalized at the Equine Perinatology Unit (Department of Veterinary Medical Sciences, University of Bologna) were enrolled in the study during two breeding seasons (2017-2018). The mares were hospitalized because the owners requested an attended parturition. The mares were hospitalized at about 310 days of pregnancy and remained under observation for at least 7 days postpartum. They were housed in separate wide straw-bedded boxes and fed hay ad libitum and concentrates twice a day; they were allowed to go to pasture during the day. At admission, information about mare's age, parity and last breeding date were recorded. After delivery, the following data were recorded: APGAR score [34], foal's weight and sex, gestation length. Foals were classified as healthy when they had a normal clinical evaluation during the course of hospitalization, including a complete blood count and serum chemistry at birth and an IgG serum concentration ≥800 mg/dL at 12-24 h of life [13].

2.2 Amniotic fluid and amniotic membrane collection

At parturition, AF was collected (average volume 50 mL) within 5 min after the appearance of the AM through the vulva by needle puncture, using a 60-mL sterile syringe (Figure 1a). After AF sampling, AM was collected using clean gloves and sterile scalpel, holding the membrane stretched between the thumb and the forefinger (Figure 1b), to prevent the membrane from touching the floor.

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Both for AF and AM samples, two aliquots were prepared for each animal: one for LM and one for TEM observation. An informed consent about the sampling was given to each owner.

2.3 Amniotic fluid processing

One aliquot was used for cytology. Cytology of the fluid was performed by direct smear and cytocentrifuged preparations (Rotofix 32A Centrifuge; A. Hettich GmbH & Co.KG, Germany) of fresh AF samples were prepared. Air-dried glass slides were stained with May-Grünwald Giemsa (Merck KGaA, Darmstadt, Germany). All slides were examined by LM (Nikon Eclipse E800) and by the same two clinical pathologists.

For TEM observation, the second AF aliquot, processed within 1 hour from collection, were centrifuged at 260 *g* for 15 min (EBA 12 Centrifuge; A. Hettich GmbH & Co.KG, Germany), diluted with Lactate Ringer Solution® (S.A.L.F. S.p.A, Bergamo, Italy) and centrifuged for 30 min at 1038 *g* with Ficoll-Paque plus® (GE Healthcare S.r.l., Chicago, USA). The AF cells were collected at the interface between the Ficoll-Paque plus® and the Lactate Ringer Solution® layers and transferred into one new tube. Cells were suspended in Lactate Ringer Solution® (1:1, v/v) and re-centrifuged at 42 *g* for 10 min. Supernatant was discarded and cytocentrifuged preparations were prepared on glass slides.

Samples were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer for 1 h at 4°C and post fixed with a solution of 1% (w/v) osmium tetroxide in 0.15 M cacodylate buffer for 1 h at room temperature. Then, samples were embedded in epoxy resins after graded-acetone serial dehydration steps. After 72 h, the embedded samples were sectioned into ultrathin slices, stained with 3% uranyl acetate in ethanol and lead citrate solutions, and then observed by TEM CM10 Philips (FEI Company, Eindhoven, The Netherlands) at an accelerating voltage of 80 kV. Images were recorded by Megaview III digital camera (FEI Company, Eindhoven, The Netherlands).

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2.4 Amniotic membrane processing

Amniotic Membrane samples were collected and washed several times with Lactate Ringer Solution® (S.A.L.F. S.p.A, Bergamo, Italy).

One sample was used for histology. Samples were 10% formalin fixed for 24 h, then embedded in paraffin after graded-ethanol serial dehydration steps. Glass slides of 6-8µ sections were stained with Hematoxylin-Eosin for LM observation.

The other sample was used for TEM observation. AM samples were fixed with 2.5% (v/v) glutaraldehyde in 0.1M cacodylate buffer for 2 h at 4 °C and post fixed with a solution of 1% (w/v) osmium tetroxide in 0.1M cacodylate buffer for 1 h at room temperature. Then, samples were processed as described for AF.

3. Results

Thirty-four healthy mares delivering healthy foals after normal pregnancy were included in the study (28 Standardbred, 5 Italian Saddlehorse and 1 Holstein). The mean age and parity were 10 ±4 years and 4 ±3 pregnancies, respectively. The mean gestation length was 340 ±8 days and foals were 8 males and 26 females. The mean APGAR score was 9 ±1 and the mean weight at birth was 50 ±6 Kg.

3.1 Amniotic fluid smears

At the observation of AF smear, the cells found were: nucleated and anucleated squamous cells with basophilic cytoplasm; intensely basophilic cornified epithelial cells (scales) often gathered in cluster of tens; PMNs, mostly neutrophils with segmented or hypersegmented nucleus and little cytoplasm; mononucleated leukocytes of varying diameter, between 8-15µm, attributable to small and large

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lymphocytes. In addition to the cellular component, clusters of eosinophilic amorphous substance, attributable to proteic substance were observed (Figure 2).

