

Embryonic and Fetal Development of the Cardiorespiratory Apparatus in Horses (*Equus Caballus*) from 20 to 115 Days of Gestation

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Abstract

Horses have always sparked social and economic interest, awakening in the scientific community an interest in embryology and reproductive biology. To date, little is known about horse embryology because this species has several morphological and physiological characteristics that are peculiar to its own reproductive process. This study describes morphological aspects of developing cardiorespiratory apparatus in equine embryos and fetuses during 20-115 days of gestation. At 21 days, the equine embryo had a forming heart with two chambers and a distinct atrium and ventricle. There was a clear cellular protuberance in the lumen of both chambers and in the region of the endocardium. During this phase, the embryo had a nose rostral to the eyes and dorsal to the mouth, but no species-specific characteristics. Only at 40 days did it show external characteristics that were specific to the species, such as a nasal diverticulum. The larynx and pharynx were observed at 26 days of gestation. Additionally, at 26 days, the formation of the epiglottal protrusions was noted, and as embryonic development advanced, cartilage formed, while over the larynx, a non-uniform epithelial lining was observed. At day 30 of gestation, the thyroid and cricoid cartilage were seen, and the formation of the epiglottis was clear. At 45 days, the larynx was completely formed, and the thyroid, cricoid, and cricoarytenoid muscle were identified. Lung tissue was observed in embryos from 24 to 49 days, and then a pseudoglandular transitional phase started between days 50 and 60. At this time, light bronchi and terminal bronchioles were observed and became wider as the lung tissue became more vascularized. Between days 90 and 105, the primary and secondary bronchi were tubular structures composed of columnar epithelium layers. Blood capillaries were observed in the lung. Structures similar to alveoli were not seen. This work contributes to advance knowledge regarding the organogenesis of the cardiorespiratory apparatus in horses.

Keywords: Cardiorespiratory apparatus; Embryo; Equine; Fetus; Horse

Introduction

Embryology in the horse (*Equus caballus*) is still not well understood because this species has several morphological and physiological characteristics that are peculiar to its reproduction. Determining the developmental stages of an equine embryo is an important issue because it is through these stages that the chronological events inherent to this species are established. Sharp [1] reported that processes that cause abortion, as well as growth and developmental problems, continue to be misinterpreted.

In horses, early development occurs in the oviduct, where the fertilized egg undergoes symmetrical cleavage to form a compact morula [2-4]. At approximately 5.5 days (ovulation day = 0), the conceptus enters the uterus and moves freely within the uterine lumen, where it connects to the posterior segment of one of the uterine horns around day 16 [5,6]. Starting at this time and continuing for 60 to 70 days, the conceptus undergoes a series of morphological alterations that leads to its embryogenesis and organogenesis [7,8] with the formation of a diffuse and epitheliochorial placenta [9].

Although humans have used equine species widely and for a long time for different purposes, such as for the production of vaccines, a

protein source, locomotion, and hobbies, data in the literature on their embryonic and fetal stages are rather scarce [10-13]. Thus, the present study aimed to describe the development of the main structures that compose the cardiorespiratory apparatuses in horse embryos and fetuses at different gestational ages (e.g., period of 20-115 days). The findings of this study should help clarify the stages related to embryonic and fetal development in equine species.

Materials and Methods

In this study, 10 equine embryos and 10 equine fetuses between 20 and 115 days of pregnancy were used. Embryos and fetuses were obtained at a horse slaughterhouse in Brazil and brought to the Department of Anatomy of the School of Veterinary Medicine and Animal Science of the University of São Paulo, FMVZ-USP. The present study was approved by the Animal Ethics Committee (Number 1475/2008).

Biometric data

The “Crow-rump” of the embryos and fetuses was measured with the aid of a stainless steel calliper to estimate the gestational age, by a method proposed by Evans and Sack [14]. Measurements of the head used as reference the nuchal ridge in one extremity and the last sacral vertebra at the opposite extremity. Weight was determined using a

digital scale (accurate to 0.001 g; model AD1000 MARTE, São Paulo, Brazil).

Macroscopic description

Morphological features were analyzed using the embryos' and fetuses' external characteristics during the different gestational stages. For this procedure, a stereomicroscope (Zeiss Stemi SV6, Germany) was used to characterize the body regions of the embryos and fetuses. All data were photodocumented using a Sony MVC-CD5000. The anatomical nomenclature used followed the International Committee on Veterinary Gross Anatomical Nomenclature [15].

Processing for light microscopy

After the anatomical description, fragments of the heart, lung, trachea, larynx, and vessels were collected for histological analyses. The collected fragments from these regions during the different stages were then fixed in 10% formaldehyde for at least 48 hours. After fixation, the samples were washed in phosphate-buffered saline solution to remove the fixative, dehydrated in an increasing series of ethanol (70% to 100%) for 1 hour in each concentration, diaphonized in xylene for 2 hours, and embedded in paraffin [16]. The blocks were then subjected to microtomy in an automatic microtome (Leica, RM2165) to obtain 5- μ m sections. The sections were affixed to histological slides and placed in an oven at 60°C. After being deparaffinized, the sections were stained with hematoxylin and eosin [16]. The material was then analyzed and its morphological characteristics photo-documented.

Processing for scanning electron microscopy

For scanning electron microscopy, the samples were fixed in 2.5% glutaraldehyde solution and washed in 0.1 M phosphate-buffered saline at pH 7.4 and postfixed in 1% osmium tetroxide and then dried in a critical point dryer device (Balzers CPD-020). Next, the material was placed in a metal support for gold plating ("sputtering" Emitech K550). The results were observed using an electron microscope (ME Leo 435 VP).

