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Prenatal developmental sequences of the esophageal epithelium in the New Zealand white rabbits: Light and electron microscopic analysis

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Abstract

Several morphogenetic sequences occur during esophageal development and birth defects occur due to defects in foregut morphogenesis. This work aimed to record the cellular events in the morphogenesis of rabbits' esophageal epithelium. On the 16th day of gestation, the esophageal epithelium varied from stratified ciliated columnar to stratified squamous type. The surface epithelium presented mucous cells with mucigen granules of various sizes occupying their supranuclear cytoplasm. Cytoplasmic vacuolation was evident in all layers of the esophageal epithelium at this age. On the 18th gestational day, some light cells could be detected in the middle portion of the epithelium, while others occupied the whole epithelial length. On the 21st day, mucous cells are more frequently observed at the apical esophageal part as well as at the surface epithelium. Numerous elongated dark cells could be distinguished embedded between the basal cells. On the 24th gestational day the number of the mucous cells reached its peak. Reaching the 30th gestational day, several lamellar bodies, a keratinized layer and mitotic divisions could be demonstrated, and the number of both mucous and dark cells was greatly decreased. Collectively, detection of surface mucous and dark cells together with the non-cornified surface in some regions of the rabbit esophageal epithelium at the end of gestation ensure a postnatal development to reach the adult epithelium essential to sustain the passage of the harsh raw food. Future immunohistochemical studies are recommended to investigate the components of secretions in mucous cells and functional studies to highlight the dark cells significance.

Research Highlights

- Esophageal epithelium of fetal rabbit was analyzed by light and transmission microscopy.
- Surface epithelium presented mucous cells with mucigen granules of various sizes. They reached their maximum number on 24th day then decreased.
- On the 16th day, cytoplasmic vacuolation was evident in all epithelial layers.

- On the 21st day, numerous elongated dark cells could be distinguished embedded between the basal cells.
- Before birth, several lamellar bodies, a keratinized layer and mitotic divisions could be demonstrated, and the number of both mucous and dark cells was greatly decreased.

KEYWORDS
cytoplasmic vacuolation, dark cell, keratinized cells, lamellar bodies, mucous cells

1 | INTRODUCTION

Mammalian embryonic foregut development encompasses changes in the behavior of both epithelial endoderm and adjacent mesoderm. Several morphogenetic sequences occur during development comprising formation of the midline notochord cells from the epithelial definitive endoderm, the folding of the endoderm into a foregut tube, and the consequent division of the foregut tube into trachea and esophagus. Birth defects occur due to defects in the foregut morphogenesis (Que et al., 2006).

While connective tissue and muscle are derived from the mesoderm, the endoderm is the source of the epithelial components of the digestive system's organs. The proliferation, migration, and differentiation of stem/progenitor and mature cells result in the development of the body's organs. The esophageal lengthening, widening, and thickening mutually with histological changes characterize development of the mammalian esophagus (Eşrefoğlu et al., 2017).

Wall of the adult mammalian esophagus including rabbits involves the mucosa, submucosa tunica muscularis (muscularis externa), and adventitia/serosa. The mucosa is composed of the epithelium,

TABLE 1 Age and number of used embryos and fetuses.

Age (day)	10	11	12	13	14	15	16	18	20	21	24	26	27	29	30
Number	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3

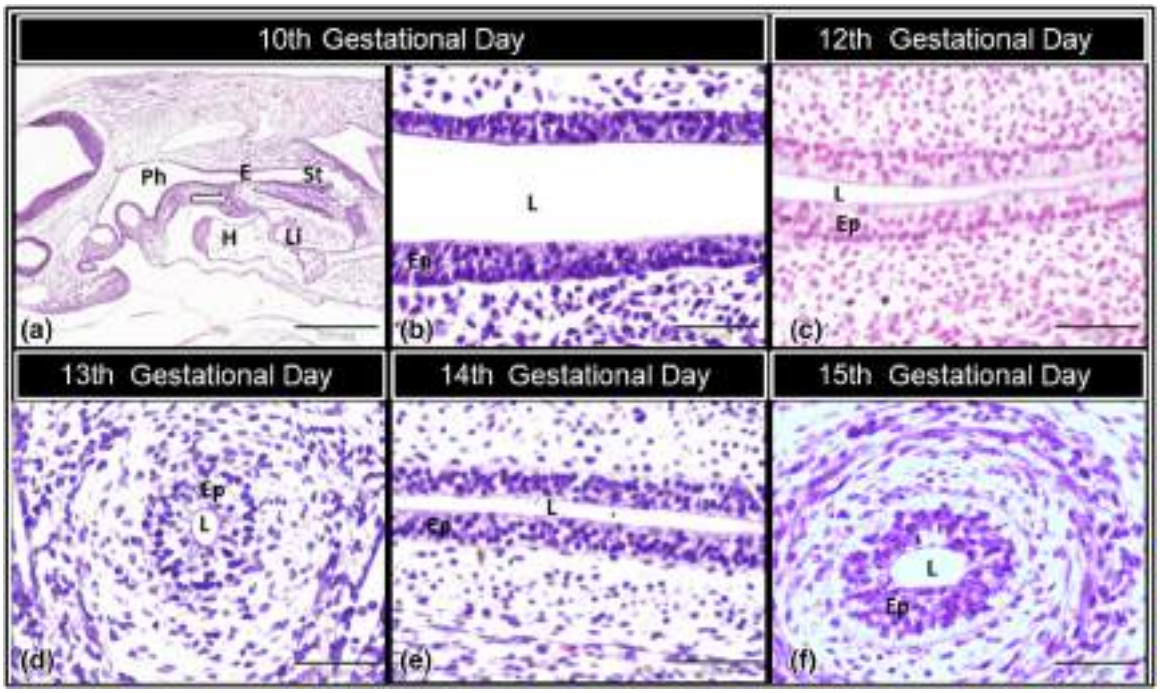

FIGURE 1 Paraffin sections of the developing rabbit esophagus (E) of 10 (a,b), 12 (c), 13 (d), 14 (e), and 15 days (f) stained with Harris hematoxylin and eosin showing the two layered-thick epithelium (Ep). In (d) cells are taller. In (e) and (f) the thickness increased to three or four layers with the nuclei of the basal layer becoming apically located. The respiratory diverticulum is indicated by arrow in (a). H, heart; L, lumen of the esophagus; Li, liver; Ph, pharynx; St, stomach.

connective tissue lamina propria, and muscularis mucosa. Keratinization of the surface epithelium varies among different animals. Moreover, esophageal morphogenesis has been evaluated in human (Eşrefoğlu et al., 2017; Que et al., 2006; Zhang et al., 2018), in rabbit (Ibrahim et al., 2019), and in rats (Rosekrans et al., 2015).

Three layers were identified in the transmission electron microscopy (TEM) of the adult mammalian esophageal epithelium: basal, polyhedral, and functional cell layers. The developing esophagus is certainly the least studied segment of the digestive tract, and our knowledge of its functional development is very limited. Ménard and Arsenault (1987), Ménard (1995), and Fu et al. (2004) verified the ultrastructural features of the developing human esophagus. The esophageal epithelium started by stratified columnar cells. The surface cell presented glycogen granules into their cytoplasm (Schaller, 1978). In rats, Gregersen et al. (2004) demonstrated that the muscle layer develops faster than the mucosal and submucosal layers.

The rabbit esophagus has not been fully explored at the ultrastructural level, specifically regarding its surface epithelium and cellular components. The current work surveys the fine structure of the esophageal epithelium of rabbit to map the cellular events in the morphogenesis of esophageal epithelium during their prenatal life.

2 | MATERIALS AND METHODS

2.1 | Ethical approval and ARRIVE guidelines

The present work was done in accordance with the Egyptian laws and University animal care guidelines. All the procedures in the current research have approved by the National Ethical Committee of the Faculty of Veterinary Medicine, South Valley University, Egypt (approval no. VM/SVU/23(1)-02).

