Prenatal Development of the Digestive System in the Horse

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Abstract

Since the horse has a highly precocial reproductive strategy, most organs are functionally well developed at birth and thus, embryonic and fetal life is interesting. Data on the development of important organs are very limited. Here, we detailed macroscopically and histologically the equine digestive system, focusing on the first third of gestation. At 21 days, the oral cavity was an empty space, and the liver contained proliferating endodermal cells. At 25 days, a fusiform stomach and the pancreatic bud were present. At 28 days, a small tongue and the esophagus occurred. At 30 days, primary and secondary palates were developed, the liver contained cords of hepatocytes, and the pancreas was triangular. At 40 days, crypts had formed in the intestinal loops, cell differentiation was observed in the hepatic parenchyma, and the pancreas was elongated. Pancreatic acini and islets were observed in fetuses of 50 days and intestines were highly convoluted. Three segments of the pharynx were distinguishable at 75 days. At 105 days, the intestinal villi were wide with round tips; especially, the liver, stomach, and oral cavity showed key steps of anatomical and cellular differentiation in early fetuses, whereas other areas, such as pancreas or pharynx were still immature in the investigated phase. Pluripotency analysis using Oct4 showed initial intense staining in all of the digestive system tissues and a later decreased becoming restricted to specific cell layers. In conclusion, our data may contribute to perform a chronological reference of developmental events for approaches predicting pregnancy disorders in horses. Anat Rec, 297:1218–1227, 2014. © 2014 Wiley Periodicals, Inc.

Key words: development; embryology; pregnancy alteration; equine; mare

The early embryonic and fetal development of the horse (Equus caballus) is a matter of interest to better understand the chronological events that establish specialization of this species, especially regarding the development of reproductive characteristics. Furthermore, processes that cause early pregnancy losses as well as growth and developmental problems remain poorly understood (Sharp, 2000). Important progress has been made especially on early implantation and placentation...
The digestive system is one of the organs that are under constant exposure to pathogens from the outer world. Therefore a strong barrier needs to be developed during the time where the foal is still protected by maternal immunity and will play a crucial role in the horses' immune system in later stages of its life. With ingestion of milk, the digestive tract increases in length and diameter and with that increases in density and height of villi and differentiation of enterocytes. This development increases the efficiency of nutritional uptake (Ousey et al., 1995) and builds the base of microbial colonization in the intestines. Murray et al. (1994) suggest that the posterior esophageal glycoproteins could be involved in the pregastric digestion. Also, the epithelial surfaces and their secreted glycoproteins play a fundamental role in mediating the relationship between environment and organism (Domeneghini et al., 2005).

The development of the intestine coincides with the development of the microbial ecology and microbial digestion. The succession of microbial colonization is most marked in the early stages of life. From suckling to weaning, the dramatic shifts in host-produced enzymatic digestion to anaerobic fermentation are directly associated with the changes in the type and quantity of feedstuffs ingested by the growing foal. Dynamic balances exist between the gastrointestinal microbiota, host physiology, and diet that directly influence the initial acquisition, developmental succession, and eventual stability of the gut ecosystem (Mackie et al., 1999). When occur morphological changes can also occur structural changes, including development of villi, crypts, blood vessels, and nerves (Trahair and Songild, 1997).

The histological analysis of the digestive system, particularly of the liver and the digestive tract, represents a sensible index of nutritional condition and can be employed to estimate the inanition incidence in fetal mortality along the ontogenetic development. Several aspects of equine organogenesis, such as the digestive system have not been well investigated and only limited information is available on the occurrence of internal features during ontogeny or limited data are available on the intrauterine development of organ systems (Allen and Wilsher, 2009; Franciolli et al., 2011a; Ginther, 1993; McGeady et al., 2006). However, the basic knowledge of the digestive system development of the equine is insufficient. The purpose of this paper was to describe the macroscopic and histological development of the digestive system in equine embryos and fetuses to indicate the questions about organogenesis that remaining unsolved to contribute to a better understanding and for further investigation of equine developmental biology, organogenesis, cell differentiation and nutrition.