Results

Circulatory system

The heart was the first embryonic organ that differed functionally, shortly after the closure of the cephalic neural tube during the formation of somites. The cardiac tube laid ventromedially (Figure 1A) and seemed to be responsible for the rhythmic contractions. The pericardium developed simultaneously with the heart and was composed of two laminae. The internal lamina was attached to the surface of the heart visceral pericardium or epicardium; the external lamina, called parietal pericardium, was continuous with the visceral lamina at the base of the heart and was reinforced by a fibrous superficial lamina or fibrous pericardium (Figure 1B-F).

The equine embryo at 21 days had a forming heart with two distinct chambers, an atrium and a ventricle (Figure 1A-C). A cellular bulge in the lumen of the chambers and in the endocardium region was

observed. At day 28, the embryos had a partially compartmentalized heart with several mature structures; the dorsal aorta still consisted of paired vessels, separating the atria (Figure 1B and 1C).

In equine embryos at 30 to 45 days, the heart assumed a mature position in the chest cavity along with the pericardium. The heart also acquired its typical format of two atria and two ventricles with a prominent interventricular groove, endocardial cushions, and tissues for cellular transport. After this period, the equine embryo underwent significant changes, such as the appearance of a rudimentary aorta and pulmonary valve maturation (Figure 1E and 1F).

At 45 days, the fetuses exhibited more characteristics of future horses, with a well-developed face, eyes with eyelids, and visible pelvic and thoracic limbs. The heart at this stage had four clearly defined chambers. The cusps of the forming atrioventricular valves and cardiomyoblasts were observed.

At 105 days, the heart showed right and left atrioventricular valves. In the right atrium, the pectineus muscle was evident in the internal surface. From the cranial view, the aorta showed a bronchial cephalic trunk as well as the bifurcation of the pulmonary artery until the end of gestation (Figure 1G and 1H).

Regarding vessels, in 40-day-old equine fetuses the arteries presented three layers: external tunic, adventitia, and intima; these structures were identical in older fetuses and maintained their vascular pattern. The veins in equine fetuses had a layer of smooth tissue featuring irregular lightness, indicating the vessels were fragile. The vein walls of 40-day-old fetuses were thinner than the arteries, and the composing layers could not be clearly identified.

Respiratory system

The respiratory system consisted of structures that facilitate the passage of air into the lungs, such as nostrils, nasal cavity, pharynx, larynx, trachea, and bronchi.

The nose covered the parts of the face rostral to the eyes and dorsal to the mouth and was observed in embryos at 20 days, but at this time, the facial characteristics of the equine were not apparent. At 40 days, the fetus started to show facial characteristics of an equine. Its nostrils were closed, and the diverticulum was very rudimentary (Figure 2A). At 60 days, the nostrils had acquired the unique form for this species, including alar support cartilage in the superior part of the nasal opening called the blind diverticulum (Figure 2B). However, at 100 days, this structure was completely formed in both sides of the nose (Figure 2E and 2F).

The nasal cavity is important in the equine species because of the region occupied by the molar teeth and the extensive development of the paranasal sinuses; in embryos at 20 days, these structures were not completely formed. In 40-day-old fetuses, these structures were forming, the dorsal nasal concha was starting to develop, and the ventral nasal concha had formed, as had the alar and basal folds (Figure 2C). At 80 days, the dorsal nasal concha had completely formed, as had the middle and dorsal nasal meatus (Figure 2C-E). In equine fetuses at 100 days, the dorsal and ventral nasal conchas and the meatus were completely formed (Figure 2F).