2.2 | Rabbit embryos specimens' collection

Eighty-three apparently healthy New Zealand white rabbit embryos and fetuses were collected from the Research Farm of Faculty of Agriculture,

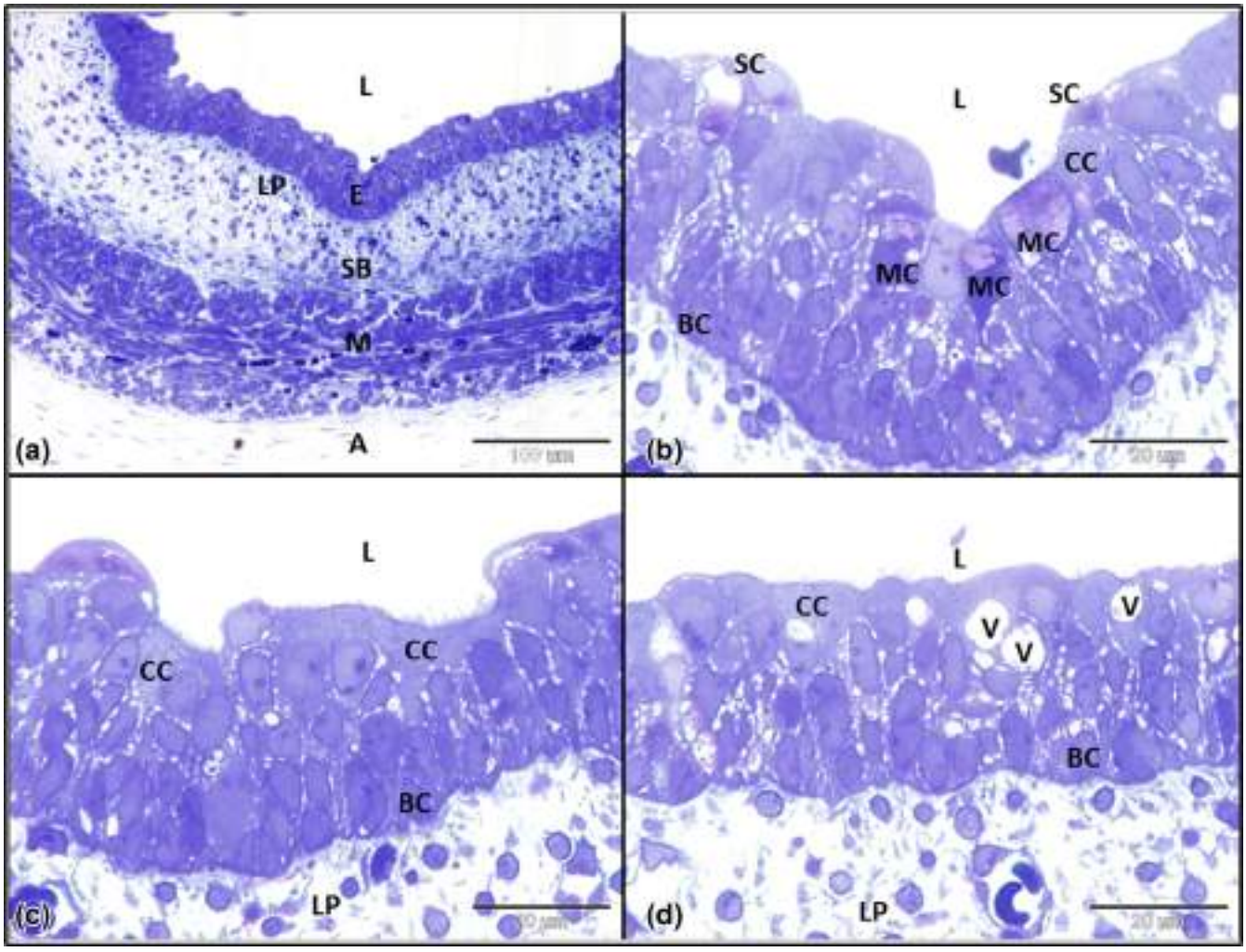

FIGURE 2 Semi-thin sections of the 16 days rabbit esophageal epithelium stained with toluidine blue showing various layers of the esophageal wall (a) and different cellular elements constituting the stratified esophageal epithelium (E) in (b–d). A, adventitia; BC, basal cell; CC, ciliated cell; L, lumen; LP, lamina propria; M, muscularis; MC, mucous cell; SB, submucosa; SC, squamous cell; V, vacuole.

South Valley University, Egypt at embryonic days (E) 10–30 (Table 1). The minimum number of animals required to obtain valid results was selected. The pregnant mothers were sacrificed at the required periods of pregnancy and the embryos and fetuses were collected shortly after evisceration.

2.3 | Histological preparations

The collected fetuses were fixed in 10% neutral buffered formalin, washed by water, dehydrated in ethanol, cleared in methyl benzoate, embedded in paraffin, serially sectioned at 5 μm thickness and finally stained by Harris hematoxylin and eosin to identify the general structural features of the developing rabbit esophagus (Bancroft & Layton, 2013).

2.4 | Semithin sections preparation

Small pieces from the rabbit esophagus were used for semi-thin sections. First, immersion fixation in 2.5% glutaraldehyde in sodium phosphate buffered saline at 4°C (overnight) and processing according to Ibrahim et al. (2019) were achieved. Then, the specimens were washed in sodium phosphate buffer (0.1 M, pH 7.2, 15 min, 4 times) and fixed in 1% osmic acid in 0.1 M Na-phosphate buffer at 4°C for 2 h. The specimens were washed once more in phosphate buffer (0.1 M, pH 7.2, 20 min, 3 times), were dehydrated in ascending grades of ethyl alcohol to propylene oxide. Propylene oxide (Merck, Darmstadt, Germany) and/or epon (Polysciences, Eppelheim, Germany) were used to accomplish resin embedding. The resin blocks were incubated for 3 days and were cut to semi-thin sections (1 μm-thick) using an ultramicrotome (Reichert–Jung Ultra cut E; Leica Microsystems) and finally were stained with toluidine blue stain.

2.5 | Electron microscopic preparation

Ultra-thin sections of 70 nm thickness were obtained from 16th, 18th, 21st, 24th, and 30th days of gestation by a Reichert ultramicrotome. The tissue sections were stained by Uranyl acetate and lead citrate, examined by a JEOL100CX II transmission electron microscope (JEOL, Tokyo, Japan), and images were captured using Kodak Megaplus Camera, Model 1.6 I with image analysis and processing software (AMT, Ford City, Pennsylvania) at the Electron Microscopy Unit of South Valley University, Qena, Egypt.

2.6 | Morphometric analysis

Images of semithin sections of the 21st, 24th, 27th, and 30th prenatal ages were used for morphometric and quantitative assessments using Image J software (1.45 s, Wayne Rasband, National Institutes of Health, USA) (Duszynski & Wilber, 1997). The number of and size of the surface mucous cells and the number of dark cells were measured per constant

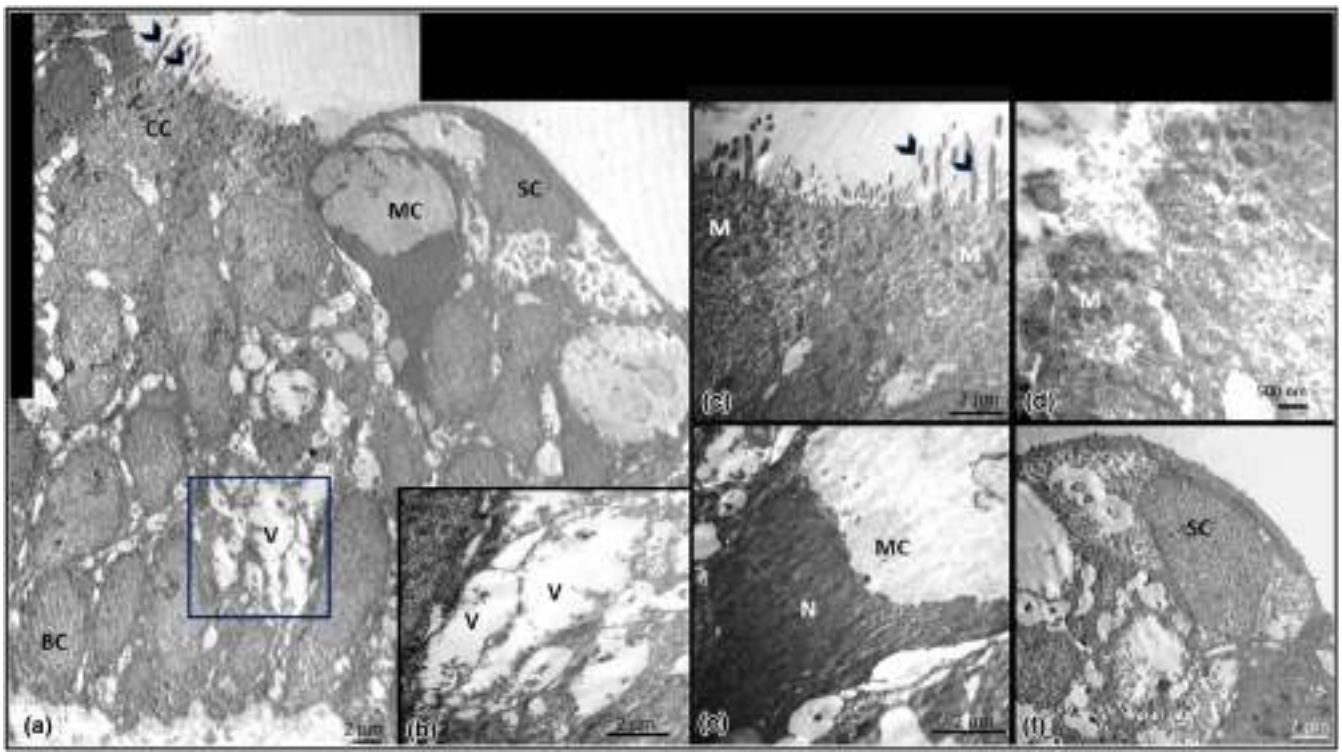

FIGURE 3 Electron micrographs of the 16 days rabbit esophageal epithelium showing the fine structure of the basal cells (BC) in (a), numerous vacuoles in (b), mucous cells (MC) in (a) and (e), surface squamous cell (SC) in (a) and (f), ciliated cells (CC) containing numerous mitochondria (M) in (a), (c), and (d). Cilia are indicated by arrowheads. N, nucleus of the mucous cell; V, vacuoles.

area of 50 μm^2. All these measurements were performed on five randomly selected sections per developmental age were examined (from each section, three randomly selected regions were measured).