Fig. 1. Histogram plot to show the distribution of the samples per time point correlating the embryos and fetuses ages with crown-rump.
MATERIALS AND METHODS
Embryos and Fetuses
A total of 10 embryos and 10 fetuses from gestational ages between 21 and 105 days were provided from embryo and fetuses collection of the Department of Domestic and Wild Animal Anatomy of the School of Veterinary Medicine and Animal Science at the University of Sao Paulo, Brazil. All mares were adults, but details regarding their age and breed were unknown. Conceptions (embryos or fetuses and their membranes) were carefully accessed by dorsal incision of the uteri, starting from the cervix, to avoid rupturing the membranes. Conceptions were placed on a glass plate for analysis of physical characteristics, and then dissected.

The age determination of the embryos and fetuses was based on the crown-rump length using a method developed by Evans and Sack (1973) and recently used in a horse embryogenesis study (Franciolli et al., 2011a). A histogram plot to show the distribution of the samples per time point was created (Fig. 1). The developmental process was based on the International Committee on Veterinary Embryology and the International Committee on Veterinary Gross Anatomical Nomenclature (1994) and the International Committee on Veterinary Embryological Nomenclature (1994). The study was approved by the Animal Ethical Committee protocol (N° 1475/2008).

Individual embryos and fetuses were previously fixed in a 10% formaldehyde solution and measured using a stainless steel caliper to determine the crown-rump length. Measurements were performed by measuring the head from the nuchal crest to the last sacral vertebra. Weight (g) was determined using a digital scale (0.001 g—MARTE, Rio de Janeiro, Brazil). An incision was made along the median sagittal plane to better visualize the organs of the digestive system. A stereomicroscope (Zeiss Stemi SV6, Germany) was used for visualization. Photographic data were obtained using a Sony MVC—CD500 camera (Sony, Beijing, China).

For light microscopy, embryos and fetuses were fixed in 10% formaldehyde or 4% paraformaldehyde. Samples were washed in phosphate buffer or distilled water, followed by dehydration in a series of ethanol solutions at increasing concentrations (70–100%) for 1 h in each solution. Sections were paraffinized in xylene for 2 h and embedded in paraffin (Histosec—MERCK, Sao Paulo, Brazil). The paraffin blocks were sectioned at 5 μm on an automatic microtome (Leica, RM2165, Nussloch, Germany), mounted on histological slides, and incubated at 60°C. The sections were then deparaffinized and stained according to the Hematoxylin and Eosin (HE) technique (Tolosa et al., 2003).

For immunohistochemistry, embryological sections at 21–38 days of gestation were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Next, they were boiled in citrate buffer (0.384 g of citric acid monohydrate, 2.352 g of sodium citrate tribasic dihydrate and 1 L of distilled water, pH 6.0) for 5 min in a microwave oven for antigen retrieval; the process repeated three additional times, and the sections were cooled to room temperature. Endogenous peroxidase was blocked by incubation in a 3% hydrogen peroxide solution in 1 M Tris—HCl buffer, pH 7.5 (TBS, 60.57 g of Tris in 500 mL of ultrapure water) for 30 min followed by treatment with 3% hydrogen peroxide for 30 min. To amplify the signal, incubation with a streptavidin–horseradish peroxidase complex (Dako-advance, CA) had been done. Samples were developed using DAB (3,3’ diaminobenzidine chromogen solution) for up to 2 min, and the development was stopped by washing with distilled water. Finally, the slides were counterstained with hematoxylin, dehydrated, diaphanized, and mounted.

RESULTS
Oral Cavity and Pharynx
Between 21 and 25 days, the primitive oral cavity (stomodeum) was observed as an empty space without visible structures. Microscopically, there was a predominance of disorganized mesenchymal tissue (Fig. 2a). By day 25, the palate and larynx were still not developed; however a small tongue and an organized epithelium of cuboidal cells were present at the region of the future palate and cheeks, as well as pharyngeal arches located in ventral region above the cardiac prominence, in primordial region of neck (Fig. 2a). We observed positive immunolabelling in all regions of the pharyngeal arches showing that the pharyngeal arches have pluripotent cells (Fig. 2b).