Cells presumably derived from tracheal epithelium (cylindrical cells with apical microvilli; Figure 2f) and cells presumably derived from amniotic or urinary epithelium (small round nucleated cells with eosinophilic cytoplasm; Figure 2e) were occasionally found.

At TEM observation (Figure 3), all samples of AF mostly showed a degenerated cellularity. Several cells showed a not well-preserved cytoplasm, with extracted cytoplasmic components and rupture of the cell membrane. Cells in which the round nucleus was recognizable, were rare. Cytoplasm showed numerous vacuolization, sometimes optically empty, in other cases with content attributable to phagocytised material of lipid origin. Cells had round, oval or elongated morphology. Into the nucleus of most of the observed cells, the presence of condensed nuclear chromatin was clearly discernible. Epithelial cells were visible, characterized by the presence of numerous keratin filaments, and PMNs, characterized by the presence of numerous vacuoles and cytoplasmic granules (Figure 3b; 3e). Furthermore, at greater magnification, into the cytoplasm of some epithelial cells and outside cells, numerous lysosomes, and particular structures probably attributable to lamellar bodies (LBs), due to their concentric lamella patterns, were clearly noted (Figure 3e).

Cell membrane of cornified epithelial cell is characterized by the presence of numerous extroflession that are cytoplasmic folds of the cell membrane (itself).

Finally, the ultrastructural analysis of the AF confirmed the presence of a heterogeneous amorphous material. This is composed by round/ovoidal structures, likely related to lamellar bodies, and irregular dense aggregate, presumably to protein complexes (Figure 3f).

3.2 Amniotic membrane

It was possible to collect AM from 7/34 mares.

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In AM, three layers were clearly visible with both techniques: epithelial, basal and mesenchymal layers.

At LM observations, the AM showed a monolayer of cells with cubic morphology that covered a thin cellular connective lamina containing rare fibroblasts (Figure 4b).

The ultrastructural analysis of the AMs showed the monolayer of epithelial cells and the underlying connective tissue relatively well preserved, despite an initial state of degeneration. The epithelial cells had numerous cytoplasmic vacuolations and the cellular interdigitations, observable between the cells, were not tightly connected. The cellular apical surface showed a high number of microvilli. The underlying connective tissue presented fibroblasts, MSCs and rare PMNs surrounded by an abundant and well-preserved extracellular matrix, with a distribution of collagen fibres (Figure 5).

4. Discussion

This is the first report about morphologic description of equine amniotic compartment with traditional technique such as cytology and histology. The AF and the AM were also analysed through TEM to better appreciate the morphological details.

In this study, AF was directly collected by amniocentesis at II stage parturition, while in most of the studies carried out in woman, AF was mainly collected by transabdominal amniocentesis at different stages of pregnancy [17,19,24,35]. The only research group [14,15] who evaluated equine AF cytocentrifuged preparations collected it at parturition, as performed in the present study. Other methods to collect AF in women are direct puncture of amniotic sac during caesarean section and hysterotomy or transvaginally during labour [21,36-38]. In the present study, as well as in most techniques used in women, it could be possible to have a contamination with the mother's vaginal epithelium.

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There is a paucity of information about the specific cellular morphology and origin of equine AF cells derived from normal pregnancy [14,15]. The cellular population of equine AF has never been investigated. This is surprising because in human medicine, the knowledge about the cellular composition of AF is extensive, and also in small ruminants, which have been used as experimental models of humans for decades [16-25; 35-44]. Finally, the knowledge about the normal amniotic compartment is of fundamental importance in order to diagnose and promptly treat high-risk pregnancies. The results of high-risk pregnancy are inadequate foetal nutrition and consequent intrauterine growth retardation, premature delivery or abortion [45].

In a study about a safe transabdominal ultrasound-guided technique to collect equine AF, Authors showed an image of cytocentrifuged AF [33]. Moreover, Hemberg et al. [14,15] investigated the presence and the origin of PMNs, a small percentage of cellular component of equine AF. In particular, PMNs were found in AF of healthy mares with normal pregnancy, identifying their origin in the foetus and their access to amniotic compartment through the endothelium of umbilical cord vessels and pulmonary alveolar spaces [14]. It was not clear how the number of PMNs is related to the foal well-being, but it was interesting to note that samples with higher values of PMNs belonged to mares delivering sick foals [15]. It is worth noting that the authors considered as sick those foals unable to stand before 2 hours after birth [15]. Therefore, it is unclear what kind of disease these animals had and whether there was a hypoxic or inflammatory stimulus during pregnancy that could have aroused a foetal inflammatory response syndrome.