2.7 | Digital coloring of TEM images

To distinguish the numerous cellular elements within the developing esophageal cells, coloring of the TEM images was presented digitally using the Adobe Photoshop 7.0 software. The coloring procedure required to change the image mode to RGB mode followed by selection of the objective cell. Then, the color balance was changed to the desired color to color the objective cells. Finally, hue and saturation were adjusted.

3 | RESULTS

The primitive esophagus represented the proximal part of the foregut just caudal to the respiratory diverticulum at 10 days old rabbit embryo. It continued caudally with the stomach without any definite line of demarcation. It was lined by stratified epithelium surrounded by loose mesenchyme. At 10 and 12 days, the epithelium was two cells deep. On the 13th day, it increased in thickness as the cells became taller. On the 14th and 15th gestational days, the epithelium turned into three layers due to the mitotic division, with the nuclei of the basal layer becoming apically located (Figure 1).

Later at the 16th day of gestation, the esophageal epithelium became 4–5 layers in the abdominal part. The epithelium nature varied from stratified ciliated columnar to stratified squamous type (Figure 2).

Ultrathin sections of 16th day old rabbit embryo revealed surface epithelium housing mucous cells with mucigen granules of various sizes occupying their supra-nuclear cytoplasm. The surface epithelial cells were mostly ciliated enriched with oval or rounded mitochondria, though some squamous epithelial cells occupied part of the epithelial surface. Mucous cells with basally situated dark nuclei and mucigen-rich apical cytoplasm could be distinguished. The vacuolation process could be observed for the first time on the 16th gestational day where it was restricted to the abdominal part of the rabbit esophagus. These

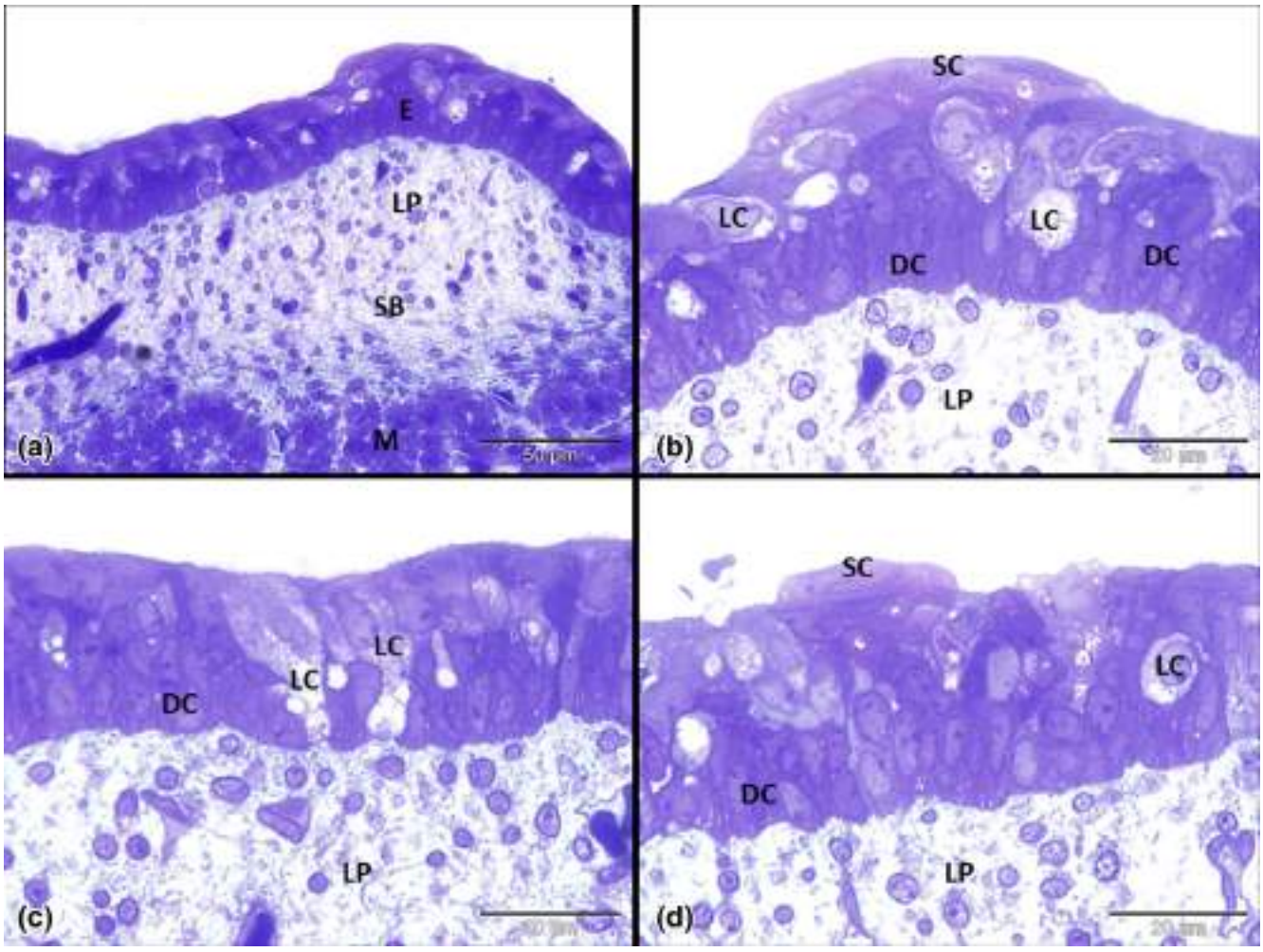

FIGURE 4 Semi-thin sections of the 18 days rabbit esophageal epithelium stained with toluidine blue showing various layers of the esophageal wall (a). In (b–d) both dark cells (DC) located basally, and light cells (LC) located in the intermediate layers of the esophageal epithelium (E) are evident. Notice the two LCs extend the whole epithelial thickness in (c). LP, lamina propria; M, muscularis; SB, submucosa; SC, squamous cell.

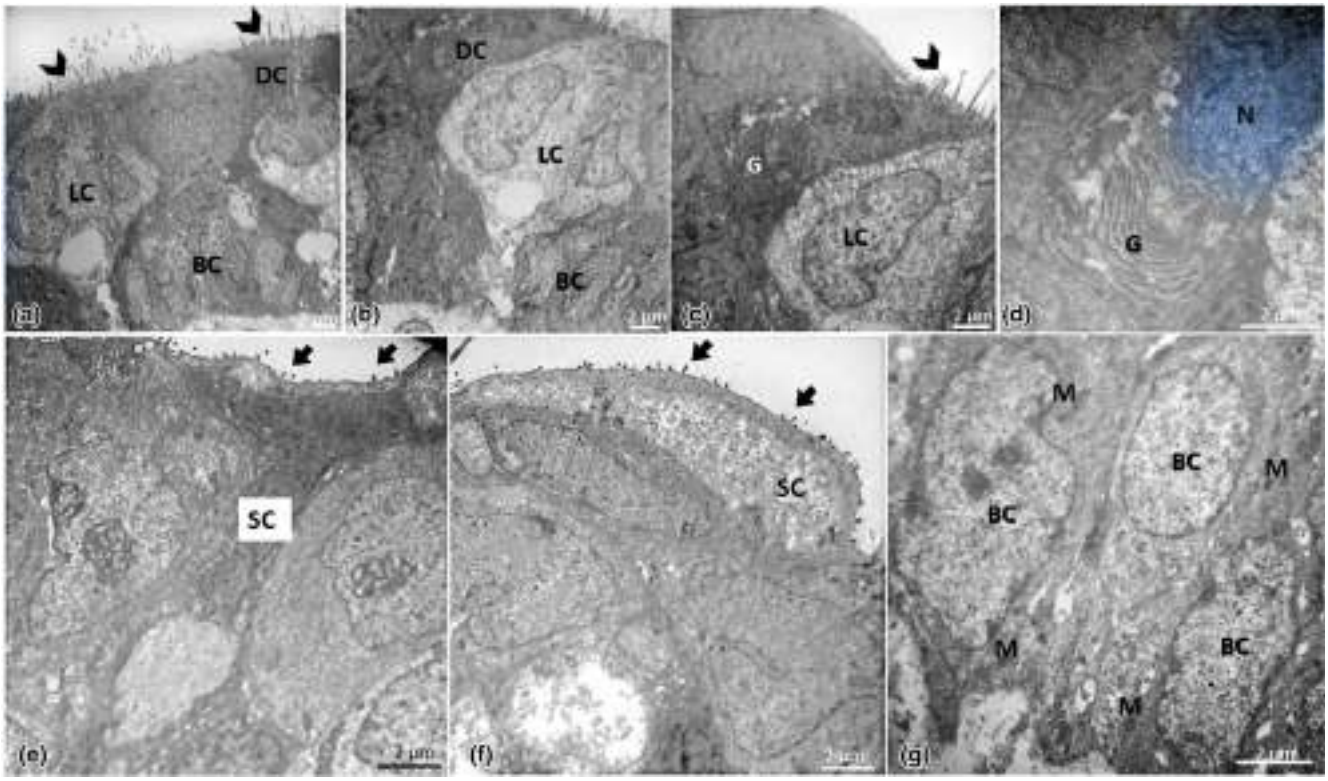

FIGURE 5 Electron micrographs of the 18 days rabbit esophageal epithelium showing the fine structure of the dark (DC) and light (LC) ciliated cells (a,b). In (c,d) the DCs presented well-developed Golgi complex (G) and apical nucleus (N). Arrowheads indicate cilia and arrows indicate microvilli formed on the free border of the squamous cells (SC) in (e,f). In (g) basal cells (BC) contained large nucleus and numerous mitochondria (M).