The tongue, which arises from the branchial system, was observed from day 28 onward (Fig. 2c). First, the base of the tongue was developed in the future hyoid region. The floor of the oral cavity corresponded to the internal ectodermal surface of the mandibular arch and continued caudally through the internal endodermic layers of the second, third, and fourth brachial arches. A midline projection, the medial lingual tubercle (tuberculum linguale medium), was observed on the internal or dorsal surface of the future mandibular arch. By 30 days, lateral ridges of the tongue appeared, and by 38 days they continued cephalically and ventrally. Lingual tubercles were completely fused, and the anterior mobile portion of the tongue, originated from the first brachial arch, was visible. At 40 days, cheeks were still undifferentiated with cuboidal epithelium (Fig. 2d), but in the tongue a squamous epithelium and connective and muscular tissues occurred (Fig. 2e).

The cells covering the ectoderm and the mandibular arch mesoderm that formed the lingual mucosa were present from day 25 onward and continued to migrate and form the muscular cells in the lingual musculature of the tongue until day 38. The mesoderm of the brachial arches differentiated to form the areolar and adipose tissues within the tongue muscle. Salivary glands were not observed in the studied embryos. At 28 days, the developing cartilage that gave rise to the cartilage of the larynx was seen (Fig. 2g). Undifferentiated mesenchymal cells...
Fig. 2. Photomicrography of the developing components of oral cavity and esophagus in equine embryos and fetuses. A 25-day-old embryo. In (a), pharyngeal arches [Pa], primitive oral cavity [arrow], primitive heart ventricle [H], and primitive liver [Li]. In (b), positive immunolabeling in all regions of the first pharyngeal arch [Pa] for Oct-4. (c) A 28-day-old embryo. The oral cavity was already divided; the lips [L] and tongue [T] are shown. (d) A 35-day-old embryo. The cheeks had undifferentiated cells [arrows], but the epithelium was beginning to form and had cuboidal cells [Ep]. (e) A 40-day-old fetus showed the tongue layers: epithelium (Ep), connective tissue (Ct), and muscle tissue (M). (f) A 50-day-old fetus. Note developed tongue (T), cartilage of the larynx (L), esophagus (E), and a detail of the salivary glands (circle). (g) A 28-day-old embryo with the elongation of the esophagus [E], laryngeal cartilage [*], and trachea [T]. (h) A 50-day-old fetus. Esophagus appeared to have a normal pseudostratified epithelium [arrow], smooth muscle [sm], and skeletal muscle [sk] structures. Unless otherwise noted, all slides were stained with H&E.
were also identified in the pharynx region and the primary and secondary palates, termed palatal vaults. The pharynx was first identified morphologically at 50 days, appearing as a tubular structure in the shape of a funnel which was distinct from the spinal column and contained narrow connections with the nasal passages, oral cavity, larynx, esophagus, and the salivary glands just observed in fetus (Fig. 2f). The three sections of the pharynx could be distinguished at 75 days. The nasopharynx was the widest part of the organ, extending from the base of the cranium to the posterior part of the soft palate, and was connected to the nasal fossa by its anterior surface, establishing a direct connection to the oropharynx. The oropharynx was directly connected to the oral cavity by the anterior portion and also connected with the laryngopharynx, which represents the natural continuation of the oropharynx. The laryngopharynx was connected at the front to the larynx and below to the esophagus.

**Esophagus**

The esophagus developed from the anterior intestine caudal to the pharynx. At 28 days (Fig. 2g), it consisted of a short tube with 0.7 mm (ImageJ software—Collins, 2007). As development continued, the esophagus rapidly elongated due to the growth and descent of the heart and lungs; however, at 30 days, the esophagus remained in a tubular shape. Cellular organization was observed for creating the muscular layer of the esophagus and the pseudostratified esophageal epithelium. At 38 days, the epithelium and glands were derived from the endoderm, and the epithelium proliferated blocking the lumen either partially or completely; however, the esophagus normally recanalized at the end of embryonic development. Striated muscles in the external muscle layer of the upper third of the esophagus were observed at 50 days. They were derived from the mesenchyme of the caudal pharyngeal arches. Smooth muscles developed from the surrounding splanchnic mesenchyme were also present at 50 days (Fig. 2h).