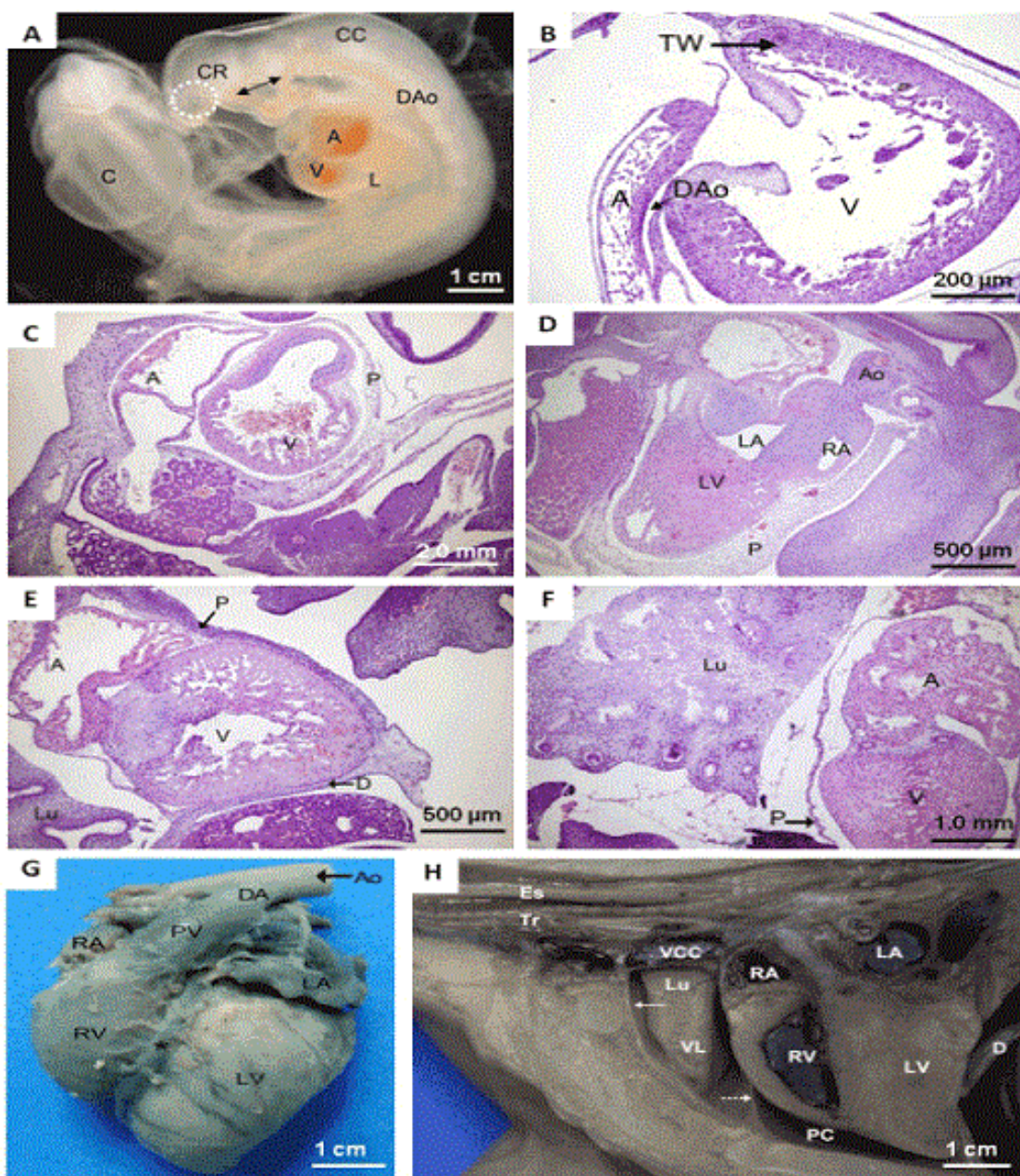


Figure 1: (A) A 21-day embryo. Observe in the cranial region (CR) the beginning of the formation of the optical placode (circle). Note the cervical curvature (CC) and the caudal region (C). At this stage, the heart chambers are forming; the ventricle (V) and atrium (A) are still undergoing torsions. Note also the syntopy with the liver (L). Observe the extent of the dorsal aorta (DAo) and its branches (arrow) in the region of the brachial arches. (B-C) Histology of the heart after 25 days of embryo development. Note the atrium (A), ventricle (V), dorsal aorta (DAo), and trabecular wall of the ventricular chamber (TW). (D-F) Observe the chronology of the development of the embryo's heart at 30 days, 33 days, and 38 days, respectively. Note the right atrium (RA) and left atrium (LA) and the ventricles (V). Throughout development, the muscular wall of the left ventricle (LV) greatly increases in thickness. Observe the pericardium (P), lung (Lu), aorta (Ao), and diaphragm (D). (G) Auricular face of the fetal horse heart at 105 days of gestation. Observe the right ventricle (RV), left ventricle (LV), left atrium (LA), right atrium (RA), pulmonary vein (PV), ductus arteriosus (DA), aorta (Ao), and paraconal interventricular branch of the left coronary artery (*). (H) Lateral view of the thoracic cavity of a horse fetus at 110 days of gestation. Note the fibrous pericardium with the pericardial blade of the serous pericardium (dashed arrow), pericardial cavity (PC), parietal pleura (continuous arrow), the open right atrium (RA) and right ventricle (RV), the left atrium (LA), left ventricle (LV), diaphragm (D), vena cava cranial (VCC), ventral lobe (VL) of the right lung (Lu), esophagus (Es), and trachea (Tr).