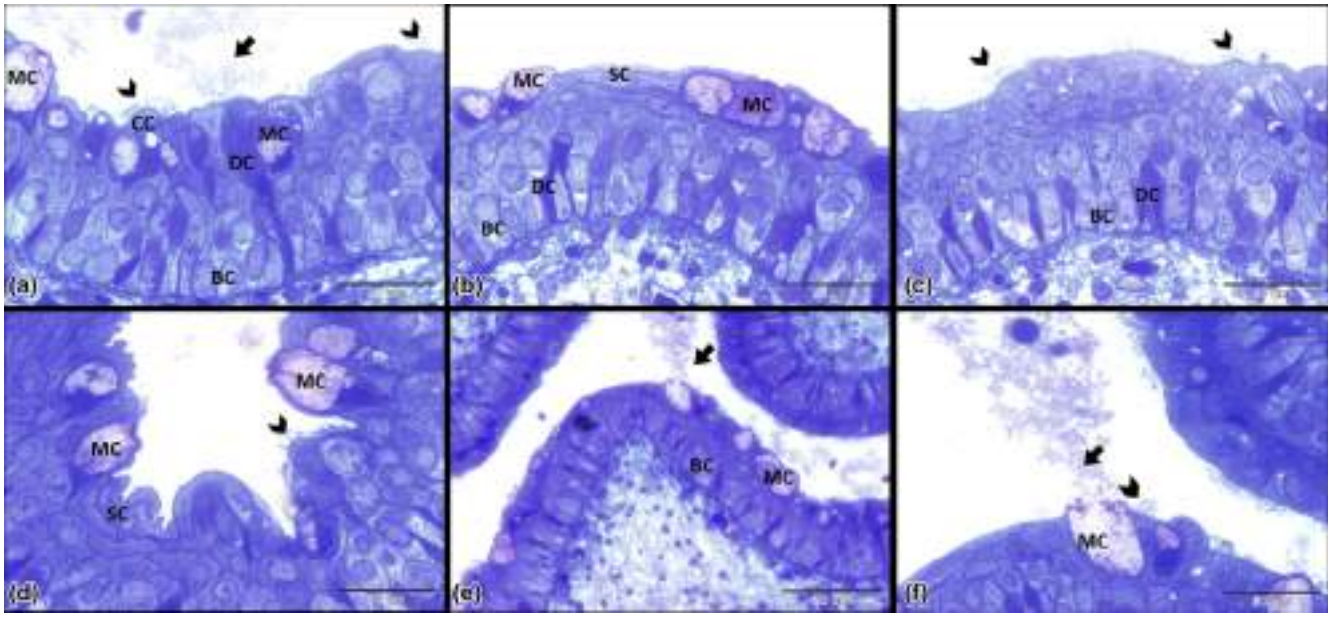

FIGURE 6 Semi-thin sections of the 21 days rabbit esophageal epithelium stained with toluidine blue (a)-(f) showing numerous surface mucous cells (MC). Some mucous cells in (e,f) could be detected discharging their secretion into the esophageal lumen (arrows). Arrowheads indicate the cilia of ciliated cells (CC). BC, basal cells; DC, dark cells; SC, squamous cells.

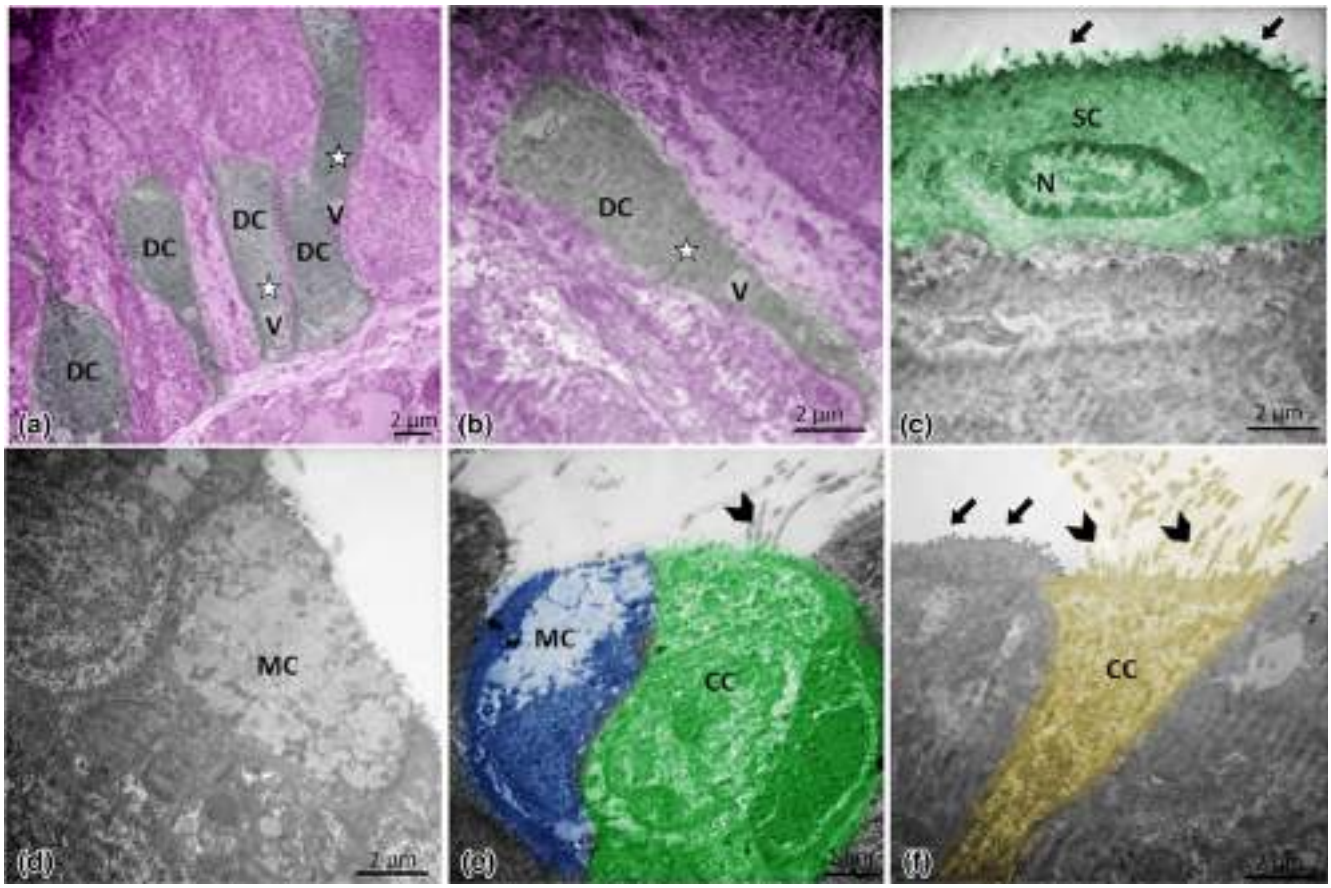

FIGURE 7 Electron micrographs of the 21 days rabbit esophageal epithelium (a)-(f) showing the fine structure of the dark cells (DC, a,b) with their characteristic vacuolation (V). In (c) the cytoplasm of the squamous cell (SC) contains mitochondria in the left to the nucleus (N) and rER on the right side of the nucleus. In the apical region, some secretory vesicles could be demonstrated. Fingerlike projections of the SCs are indicated by arrows. In (d,e) typical mucous cells (MC) with basal compressed nucleus and apical mucus droplets. Ciliated cell (CC) with distinct cilia (arrowheads) and its cytoplasm contains apical mitochondria. *, nucleus of dark cells.