**Stomach and Intestine**

The terminal portion of the anterior intestine was initially a simple tube. Posteriorly, a slight dilation marked the location of the stomach primordium. At 25 days, this primordium seemed like a fusiform enlargement of the caudal portion of the anterior intestine, and was oriented along the medial plane and characterized by the presence of a stratified pavimentous epithelium. The primordium expanded and grew dorsoventrally. At this age, the stomach began to rotate, and the intestinal loops were formed as small ducts of simple cuboidal epithelium. With the rotation of the stomach, the cranial region had moved to the left and slightly downward, while the caudal region was situated upward and to the right. The stomach assumed its final position with its major axis almost transverse to the body's major axis. The 30-day-old embryo's stomach had a distinct and differentiated formation of the epithelium, submucosa, musculature, and serosa. The formation of intestinal loops with a tubular shape was observed; these loops were microscopically differentiated with epithelium, submucosa, musculature, and serosa typical of this organ. A developing omental bursa was also observed (Fig. 3a). A normal monogastric stomach occurred at 35 days; however, glands were not observed. The intestinal loops showed no further differentiation (Fig. 3b). Crypts in the intestinal loops were present at 40 days. At 50 days, the stomach was suspended from the dorsal wall of the abdominal cavity along the medial plane by the dorsal mesentery, also known as dorsal mesogastrium. The ventral mesentery, or ventral mesogastrium, attaches the stomach and duodenum to the liver and ventral abdominal wall was observed as well as the omentum covering the stomach. In addition, the intestines were highly convoluted and contained glands, especially in the duodenum (Fig. 3c). At 65 days of gestation, villi of various lengths occurred in the intestinal segments (duodenum, jejunum, and ileum). The mesojejunum and omentum were well differentiated at 75 days (Fig. 3d). At 105 days, the cecum crypts were wide and uniformly shaped (Fig. 3e). Goblet cells were not observed at any of the ages studied. The parietal and visceral peritoneums were formed by 105 days (Fig. 3f). Glands and teeth formation was not observed during these studied stages.

Pluripotency analyses using Oct4 immunostaining revealed that the stomach and the intestinal loops were strongly stained in 21-day-old embryos, indicating high levels of activity in the germ line of primordial cells for generating the intestine's various cellular components (Fig. 3g). The staining was slightly decreased at 25 days, but the intense pluripotency activity of these cells continued. At 35 and 40 days, the Oct4 staining was decreased in the stomach and intestines, especially in the epithelial region, and was mainly restricted to specialized cell areas in the epithelial and muscular layers (Fig. 3h).

**Liver**

The liver originated from a ventral bud, the hepatic diverticulum that arose from the caudal or distal part of the anterior intestine. In 21-day-old embryos, the liver had a simple cuboidal capsular epithelium, proliferating endodermal cells, and hepatoblasts that later gave rise to the hepatocytes and the formation of a central vein lacking endothelial cells. At 25 days, the liver also had a disorganized hepatic parenchyma and hepatoblasts (Fig. 4a); the primordial sinusoids and blood cells were differentiated. Strong Oct4 staining was observed at 21 and 25 days, indicating strong cellular proliferation and differentiation of the hepatic cells. At 30 days, the liver parenchyma began to organize, and hepatocyte cords started to Anastomose near spaces covered by endothelium where hepatic sinusoids were located (Fig. 4b). Hepatoblasts, blood cells, and hepatocyte cords occurred at 35 days. The centrilobular vein was completely formed, lined by endothelial cells. Red blood cells and hepatocytes were both present (Fig. 4c). The fibrous and hematopoietic tissue and the Kupffer cells that were derived from the mesenchyme of the transverse septum were observed in 40-day-old embryos. Then, Oct4 staining was restricted to the hepatocytes (Fig. 4d).