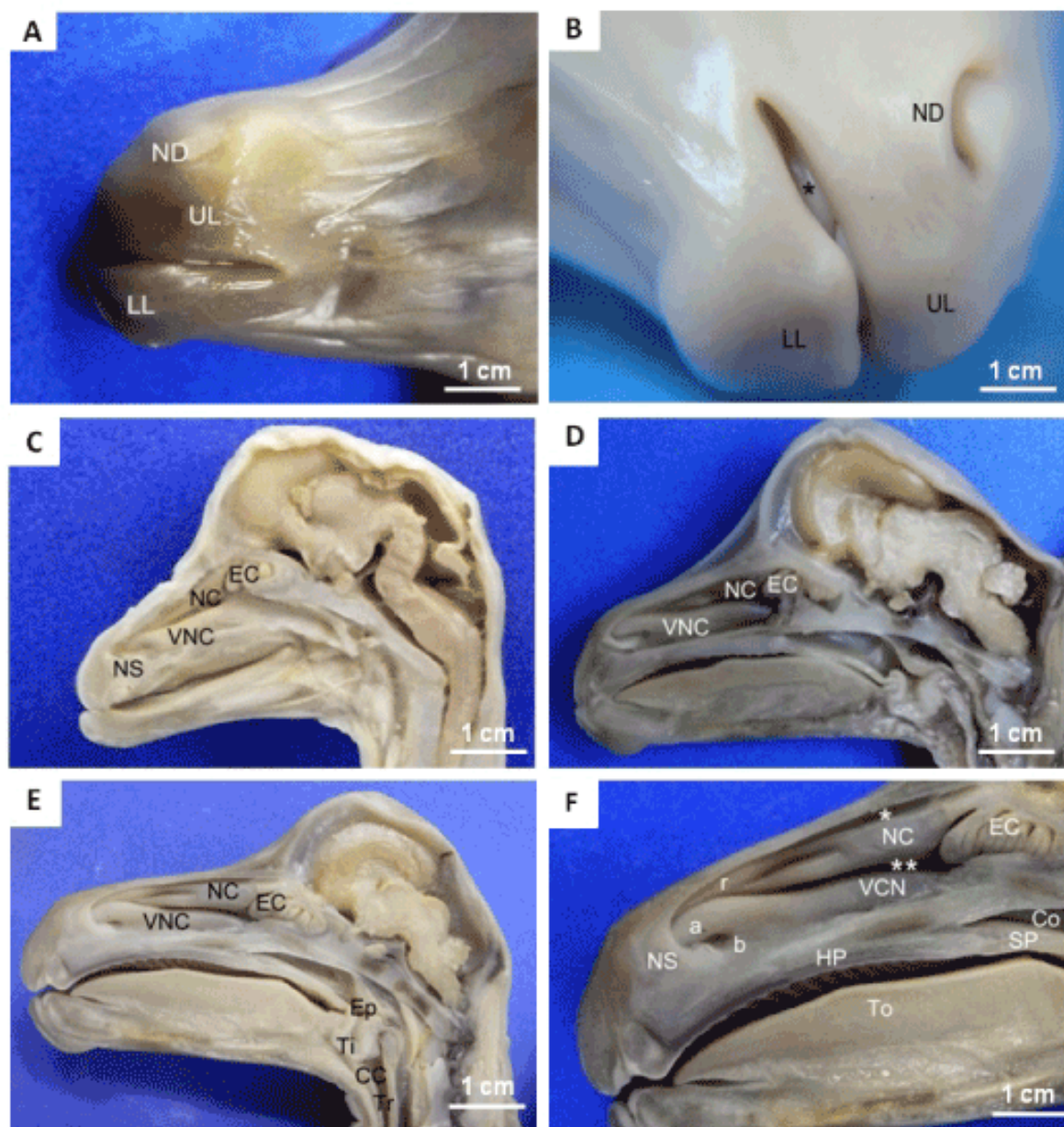


Figure 2: (A) Equine fetus at 40 days. Observe nostrils with the nasal diverticulum (ND) closed, upper lip (UL), and lower lip (LL). (B) Rostral region of the head of the equine fetus at 110 days. Note the open external nose with the nasal diverticulum (ND), upper lip (UL), lower lip (LL), and oral cavity (*). (C-F) Development of the nasal cavity between 40 and 110 days of gestation. (C) At 40 days the nasal septum (NS), nasal concha (NC), ventral nasal concha (VNC), and ethmoid concha (EC) are observed. (D-E) Observe at 80 days (D) and 110 days (E) the nasal concha (NC), ventral nasal concha (VNC), ethmoid concha (EC), cricoid cartilage (CC), thyroid (Ti), trachea (Tr), and epiglottis (Ep). (F) Detail of the dorsal region of the nasal concha (NC), ventral nasal concha (VNC), ethmoidal concha (EC), nasal septum (NS), hard palate (HP), soft palate (SP), choana (Co), and tongue (To). The dorsal nasal concha has a right fold line (r) and a dorsal nasal meatus (*). The ventral nasal concha has alar (a) and basal (b) folds, showing the middle nasal meatus (**).

In horses, the vomeronasal organs did not establish communication with the mouth, but with the nasal cavity (Figure 3A, 3D and 3E). The vomeronasal organ was well developed in 60 day-old fetuses (Figure 3D and 3E). This structure was located near the posterior extremity at the second molar level. A cartilage sheath could be seen forming a

groove at the junction of the vomer with the symphysis of the palatal processes of the superior maxillary closely adhered to the bone tissue, especially in the prior region where the grooves deepened (Figure 3A and 3D).