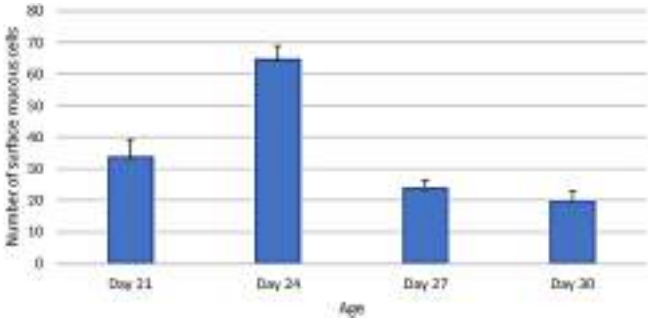

FIGURE 8 The number of the surface mucous cells on days 21, 24, 27, and 30 indicates increase of their number on the 24th day and then decreased.

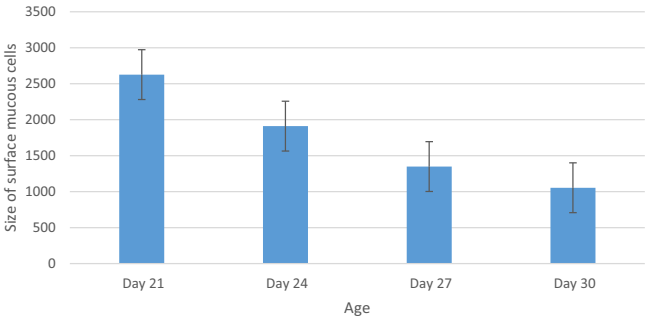

FIGURE 9 The size of the surface mucous cells in days 21, 24, 27, and 30 indicates its gradual decrease with the advancement of age.

cytoplasmic vacuoles were evident in all cell layers of the esophageal epithelium at this age (Figure 3).

On the 18th gestational day, this increase in the number of the epithelial layers extended cranially to include the thoracic and cervical parts. The surface squamous cells were more frequently observed in esophageal sections. Some light cells occurred embedded in the middle portion of the esophageal epithelium, while others occupied the whole epithelial thickness (Figure 4).

At the level of the ultrastructure, vacuolation has been demarcated to the light cells. Ciliated cells with well-developed Golgi complex and apically situated nucleus could be identified. Basal cells rich in mitochondria and some free ribosomes were characterized by large

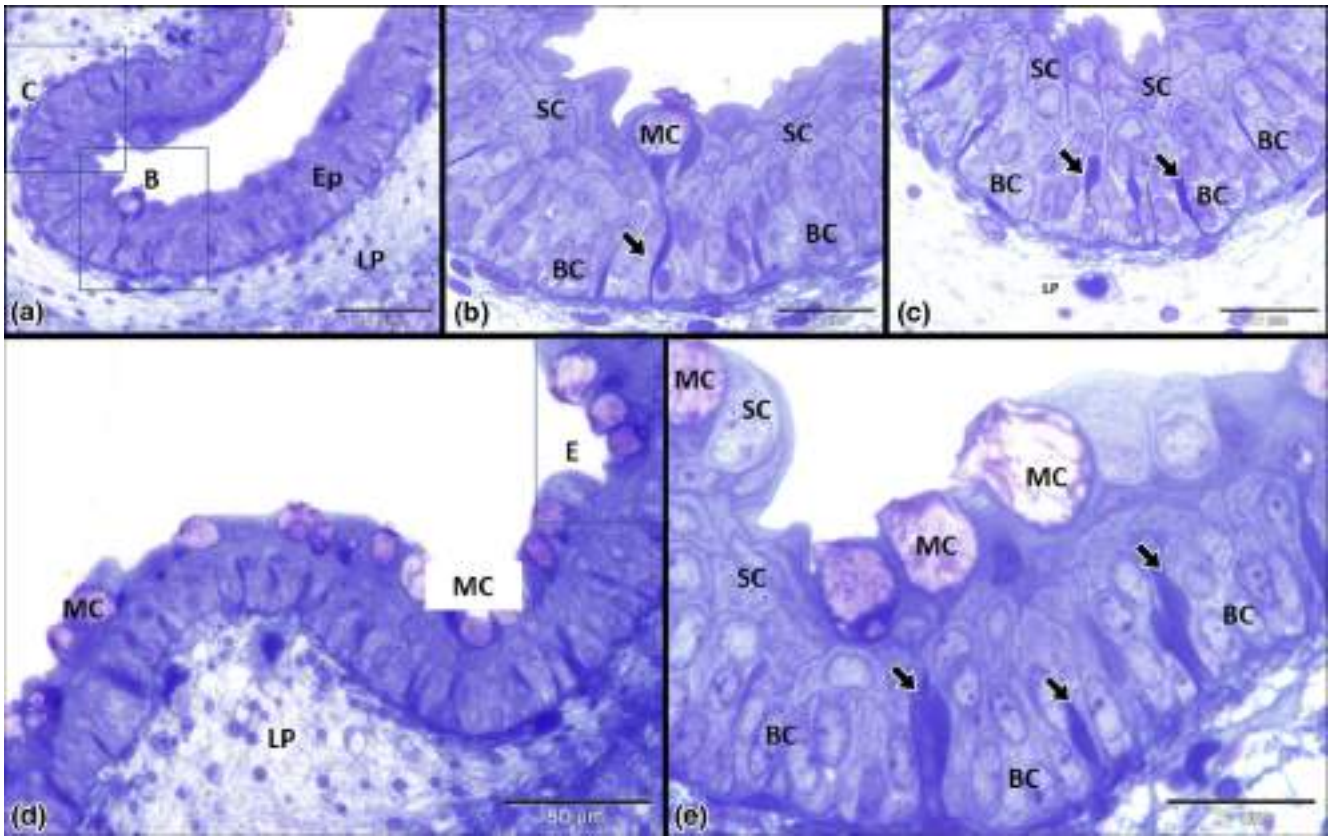

FIGURE 10 Semi-thin sections of the 24 days rabbit esophageal epithelium (Ep) (a)-(e) stained with toluidine blue showing increased number of mucous cells (MC) with their characteristic foamy cytoplasm. Fewer dark cells (arrows) could be demonstrated at the lower half of the epithelium. BC, basal cells; LP, lamina propria; SC, squamous cells.

nuclei with slight indentations and distinct nucleoli. Some polygonal non-ciliated surface cells could be distinguished. Surface of the squamous cells was characterized by numerous microvilli. Large light ciliated cells with large apical nucleus and basal vacuolated cytoplasm were also evident (Figure 5).

Moving onwards, at the 21st gestational day, mucous cells were more frequently observed at the apical esophageal part in addition to the surface epithelium. These mucous cells were characterized by highly vacuolated cytoplasm of mucigen granules and basally located nuclei. Numerous slender elongated dark cells could be distinguished embedded between the basal cells. Some surface mucous cells could be identified releasing their cytoplasmic contents into the esophageal lumen (Figure 6).

At the ultrastructure level, the electron-dense undifferentiated dark cells extended from the basal lamina to the luminal surface of the esophageal epithelium. Their morphology varied, some cells had a wide base and narrow apex while others had a wide apex and narrow base. One or two large vesicles were frequently identified in the basal cytoplasm. The cytoplasmic organelles of the dark cells as well as their nuclei could hardly be recognized. Surface squamous cells with numerous fingerlike projections on their cell surface and large nucleus containing distinct nucleolus and dispersed chromatin were characteristic. Their cytoplasm represented rough endoplasmic reticulum and mitochondria. In the apical region, some secretory vesicles could be demonstrated. Ciliated cells with their distinct cilia or apical basal bodies of the cilia could be identified. Their cytoplasm contained numerous mitochondria and free ribosomes. Moreover, typical mucous cells with basal compressed nucleus and apical mucus droplets were evident (Figure 7).

On the 24th day of gestation the number of the surface mucous cells with their characteristic foamy cytoplasm and basally situated nucleus was greatly increased (Figure 8) and their size was decreased (Figure 9). The dark cells diminished in their length, becoming located at the lower half of the esophageal epithelium (Figure 10). TEM images revealed the appearance of 2–3 rows of squamous cells underlying the superficial ciliated, mucous or surface squamous cells. The size of the fingerlike projections on the surface of the squamous cells was obviously reduced. The basal cytoplasm of some dark cells was enriched with round or oval mitochondria (Figure 11).