**Pancreas**

The dorsal pancreatic bud, which was large and cranially located, gave rise to the majority of the pancreas...
Fig. 3. Photomicrography of the developing stomach and intestine in equine embryos and fetuses. In (a) A 30-day-old embryo. The stomach [S], primitive omental bursa [OB], and intestinal loops [I] were observed. (b) Typical layers [Ep, Sm, M] of the loops were seen at 35 days. (c) A 50-day-old fetus. Duodenal glands were present [circles]. (d) A 75-day-old fetus with differentiation of the mesojejunum [Mj] and the omentum [arrow]. (e) A 105-day-old fetus. The crypts [arrows] were present in the cecum. (f) A 105-day-old fetus with the parietal peritoneum [Pp] and the visceral peritoneum [Vp]. (g) A 21-day-old embryo showing strongly stained for Oct4 which revealed intense cell proliferation in all tissues [arrows]. (h) A 40 day-old fetus, the Oct4 staining was decreased restricted to specialized cell areas in the epithelial and muscular layers [arrows]. Unless otherwise noted, all slides were stained with H&E.
Fig. 4. Photomicrography of the developing liver and pancreas in equine embryos and fetuses. (a) A 25-day-old embryo. The simple cuboidal epithelium of the capsule [red arrow], proliferating endoderm [circle], central vein [V], and hepatoblasts [yellow arrows] were shown. (b) A 30-day-old embryo. Hepatoblasts [H], sinusoid [S], and blood cells [circle] were observed. (c) A 35-day-old embryo. Hepatoblasts [H], blood cells [circle], and hepatocyte cord [HC] were seen. (d) A 25-day-old embryo, indicating strong cellular proliferation and differentiation of the hepatic cells stained by Oct-4 [arrows]. (e) Stomach [S] and pancreatic bud [PB] in a 25-day-old embryo. (f) Pancreatic islets [PI] beginning to organize in a 30-day-old embryo. (g) A 30-day-old embryo showing strong Oct4 staining especially in regions of the developing pancreatic acini [arrows]. (h) Specialized acinar cells [arrows], and B cells [circle] in a 50-day-old fetus. Unless otherwise noted, all slides were stained with H&E.
and grew between the dorsal mesentery layers; it was first observed in embryos at 25 days (Fig. 4e). There was also strong Oct4 staining. At 30 days, the pancreas had a triangular shape and was located between the intestinal loops. The ventral pancreatic bud formed the uncinate process and parts of the pancreas head, growing between ventral mesentery layers (Fig. 4f). Oct4 staining was strong at 30 days; especially, in regions of the developing pancreatic acini (Fig. 4g). At 40 days, the pancreas was more elongated and contained cells that were morphologically similar to acini, but did not have pancreatic islets. The endoderm of the pancreatic buds developed into the pancreatic parenchyma to form the acini and pancreatic islets in 50-day-old fetuses (Fig. 4h).

A chronological listing of developmental events in the horse in relation to stage of gestation is shown in the Table 1 as well as the crown-rump and weight measurements to better represent the development of the different segments of digestive system.

## DISCUSSION

Embryogenesis in some species is correlated with a series of continual changes similar to those that occur during prenatal development in other species (Beaudoin et al., 2005; Knospe, 2002). To overcome shortcomings and to provide a better basis for comparative embryology, we described the development of the digestive system in the equine, focusing on the first third of gestation when essential developmental steps could be expected. The age determination of ontogenetic stages in this study followed the method established by Evans and Sack (1973), using crown-rump length to estimate the age of equine embryos and fetuses. Previously, Francioli et al. (2011a) applied this technique on the same material to describe the overall development of the main organ system. Other studies in horses have used other methods to determine embryonic age, including Acker et al. (2001); however, these authors used small horses (ponies). For other large and precocial mammals, Morini (2009) also reported the importance of crown-rump length in the buffalo for determining the age of embryos and fetuses, and Assis Neto et al. (2010) referred to morphological characteristics in cattle for age determination.

In horses, a division of the primitive intestine as a derivative of the endoderm (Brewer et al., 2004) into the components of the digestive system was recognized at 21 days of gestation, when the embryonic liver was present, consisting of hepatoblasts and disorganized parenchyma. In addition, the presence of an undifferentiated oral cavity at this period confirmed previous preliminary findings in mares (Ginther, 1993). Likewise, a division of the primitive intestine into compartments of the digestive system such as the esophagus, stomach, duodenum, liver, and pancreas during very early gestation has been described in the cattle (Lima, 2007), buffalo (Morini, 2009), human (Montgomery, 1999) and paca (Francioli et al., 2011b). Similar to the equine embryos studied here, the liver was the relatively largest organ in other species including the bovine (Lima, 2007) and human (Junqueira and Carneiro, 2008).