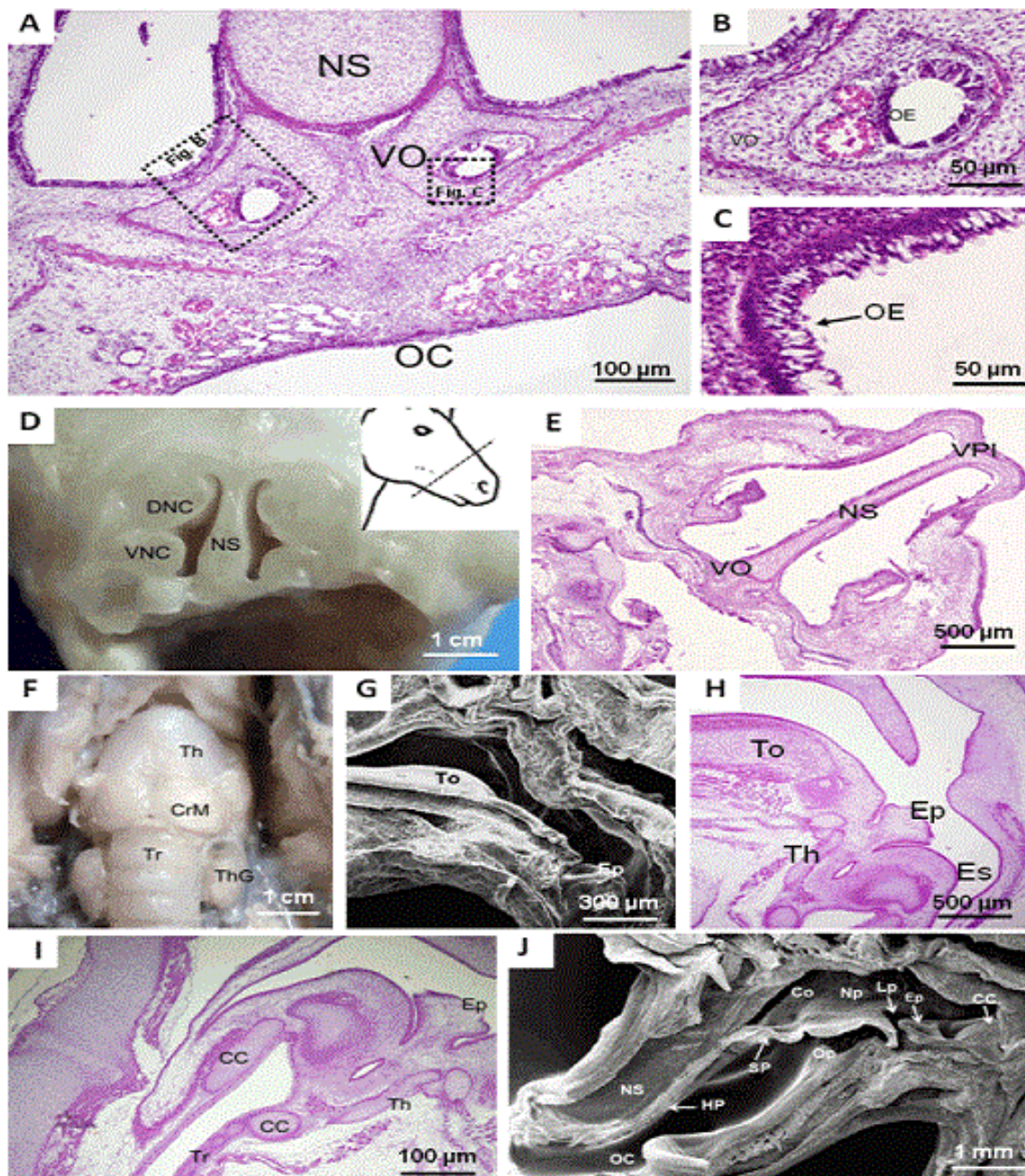


Figure 3: (A-J) Region of the vomeronasal organ (VO) of equine fetuses at 60 days. (A) Observe the nasal septum (NS) consisting of hyaline cartilage and the region of the oral cavity (OC). (A-C) Olfactory epithelium (OE) is found in the region of the nose and the vomeronasal organ. In the region of the triangle, there is a high olfactory epithelium pseudostratified structure, with specialized cells. (D-E) Transversal sinus cut of an equine embryo at 64 days. Note the venous plexus (VPI) of the nasal concha, dorsal nasal concha (DNC), ventral nasal concha (VNC), nasal septum (NS) consisting of cartilage, and vomeronasal organ (VO). (F-J) Development of the larynx during days 26-45 of gestation. (F) Ventral view of the cervical region of the equine fetus at 115 days of gestation. Observe the structures of the larynx: cricoarytenoid muscle (CrM), thyroid (Th), trachea (Tr), and thyroid gland (ThG). (G) Scanning electron microscopy of the oral cavity showing the protrusion of the cartilage of the epiglottis (Ep) and the larynx in relation to the tongue (To). (H-I) Histology of the pharyngeal region in embryos at 30 days of gestation, in which the cricoid cartilage (CC), thyroid (Th), trachea (Tr), epiglottis (Ep), esophagus (Es), and tongue (To) are seen. (J) Scanning electron microscopy of the oral cavity (OC) in fetuses at 45 days of gestation: hard palate (HP), soft palate (SP), choana (Co), nasopharynx (Np), oropharynx (Op); lingopharynx (Lp), nasal septum (NS), epiglottis (Ep), and cricoid cartilage (CC).