Reaching the 27th gestational day, several mitotic divisions could be demonstrated clearly at the basal cell layer of the esophageal epithelium and the number of dark cells decreased (Figure 12) and diminished in its length. In addition, a cornified layer could be identified covering the epithelial surface (Figure 13). These changes continued at the 30th day of gestation and the three portions of the esophageal

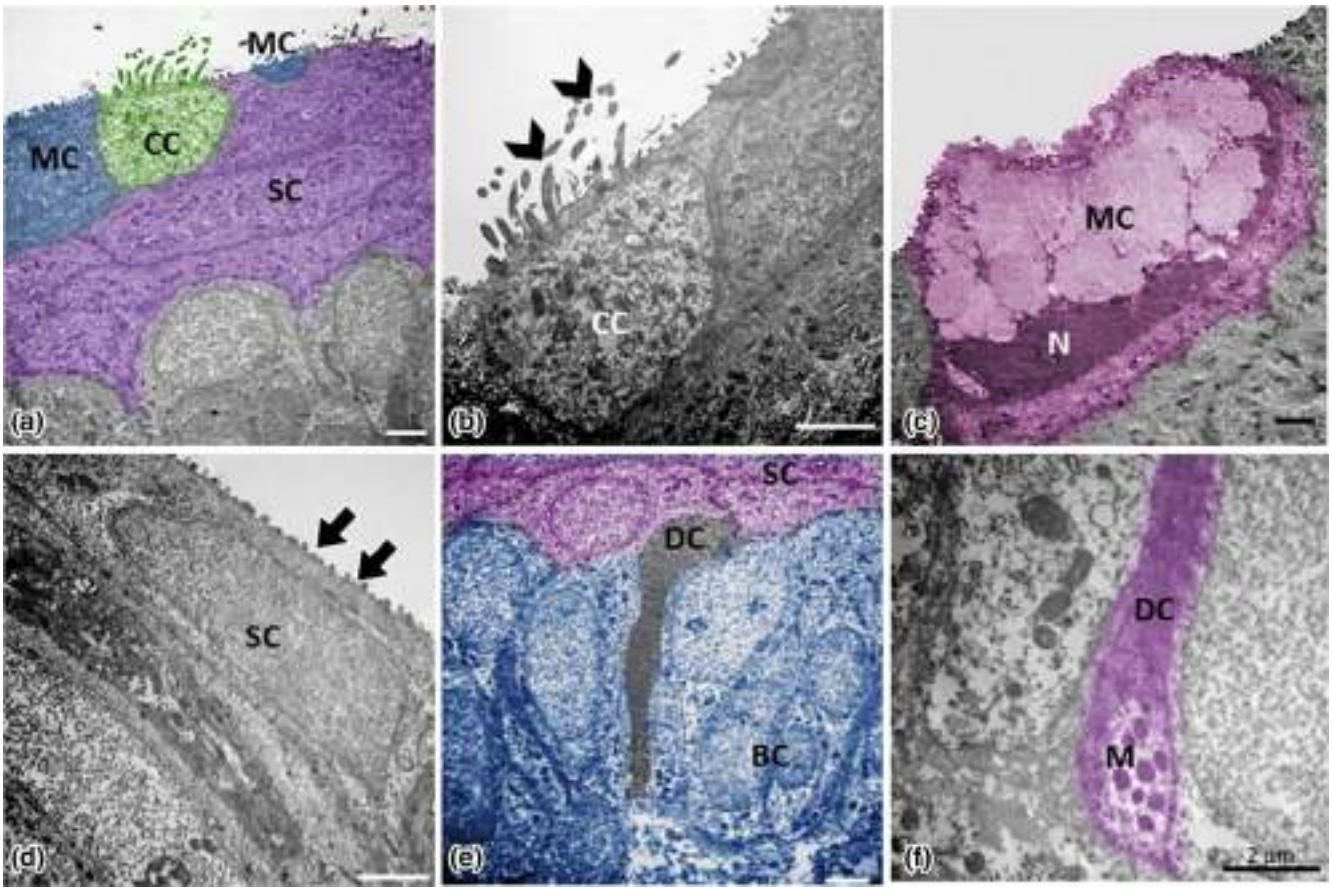

FIGURE 11 Electron micrographs of the 24 days rabbit esophageal epithelium showing the fine structure of its cellular components (a). The surface ciliated cells (CC) with their cilia (arrowheads) and numerous mitochondria (b). Mucous cells (MC) with their basal nucleus (N) and apical mucin droplets (c). The squamous cells (SC) with fingerlike projections (arrows) (d). The dark cell (DC) is enriched with mitochondria (M) in its basal cytoplasm (e,f). BC, basal cell.

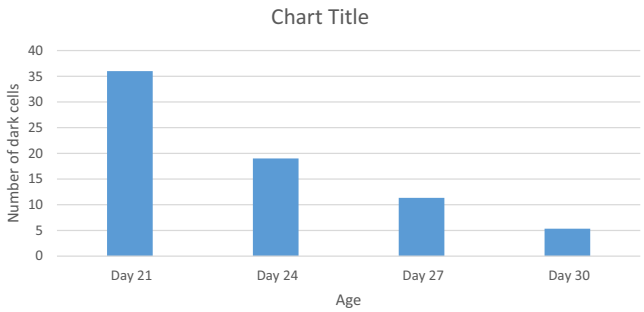

FIGURE 12 The number of the dark cells on days 21, 24, 27, and 30 shows the highest number on the 21st day and then decreases till the end of gestation.

epithelium became easily distinguished from each other. The basal portion contained both basal and dark cells, the intermediate portion housed the polyhedral cells, and the upper most portion composed of several squamous epithelial cell layers. Some cornified cells could be distinguished above the squamous cells (Figure 14).

At the electron microscopic level, the squamous cells represented various nuclear morphology and they were separated from the underlying polyhedral cells by continuous basal lamina with characteristic interdigitations. The polyhedral cells demonstrated spherical nucleus with distinct nucleolus with numerous electron-dense bodies located in cytoplasm, near the cell membrane with bundles of intermediate filaments demonstrated around the nucleus. Polyhedral cells were tightly connected to each other through desmosomes (Figure 15). The basal cells represented oval nucleus and high mitochondrial content. Some cells presented several lamellar bodies and centrioles (Figure 16).

4 | DISCUSSION

The developing esophageal epithelium of New Zealand white rabbits using semithin and ultrathin sections at the second half of pregnancy revealed several morphogenetic sequences. At the 16th gestational day, the epithelium became 4–5 layers in the abdominal part. This

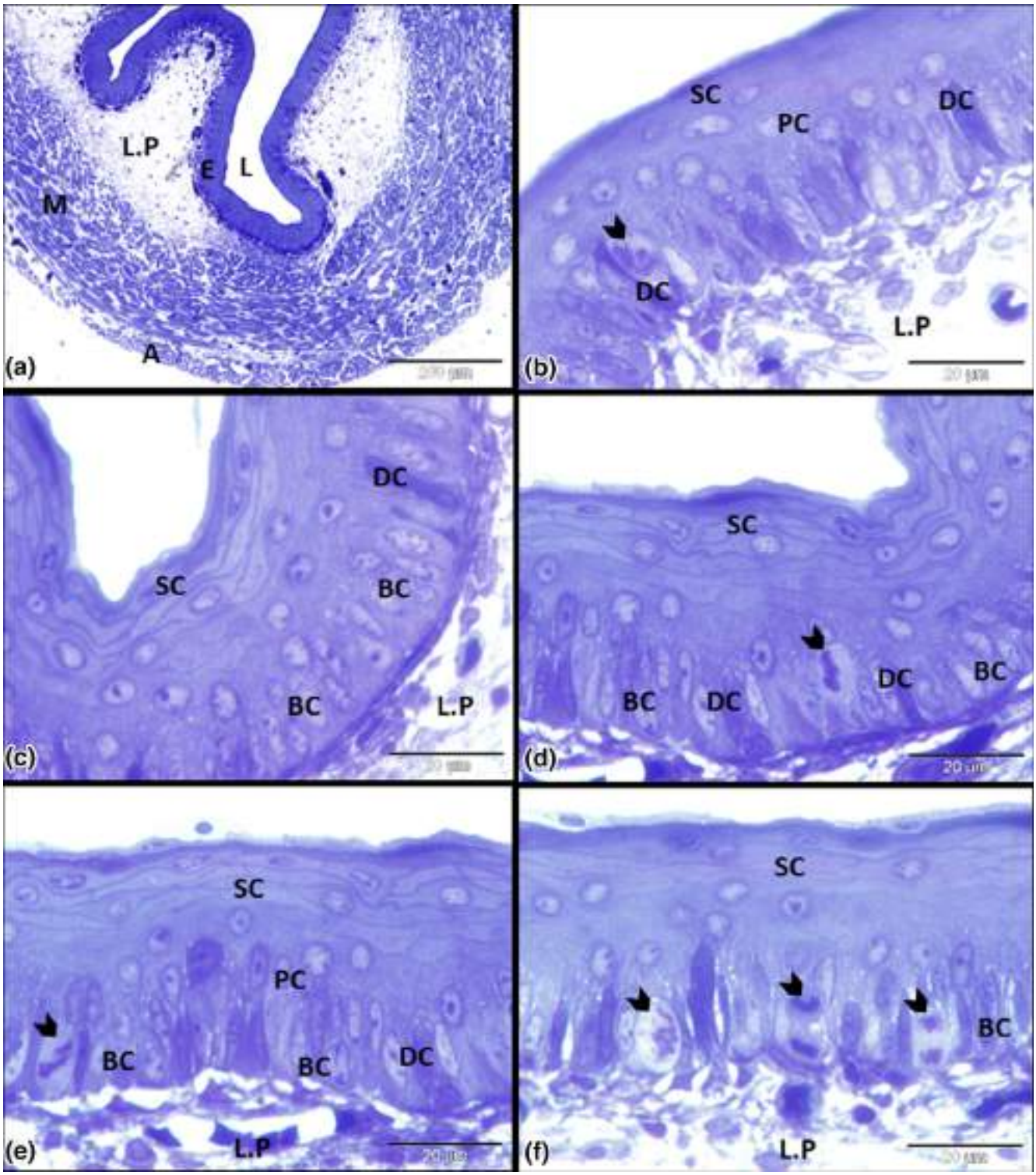

FIGURE 13 Semi-thin sections of the 27 days rabbit esophageal epithelium (E) (a)-(f) stained with toluidine blue showing prevalence of the surface squamous cells (SC). The number and size of the dark cells (DC) are diminished. Numerous mitotic figures are indicated by arrowheads. Notice the keratinized layer on the free surface. A, adventitia; BC, basal cell; L, lumen; LP, lamina propria; M, muscularis; PC, polyhedral cells.

increase extended cranially to include the thoracic and cervical parts at the 18th day of gestation.