In bovine and human medicine, in order to establish the AF cells origin, the epithelia in contact with the amniotic compartment have been cytologically and histologically examined, such as foetal skin, amnion, umbilical cord and mucosa of digestive, urinary, and respiratory tracts [20,24,35,39,46]. These studies showed the abundant presence in the foetal oral cavity of squamous cells like those found in the AF [21,46]. Round cells seem to originate from amnion [20,35,36,38,40] or from foetal

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urinary tract [24,39] and polygonal cells seem to derivate from the foetal cornea [21,22]. In the present study, equine AF cells were described examining cytocentrifuged preparations and resulted similar to those described previously [20-24, 35-40,46] even if the origin of the AF cells was not investigated. It could be only supposed that nucleated, anucleated and cornified cells derive from desquamation of the epithelium of the foetal skin and foetal oral cavity, while rounded cells could derived from amniotic membrane or foetal urinary tract.

The cells were also studied through TEM, obtaining similar results compared to those obtained with LM, as reported in human studies [26-30]. In fact, the cellular details of round cells, PMNs and epithelial cells were observed.

However, a surprising result was the presence of structure similar to LBs inside some epithelial cells. Lamellar bodies are surfactant-containing lamellated structures and are indicators of foetal lung maturity. They are produced by type II pneumocytes in foetal lung. Then, LBs are secreted into the alveolar space, and transferred into AF [9,41,47]. In a recent study, a new and unexpected function of human AM was discovered. Authors of that study reported that AM cells contain LBs within the native membrane and express all four surfactant proteins [48]. This means that AM could be a second potential source of pulmonary surfactant that it was found inside the cells in amniotic fluid [48]. The identification of the surfactant proteins in equine AM, with immunofluorescence and membrane immunohistochemistry techniques, deserves further studies. As well as in human medicine, the functional relevance, such as a possible role of AM in foetal lung maturation or in pulmonary host defence of the growing foetus, remains to be determined.

In the bovine species, AF was collected from 10 to 24 weeks of pregnancy but the change in cellular population was not assessed over time [46]. Conversely, in women, early (9-15 weeks), mid (15-32 weeks), and late gestation (32-36 weeks) were considered in cytological evaluations. The number of AF cells has been shown to increase with gestation, while the percentage of viable cells and

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macrophages decrease [20,37,49]. In the present study, equine AF was collected at parturition. In the equine species, the possibility of collecting AF during pregnancy by a recent proposed transabdominal amniocentesis technique is currently used only experimentally and not yet in clinical practice [33]. A further study conducted on equine AF, collected at different stages of gestation, would be needed to describe more accurately the cellular population changes during pregnancy.

In the present study, the AM was analysed by histological examination and TEM, obtaining results with more morphological details regarding the ultrastructure of the AM than by AF analysis. This is due to the better preservation of the AM than the cells, probably because the tissue remains viable longer than the exfoliated cells.

The equine AM is formed from the folds of the ectoderma in the early stages of pregnancy [1] and consists of three layers: epithelial, basal and mesenchymal one. In this study, the three layers were clearly visible. The epithelial layer of the AM showed a well-preserved monolayer of cubic cells. Moreover, equine AM showed a morphological integrity such as a close lateral cell interdigitation, short microvilli on the apical cellular surface and numerous cytoplasmic vacuolization. These aspects were found similar in cattle [32], sheep [31] and deer [50].

The great number of cytoplasmic vacuolization may be interpreted as the ability of cells to produce a huge variety of secreting molecules enclosed inside vesicles of different types that could be released outside the cells, as reported in other studies regarding equine AM-MSCs observed by TEM [6]. In addition, it can be hypothesized that the several vesicular structures observed by TEM represent a mechanism to efficiently recycle cell constituents by autophagy. The intense proliferating and metabolic activity, in fact, makes it necessary to constantly renew sub-cellular components [6]. It could be interesting to investigate the nature of this cytoplasmic vacuolization and understand their function also in the equine species.

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5. Conclusions

This study described for the first time the detailed cellular morphology of equine amniotic fluid during normal pregnancy, using light and transmission electron microscopy. Different cell types have been described and a new and unexpected function of equine AM, which could be a second potential source of pulmonary surfactant.

Further studies on a larger number of samples, especially during high-risk pregnancy and at different gestational ages, would allow to expand the knowledge of the cellular population and its variation during pregnancy. This could be done if the technique of ultrasound-guided transabdominal amniocentesis could be used also in non-experimental mares. Subsequently, the possible diagnostic use of cellular evaluation of amniotic fluid in pregnant mares with high-risk pregnancy may also be investigated.

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