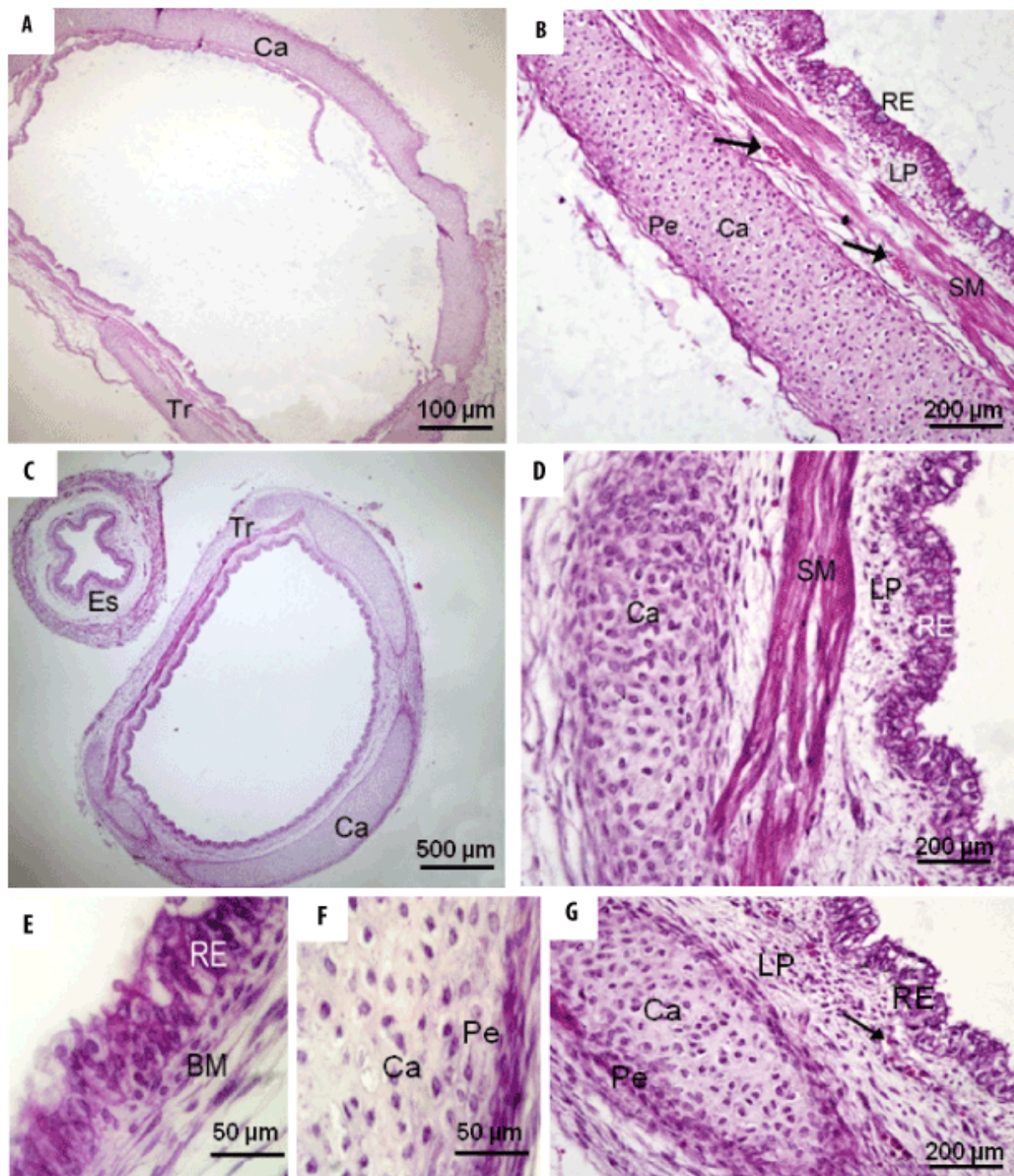


Figure 4: (A-B) Histological characteristics of the trachea (Tr) of the equine embryo at 24 days. (A) Note the absence of invagination of the cartilaginous tissue which will form the tracheal rings. (B) Observe the respiratory epithelium (RE), lamina propria (LP), smooth muscle (SM), vascularized connective tissue (arrows) containing glands and still-forming seromucous cells, and the dense tissue of the perichondrium (Pe) enveloping the hyaline cartilage (Ca). (C-G) Panoramic view of the trachea (Tr) of the equine embryo at 73 days and its syntopy with the esophagus (Es). Note the respiratory epithelium (RE), lamina propria (LP), vascularized connective tissue (arrow), dense connective tissue of the perichondrium (Pe) involving the hyaline cartilage (Ca), smooth muscle (SM), and basal membrane (BM) under the respiratory epithelium (RE).