The vacuolation process could be observed for the first time at the 16th day of pregnancy where it was restricted to the abdominal part. On the 18th day, this process extended along the whole length of the esophagus while, on the 21st day it disappeared. The process of vacuolation has been found to occur in the embryonic esophagus of pigs (Flint, 1907), rats and rabbits (Johnson, 1910), hedgehog

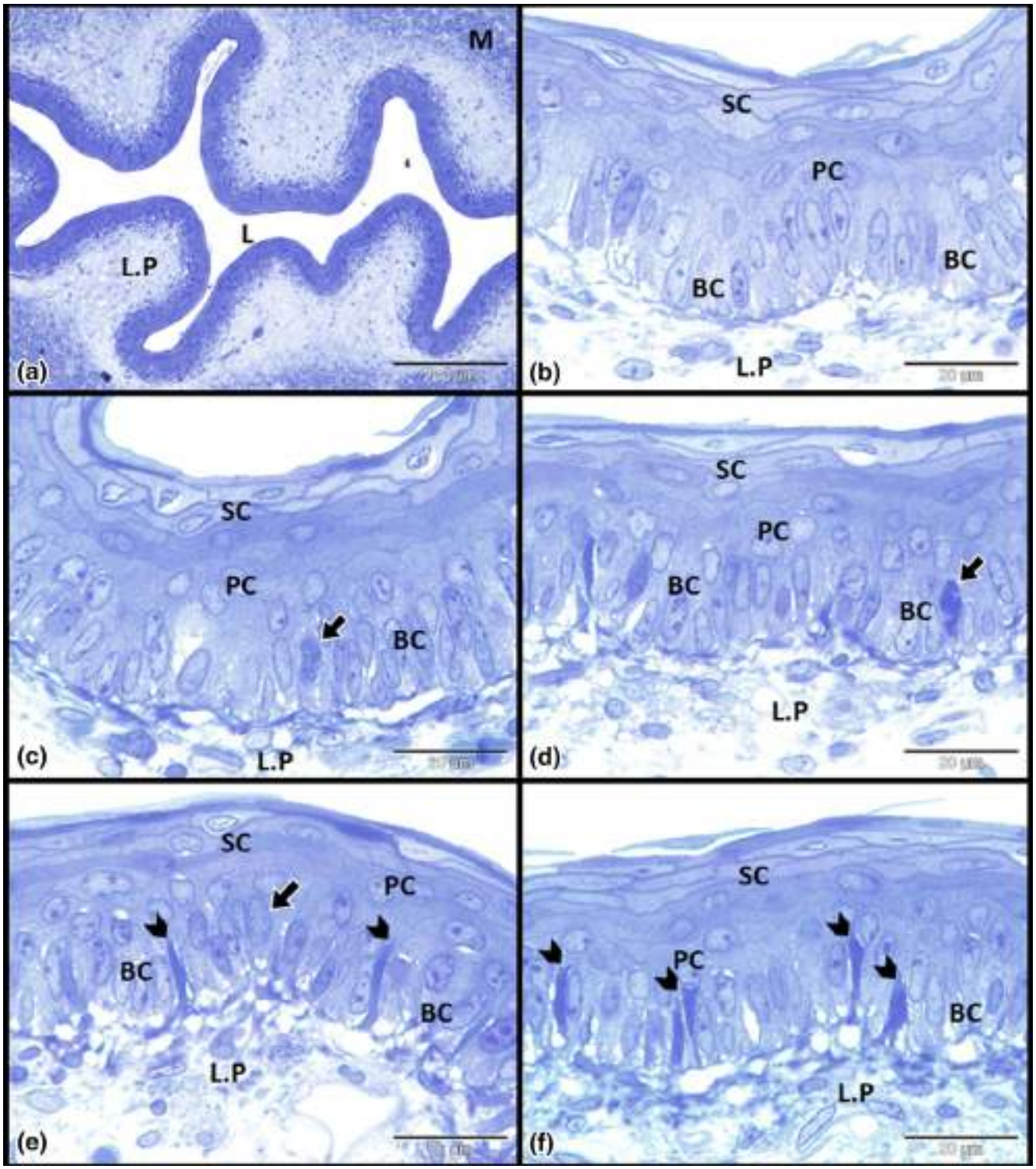

FIGURE 14 Semi-thin sections of the 30 days rabbit esophageal epithelium (a)-(f) stained with toluidine blue showing the surface squamous cells (SC) covered by a cornified layer. Mitotic figures are indicated by arrows. Dark cells are indicated by arrowheads. BC, basal cells; L, lumen; L. P, lamina propria; M, muscularis; PC, polyhedral cells.

(Forssner, 1907), and human (Johns, 1952). Although Johns (1952) mentioned that significance of the vacuolation process is not clear; we suppose that vacuolation may lead to widening of the esophageal lumen. On the other hand, Schridde (1908) supposed that true vacuolation does not occur and that histological appearance could be explained by epithelial bridges linking opposing walls of the esophagus.

On the 21st day of gestation, epithelium began to develop to ciliated stratified columnar type where some of the basal cells show

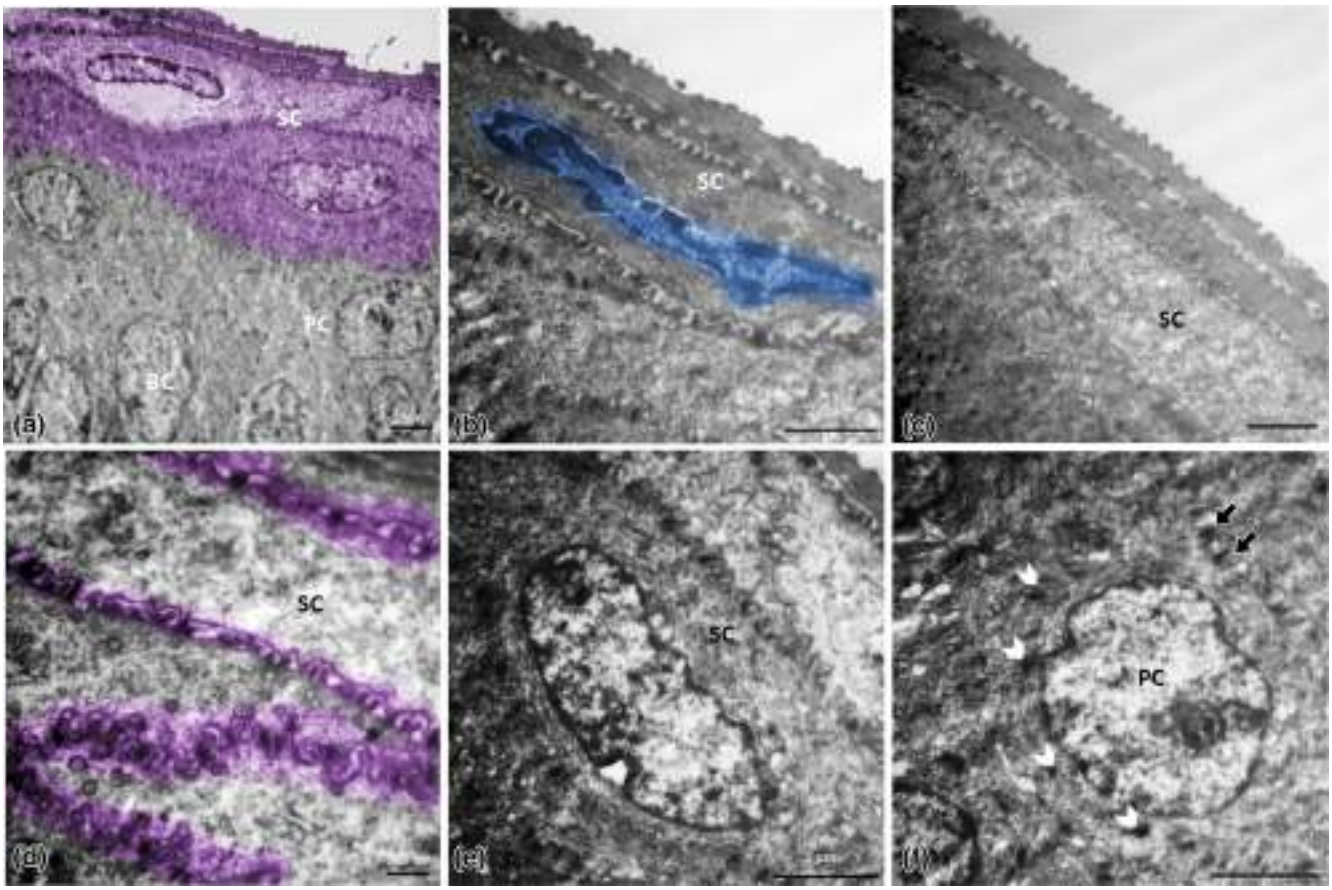

FIGURE 15 Electron micrographs of the 30 days rabbit esophageal epithelium (a)-(f) showing the fine structure of the surface squamous cells (SC) with their characteristic flattened nucleus and continuous basal lamina. Notice the interdigitation between the SCs (purple) in (d). The polyhedral cells (PC) show a central round nucleus with distinct nucleolus (f). Notice the bundles of intermediate filaments (arrowheads) demonstrated around the nucleus. Polyhedral cells are tightly connected to each other through desmosomes (arrows).