In 25-day-old equine embryos, a small tongue called medial lingual tubercle was present and the stomach was fusiform, large and in similar position and rotation than described for other species (Hoar and Monie, 1981; McGeady et al., 2006). The liver had primordial hepatic sinusoids, blood cells, and a large number of hepatoblasts, in accordance with previous reports in humans (O’Rahilly, 1978; Severn, 2005), which found that hepatic cords anastomose, near spaces covered with epithelium, and formed primordial hepatic sinusoids. Small, tubular intestinal loops covered by abundant mesenchyme were present beginning at day 25 in horses (Francioli et al., 2011a). In addition, the present study showed that the intestine consisted of a high amount of tubular sections with undifferentiated epithelium in 28-day-old embryos. Pancreatic buds occurred early and the pancreas differentiated much earlier than the seventh week of gestation as previously suggested (Hoar and Monie, 1981). The tubular structure and anterior position of the esophagus was in agreement to former studies in embryological development of the horse (Francioli et al., 2011a) and in cattle (Alberto et al., 2013).

### Table 1. Main events that occurred in each age during the horse development between 21 and 105 days of gestation

<table>
<thead>
<tr>
<th>CR (cm)</th>
<th>Weight (g)</th>
<th>Age (days)</th>
<th>Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6</td>
<td>0.5</td>
<td>21</td>
<td>Primitive oral cavity, disorganized hepatic parenchyma</td>
</tr>
<tr>
<td>1.9</td>
<td>0.8</td>
<td>25</td>
<td>Disorganized mesenchymal tissue in the oral cavity, primordium of the stomach. Intestine as a small ducts (tubes), pancreatic bud</td>
</tr>
<tr>
<td>2.3</td>
<td>1.5</td>
<td>28</td>
<td>Small tongue, esophagus as a short tube</td>
</tr>
<tr>
<td>2.5</td>
<td>1.8</td>
<td>30</td>
<td>Lateral ridges of the tongue appeared, esophagus with tubular shape, stomach showed differentiation of the layers, hepatic parenchyma start to organize, pancreas with triangular shape</td>
</tr>
<tr>
<td>3.0</td>
<td>2.5</td>
<td>35</td>
<td>Mono gastric stomach, but without glands, hepatocytes cells were differentiated</td>
</tr>
<tr>
<td>3.3</td>
<td>3.0</td>
<td>38</td>
<td>Muscular longitudinal fibers in esophagus</td>
</tr>
<tr>
<td>3.6</td>
<td>3.5</td>
<td>40</td>
<td>Connective and muscular tissue in the tongue. Intestinal crypts, Kupffer cells at liver. Pancreas more elongated</td>
</tr>
<tr>
<td>4.5</td>
<td>5.5</td>
<td>45</td>
<td>Final length of the esophagus</td>
</tr>
<tr>
<td>5.2</td>
<td>8.2</td>
<td>50</td>
<td>Appear the pharynx, striated muscle in the external muscular layer of the esophagus, acini and pancreatic islets</td>
</tr>
<tr>
<td>8.4</td>
<td>35.7</td>
<td>65</td>
<td>Villi of various lengths in the intestine, mesojejunum and omentum well differentiated</td>
</tr>
<tr>
<td>11.5</td>
<td>116.9</td>
<td>75</td>
<td>Three section of pharynx can be distinguished</td>
</tr>
<tr>
<td>18.9</td>
<td>391.4</td>
<td>105</td>
<td>Parietal and visceral peritoneum formed</td>
</tr>
</tbody>
</table>
development and maintain pluripotency in equine species such as mouse and pig. It was reported that Oct4 is a key transcription factor to control embryonic stem cell differentiation similar to described by Roballo et al. (2013) as fetuses with completely formed liver, similar to what was described by Alberto et al. (2013) for the cattle. An elongated pancreas with primordial acini, and small islets was observed. Shortly after, the esophagus had its final length, the pharynx was fully developed and the esophagus had striated and smooth muscles as previously reported for 50-day-old embryos (McGeady, 2006). In addition, this study demonstrated that at 65 days, the dorsal mesentery was suspending the stomach, and the intestines were convoluted and contained glands and villi with varying heights in the duodenum, jejunum, and ileum. Although the development of the intestinal cells was progressive, undifferentiated cuboidal endodermal cells along the differentiation axis remained until mid-gestation in the horse, likewise to several other species (Sadler, 2005; Santa-Barbara et al., 2003). Cellular differentiation seemed to be a consequence of epithelial-mesenchymal interactions that forced endodermal differentiation. In this regard, signals from the mesoderm along the anterior–posterior and dorsoventral axes seem to be important (Sadler, 2005; Santa-Barbara et al., 2003). The results on the morphological differentiation of the endoderm in horses supported this hypothesis.