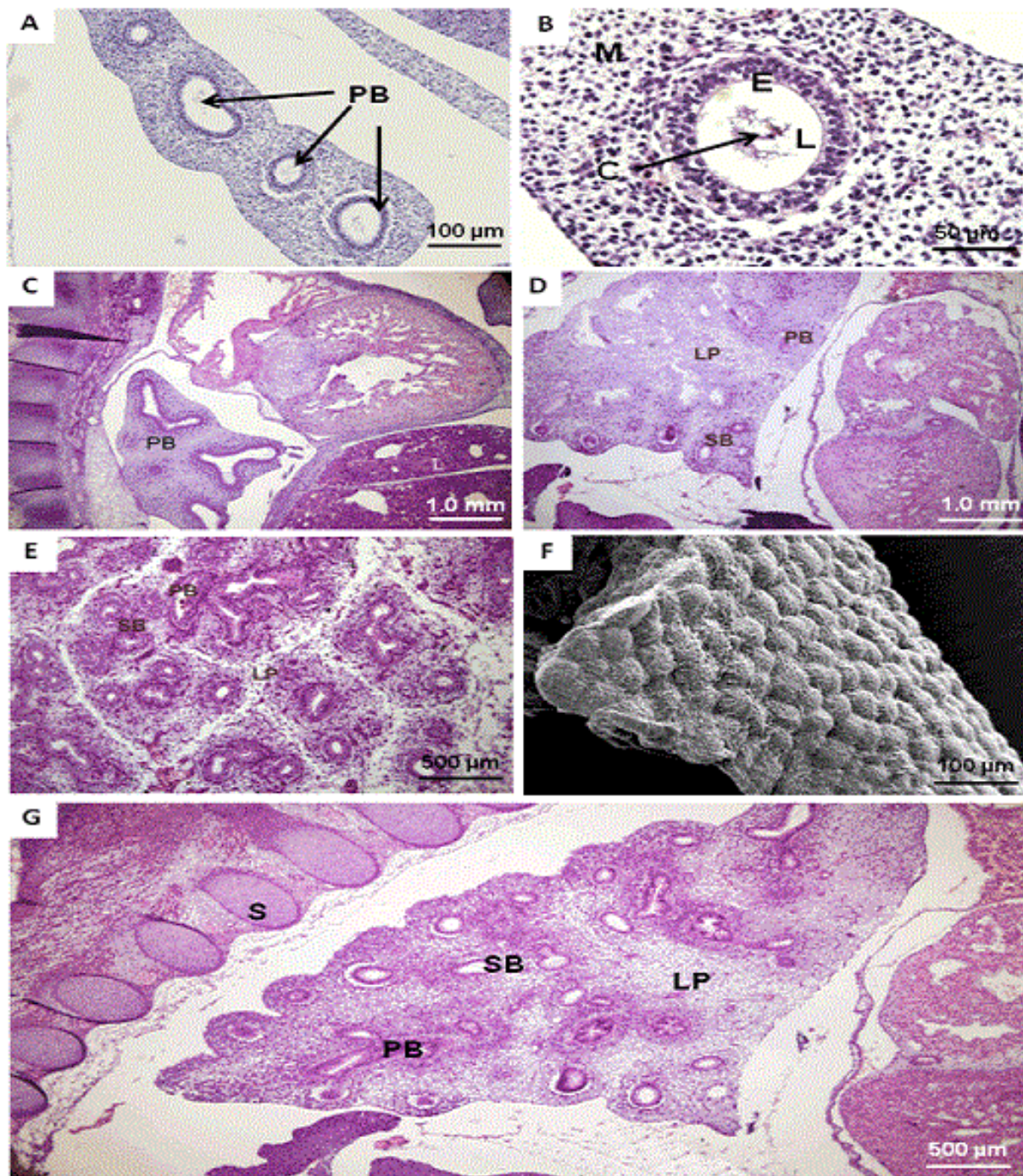


Figure 5: Development of the lung in equine embryos and fetuses. (A-B) Note the histological features of the lung bud in embryos with 21 days of gestation. Note the beginning of the formation of the primary bronchi (PB) within the parenchyma formed by mesenchyme (M). The bronchi have stratified epithelium (E) limiting their lumen (L). Blood cells (C) are observed both inside the bronchi and in the mesenchyme. (C-F) Lung development during days 24-49 of development. (D-F) During this period, the lung undergoes divisions of the branches of the secondary bronchi (SB), with lobes forming on its surface that are observed by both histology and scanning electron microscopy. (G) Note in the lung of 40-day-old embryos the presence of the somites (S), the primitive lung (Lu), and its syntopy with the liver (L), primary bronchi (PB), secondary bronchi (SB), and lung parenchyma (LP).

Larynx and pharynx

The outer layer of the larynx was of endodermal origin, while the cartilage and muscles were of mesenchymal origin (from the brachial arches). The larynx is a tubular organ, located medially, and was in close communication with the pharynx and trachea (Figure 3J). The larynx wall was composed of cartilage that was linked together by ligaments and muscles, and it was rostral to the hyoid bone (Figure 4C). Its walls were composed of irregular cartilage pieces joined together by fibroelastic tissue. The thyroid and cricoid cartilage was formed by hyaline cartilage (Figure 4A and 4B); the other cartilages, such as the epiglottis and arytenoid, were of the elastic type.

In embryos at 26 days of gestation, the formation of protrusions in the epiglottis was noted. As embryonic development advanced, the cartilage of the epiglottis formed, and along the larynx a non-uniform epithelial coating was observed (Figure 3G-I).

At 30 days the thyroid and cricoid cartilage were seen in the embryos and the formation of the epiglottis was clear (Figure 3B and 3C). In equine fetuses at 45 days of gestation onward, the larynx was fully formed, and the thyroid, cricoid, and cricoarytenoid muscles were identifiable (Figure 4A).

The development of the pharynx was anatomically visible in fetuses from 40 days onward, and their histological formation was visualized in embryos at 26 days (Figure 3J). At 26 days, the pharynx was coated by stratified squamous epithelium. In fetuses at 40, 80, and 110 days of gestation, the pharynx developed along the larynx, and the regions of the nasopharynx, oropharynx, and laryngopharynx were observed (Figure 3J).

Trachea

The trachea is a tubular organ consisting of incomplete cartilaginous tracheal rings closed by the tracheal muscle, located dorsally, with an intimate relationship with the esophagus (Figure 4A). The rings that compose the trachea have the primary function of preventing tracheal airway obstruction caused by bending of the neck. The trachea began at the larynx and ran to the thoracic cavity, giving rise to two extrapulmonary bronchi called main bronchi (Figure 4A and 4G). The trachea started to form from the invagination of the pharyngeal intestine at 21 days, where the hyaline cartilage of the trachea was observed. In 75-day fetuses the trachea had a mucosal epithelium composed of prismatic pseudostratified epithelium. The muscle layer was reduced to smooth muscle or tracheal muscle in the dorsal position and in the transversal orientation, extending between the open ends of the cartilage (Figure 4B and 4C).