basally located nuclei and some of the luminal cells become ciliated columnar. On the 24th day, most of the luminal cells of the epithelium became mucus-secreting cells with columnar cells in between.

On the 27th gestational day, the mucus-secreting cells decreased in number. At the end of gestation period, the esophagus is lined by keratinized stratified squamous epithelium throughout its course. The basal layer is columnar with basally located nuclei, followed by 2–4 layers of polyhedral cells. The superficial layer is a single layer of squamous cells. The mucus-secreting cells are rarely observed within epithelium.

The developmental stages of the esophageal epithelium observed in the present work are generally in accordance with that observed by Johns (1952) in human embryos. He stated that in the early stage of development, epithelium is stratified columnar of three cells deep then it becomes two-layered; it subsequently proliferates to become many-layered. Later on, the superficial stratum becomes ciliated columnar. Finally, it becomes replaced by stratified squamous epithelium.

The ciliated stratified columnar epithelium observed in the present study at 21st day of gestation, was also reported by Ménard and Arsenault (1987) in human at the 12th–16th week of intrauterine development. Sakai et al. (1989) observed that ciliated cells are numerous at the esophagus epithelium of human fetuses at the 8th week, but they decreased after the 14th week of development.

Ménard (1995) suggested that the middle part of the esophagus is where patches of ciliated cells first appear multifocally. As time passes, the patches get larger and expand upwards and downwards to almost line the esophagus completely. Desquamation of cells with pyknotic nuclei from the stratified columnar epithelium's surface, which occurs before the ciliated type, in these patches suggests that the ciliated cells do not arise from the surface columnar cells themselves but rather come from the deeper cells in the stratified epithelium. While maintained that the ciliated cells are desquamated in sheets, some researchers believed that the ciliated cells were immediately changed into squamous cells.

In the present work, mucus-secreting cells were detected at the last third of pregnancy. Where, at the 24th day of pregnancy, it constituted most of the luminal cells of the epithelium then it decreased until it became scarce at the end of the gestation period. These cells have been recorded by Johns (1952) and Ménard (1995) in the esophagus of human embryos. The latter author claimed that superficial esophageal glands develop from them. The reason for appearance and disappearance of the mucus-secreting cells in the embryonic esophagus is unknown. We

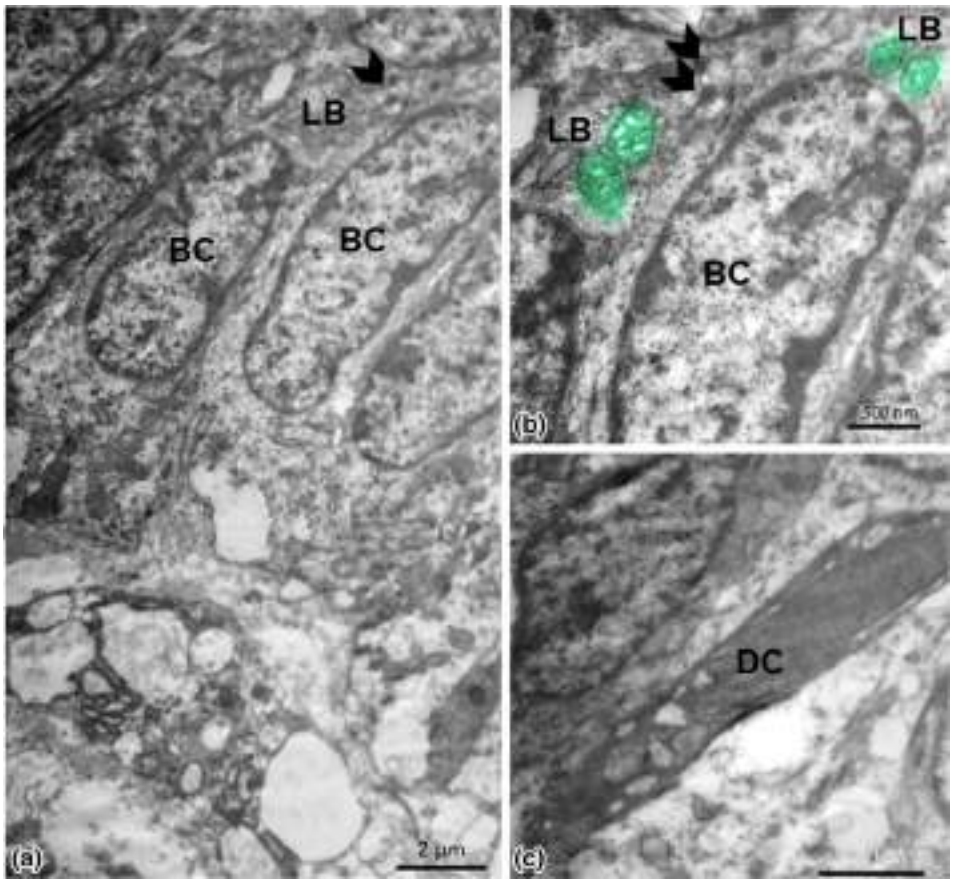

FIGURE 16 Electron micrographs of the 30 days rabbit esophageal epithelium showing the fine structure of the basal (a,b) and dark cells (c). Basal cells (BC) showing large oval nucleus and numerous mitochondria. Notice the lamellar bodies (LB) and centrioles (arrowheads). DC, dark cells.

suggest that these cells are ancestral of those present in lower vertebrates like fish (Kalhoro et al., 2018), amphibian (Zhang et al., 2015) and some reptiles as American alligator (Uriona et al., 2005), and snakes (Cundall et al., 2014).

In humans, luminescently released esophageal mucin has a substantial role in preserving the high viscosity of esophageal secretions. Mucin may be the primary target for stomach acid and pepsin, absorbing the harmful effects of gastroesophageal refluxate, as evidenced by a significant increase in the luminal release of mucin under the impact of acid and pepsin and a subsequent drop in perfusate viscosity (Namiot et al., 1994).

In the current work, the esophageal epithelium of rabbit became almost stratified squamous at the end of gestation period. In humans, it has been accepted that the esophagus epithelium becomes stratified squamous within the 4th month of development (Moore et al., 2002). Sakai et al. (1989) observed an immature stratified squamous epithelium at the 14th week and fully mature one at the 21st week of development. Schaller (1978) observed the squamous stratified epithelium after the 23rd week.

Interestingly, electron dense lamellar bodies could be distinguished in some basal cells. Such bodies were detected in the keratinized and granular layers of the esophageal epithelium of European Beaver (*Castor fiber*) (Martyniuk et al., 2023). Similar bodies were demonstrated in the developing pneumocyte type II of New Zealand white rabbits during its postnatal life (Mokhtar et al., 2019). Several functions have been elucidated for these bodies including skin barrier formation, proper desquamation of epidermis and skin health (Grayson et al., 1983). Detection of such bodies in the basal cells of the fetal esophagus suggests that they are the stem cells of esophageal keratinocytes.

Conclusively, demonstration of intraepithelial surface mucous cells at the last third of gestation and their number reached its peak on the 24th day indicates a potential role for these cells at this stage of development. Although immunohistochemical study to reveal the components of their secretions is essential. In addition, number of dark cells recorded its peak on the 21st gestational day and then decreased. This may signify a functional role played by these cells specific to this age.

AUTHOR CONTRIBUTIONS

Wafaa Gaber: Methodology; visualization; writing – review and editing; formal analysis; conceptualization; validation; investigation; resources; project administration; software. **Fatma Khalil:** Methodology; software; investigation; writing – review and editing; validation; formal analysis; project administration; data curation. **Dalia Mohamedien:** Methodology; investigation; visualization; software; writing – review and editing; writing – original draft; data curation; formal analysis; validation; conceptualization; project administration.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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