During the embryonic phase from 21 to 38 days of gestation, there seemed to be an interaction between the endoderm and the splanchnic mesoderm. This might be the result of cell signaling between the tissue layers that coordinate the organogenesis of the intestinal derivatives as seen in humans (Montgomery, 1999; Ramalho-Santos et al., 2000; Sadler, 2005; Santa-Barbara et al., 2003).

From day 40 onward, conceptuses have been classified as fetuses with completely formed liver, similar to what was described by Albert et al. (2013) for the bovine. The elongated pancreas with primordial acini, and small islets was observed. Shortly after, the esophagus had its final length, the pharynx was fully developed and the esophagus had striated and smooth muscles as previously reported for 50-day-old embryos (McGeady, 2006). In addition, this study demonstrated that at 65 days, the dorsal mesentery was suspending the stomach, and the intestines were convoluted and contained glands and villi with varying heights in the duodenum, jejunum, and ileum. Although the development of the intestinal cells was progressive, undifferentiated cuboidal endodermal cells along the differentiation axis remained until mid-gestation in the horse, likewise to several other species (Sadler, 2005; Santa-Barbara et al., 2003). Cellular differentiation seemed to be a consequence of epithelial-mesenchymal interactions that forced endodermal differentiation. In this regard, signals from the mesoderm along the anterior–posterior and dorsoventral axes seem to be important (Sadler, 2005; Santa-Barbara et al., 2003). The results on the morphological differentiation of the endoderm in horses supported this hypothesis.

In addition, cell proliferation analysis using Oct4 showed strong protein expression in young embryos, but lost this over time. During the fetal stage, this marker was restricted to specific areas as observed in the stomach, intestine, liver, and pancreas in our study. Therefore, main proliferation and differentiation processes of the horse digestive system components can be related to the embryonic phase once that the high proliferation was observed in the pharyngeal arches during early development similar to described by Roballo et al. (2013) in carnivores. Oct4 is commonly used to identify cell proliferation: According to Vetsted et al. (2006), it is a pluripotency marker for the germ line of primordial cells in species such as mouse and pig. It was reported that Oct4 is a key transcription factor to control embryonic development and maintain pluripotency in equine embryos (Choi et al., 2006). The Oct4 is expressed in pluripotent cells such as blastomers during cleavage, the internal cell mass of the blastocyst and the young embryonic epiblast after implantation as well as in embryonic stem cells (Kurosaka et al., 2004). These authors also found that trophodermal differentiation is correlated with Oct4 regulation and that increases or decreases in the levels of expression of this marker result in the differentiation of stem cells into the primitive endoderm and trophoderm, which explains the progressive down-regulation of Oct4 in the fetuses observed in our study.

**CONCLUSION**

Cellular differentiations that lead to specific functions of organs involve many steps. An understanding of basic morphological changes that occur during such processes is crucial for directing developmental biology studies. The macroscopic and microscopic analysis of equine embryos and fetuses shed light on the organogenesis of the digestive system in addition to a cell proliferation analysis using Oct4 expression. The results provide a reference on chronological events during the early intraterine phase that may contribute to better understand normal versus pathological development for both in vivo breeding and the usage of assisted reproductive techniques in horses as well as a basis for studies with nutrition and comparative embryology in a highly precocial environment.

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