Lungs

Horses have two lungs, with the right lung having more lobes than the left because the heart leans slightly to the left, taking up some lung space. In the present study, lung tissue traces were found in embryos over 24 days of age (Figure 5A and 5C). The lungs originated from an invagination of the ventral wall of the primitive intestine in its cranial part; this occurred after the fourth week of gestation. Lung tissue was observed in embryos from 24 to 49 days, and then a pseudoglandular transitional phase started between days 50 and 60, characterizing the canalicular phase, which ended with the terminal phase (Figure 5F and 5G). At this time, light bronchi and terminal bronchioles were observed and became wider as the lung tissue became more vascularized (Figure 5B). Between days 90 and 105, the primary and

secondary bronchi were tubular structures composed of columnar epithelium layers (Figure 5E). Blood capillaries were observed in the lung. Structures similar to alveoli were not seen.

Discussion

Several continuous modifications correlate with the process of embryogenesis, which is similar in many species [17,18]. Although they include several similarities, there is a great variety of systems for the classification of embryos. These include the Nomina Embryologica Veterinaria [19] system, which establishes 15 stages of development; the Carnegie system [19], which names 23 stages; the Theiler system [20], which considers 27 stages for mice; and the Dyban et al. [21] system, which establishes 27 stages for rodents and laboratory rabbits. In general, the description of embryonic and fetal development is based on stages established according to age, morphological changes that occur by gradual differentiation, increases in body size and weight, and the total development of systems and organs [17,18,20].

Embryos have a letter C shape [22] and develop in the cranio-caudal direction. Marrable and Flood [23], studying embryology in horses, found that at 34 days of gestation the conceptus has the shape of a flattened ellipse.

The cardiovascular system of the equine embryo arises from the need for the embryo to meet its nutritional needs by diffusion only. According to Junqueira and Zago [24] and Sadler [22], the heart is the first embryonic organ to differentiate in many species, and the neural cardiac tube is located ventromedially. This structure is responsible for the rhythmic contractions that are observed in equine embryos. The distinct cardiac prominence observed in this study agrees with the findings of Winters et al. [25] in cattle. During this phase, the caudal and cranial somites are visible and number 18 to 19 in the bovine species. In 20-day-old horse embryos, the external characteristics include a translucent tegument with small blood vessels visible [23]. During this phase in horses, thoracic and pelvic buds and cardiac morphology can be observed [3,26].

At 21 days, the heart shows two distinct chambers (an atrium and a ventricle), and there is also a cellular protuberance in the lumen of the two chambers and in the endocardial region [12,13,27-30]. At 28 days, the heart is partially compartmentalized with several structures in various stages of maturation, but the dorsal aorta still consists of paired veins, separating the atria.

Winters et al. [25] sectioned bovine embryos at 22 days and visualized the bulb, ventricle, dorsal aorta, and first loop of the aortic arch; they observed the cardiac atrium and ventricle chambers, which differed by the thickness of their walls (the ventricle is thicker), in addition to the other characteristics mentioned above. They also observed the histological layers of the heart, such as epicardium, myocardium, and endocardium. In 26-day-old embryos, a dorsal artery is observed along the body of the conceptus [22,31,32]. Our findings corroborate all of these findings, as the heart of equine embryos/fetuses showed an atrial/ventricular division, constituent layers, and a dorsal aorta.

During the period of 30 to 45 days, the heart assumed a mature position in the thoracic cavity and acquired the typical format of two atria and two ventricles, with a prominent interventricular groove and the maturation of a rudimentary aorta and pulmonary valves, corroborating the previous findings [13,28]. At this time, we observed that equine fetuses began to assume equine characteristics. Eyes with

eyelids and pelvic and thoracic limbs were visible, and the heart had four clearly defined chambers as well as the cusps of the atrioventricular valves and cardiomyoblasts, observations not previously described by Francioli et al. [13].

From 75 to 115 days, the heart had right and left atrioventricular valves formed. On the inner surface of the right atrium the pectineus muscle is evident. In the cranial view, the aorta shows a bronchiocephalic trunk, as well as a bifurcation of the pulmonary artery, until the end of gestation [13,28].

The bronchi are made of tubes with epithelial walls lined by condensed mesenchyme and are usually surrounded by blood vessels. In this study, bronchial buds started to appear in equine embryos at or shortly after 25 days of gestation, and were considered pseudoglandular. Moore and Persaud [32] reported that in humans, the development of segmented buds starts at 41 days of gestation and the subsegmental buds at 44 days. In the equine embryos we studied, the lung lobes were already in the process of division between days 20 to 30, leading us to believe that these respective gestational periods in humans and horses are alike. The expansion of the lung buds in the caudal and longitudinal direction corresponds to that described for humans [22].

In our embryos between 36 and 38 days, the presence of lung tissue was observed. At this time, the lung was in a transition phase between the pseudoglandular and canalicular periods, where the transparency of the bronchi and the terminal bronchioles becomes greater and the lung tissue becomes more vascularized [30]. The primary and secondary bronchi had tubular formations coated with simple cylindrical epithelium. Formation of blood capillaries was observed. At this age, formations equivalent to primitive alveoli were not found, which agrees with previous findings [13,33,34].

According to Alberto [34] in cattle and Francioli et al. [35] in pacas, the first lung buds consist of an epithelial tube with several epithelial layers, coated with initially condensed mesenchyme, and in the regions more remote from the tube, the mesenchyme becomes more loose. Our results corroborated these findings. We also observed the presence of blood cells, which, according to Alberto [34] in cattle and Morini [36] in buffalo, is consistent with the advanced vascularization still occurring in the pseudoglandular stage.

This work contributes to advance knowledge regarding the organogenesis of the cardiorespiratory apparatus in horses and provides information for comparative aspects with other species.

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