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Detection of MUC1^{+}/MUC2 and MUC5AC^{-} Membrane-Associated Mucins in the Intraepithelial Surface Mucous Cells of the Developing Rabbit Esophagus

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Keywords
Bleb · Basal cell · Cell migration · Membrane-associated mucins · MUC1 · Non-goblet mucous cell

Abstract
Introduction: Mucins are polydisperse molecules created to perform a variety of functions at the mucosal surface of the adult gastrointestinal tract. Two main groups of mucins could be identified: the membrane-associated mucins (MUC1, MUC4, MUC13, and MUC16), those bound to the apical plasma membrane of epithelial cells, and the secreted mucins (MUC2, MUC5AC, MUC5B, and MUC6), those secreted from the goblet cells. Little is known about the types and distribution patterns of mucins in prenatal life. ***Methods:*** We detected mucin-secreting cells in the developing rabbit esophagus though these cells are absent in the adult one. In order to identify the content and possible functions of these cells, we investigated the histochemical and immunohistochemical characteristics of their mucins. ***Results:*** Starting at 16th day of pregnancy, periodic acid Schiff (PAS), alcian blue (AB) pH (2.5), and PAS-AB combination intensely stained the mucous content, demonstrating both acidic and neutral mucopolysaccharides. Some blebs could be recognized on the free surface of the esophageal epithelium. Also, the mucous cells and some basal cells strongly immunoreacted with MUC1, but not MUC2, nor MUC5AC antibodies. ***Conclusion:*** Collectively, these data suggest that surface mucous cells are modified epithelial cells, not goblet cells, and may originate from the basal layer of the epithelial cells. A possible regulatory role for these MUC1-positive mucins in esophageal epithelial and mesenchymal cell differentiation and late organogenesis is suggested. However, future functional studies are recommended.

Introduction

The mucosal surface of the gastrointestinal tract (GIT) is covered by a thick mucus layer that represents the first line of innate host defense. Mucus is composed of high-molecular-weight glycoproteins called mucins which are

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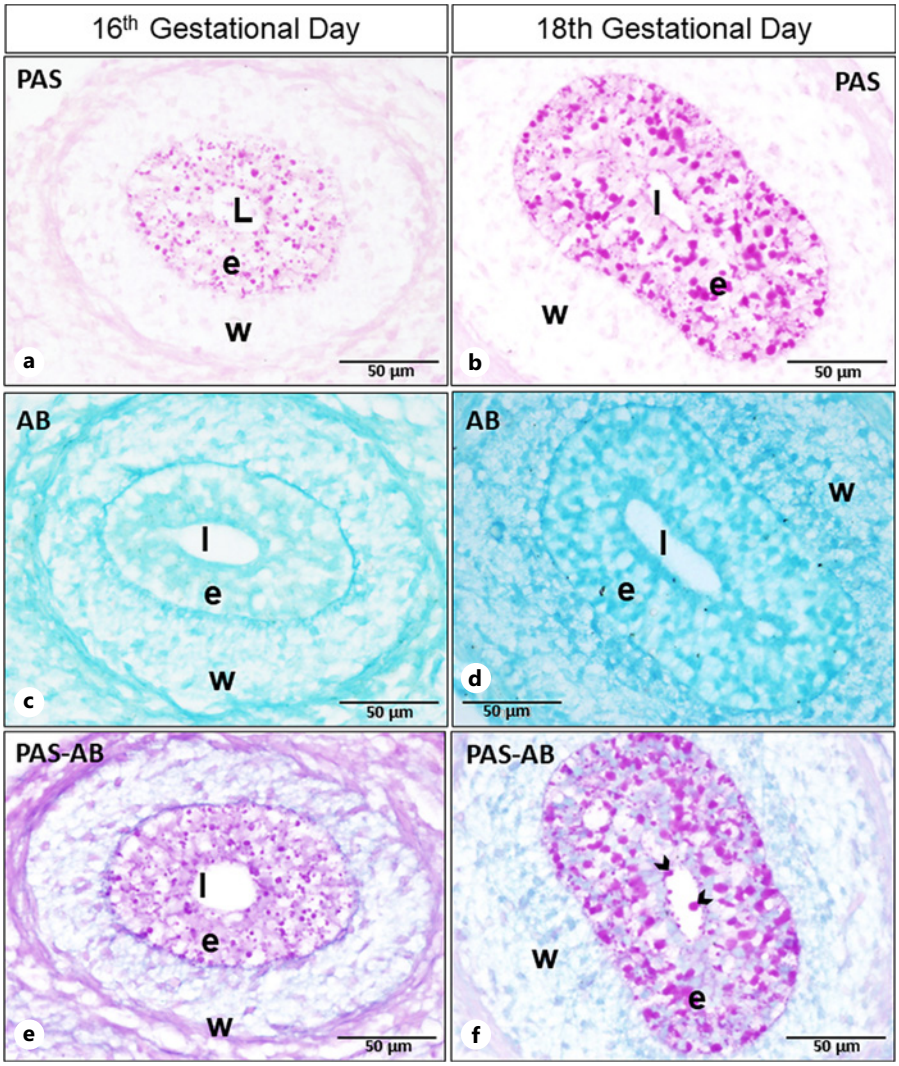

Fig. 1. Histochemical features of the rabbit esophageal wall of 16th and 18th days of gestation stained with periodic acid Schiff (PAS) (**a**, **b**), alcian blue (AB) (**c**, **d**), periodic acid Schiff-alcian blue combination (PAS-AB) (**e**, **f**) showing mucins reacted intensely with all stains and distributed within the whole epithelium (**e**). Notice the epithelial blebs (arrowheads) in **f**. l, esophageal lumen; w, esophageal wall.

produced and secreted by the goblet cells. The primary function of these mucins is to lubricate the epithelium and protect it from damage by aggressive and toxic elements [1].

Recently, goblet cells and mucins have been involved in several complex immune functions including antigen presentation and tolerance. When the body is functioning normally, goblet cells frequently produce mucins to replace and preserve the mucus barrier, but function of the goblet cell can be disrupted by numerous issues that may alter integrity of the mucus barrier [2].

Mucins are a class of polydisperse molecules; they are created to perform a variety of functions at the mucosal surface of the GIT. There are two main groups of mucins: membrane-bound or membrane-associated mucins (MAMs) (e.g., MUC1, MUC4, MUC13, and MUC16), those bound to the apical cell membrane of epithelial cells along the GIT, and the secreted mucins (MUC2, MUC5AC, MUC5B, and MUC6), those secreted from the mucous-secreting cells [3].

By contrast to secreted mucins that only have a protective effect on the mucosal surface, membrane-bound mucins or MAMs also play a role in signal transduction. The various activities of distinct mucins are related to their molecular structure. The molecular weight and size of the secreted mucins are quite large, they include a high percentage of o-glycosidically linked carbohydrates (typically between 50 and 80 percent), and they can create viscoelastic gels. MAMs are members of the same family and have many of these fundamental structural characteristics. They are monomeric, incorporated into membranes, and do not form gel [4].

Mucin 1 (MUC1) is a mucin-type glycoprotein, normally observed on the apical surfaces of epithelial cells

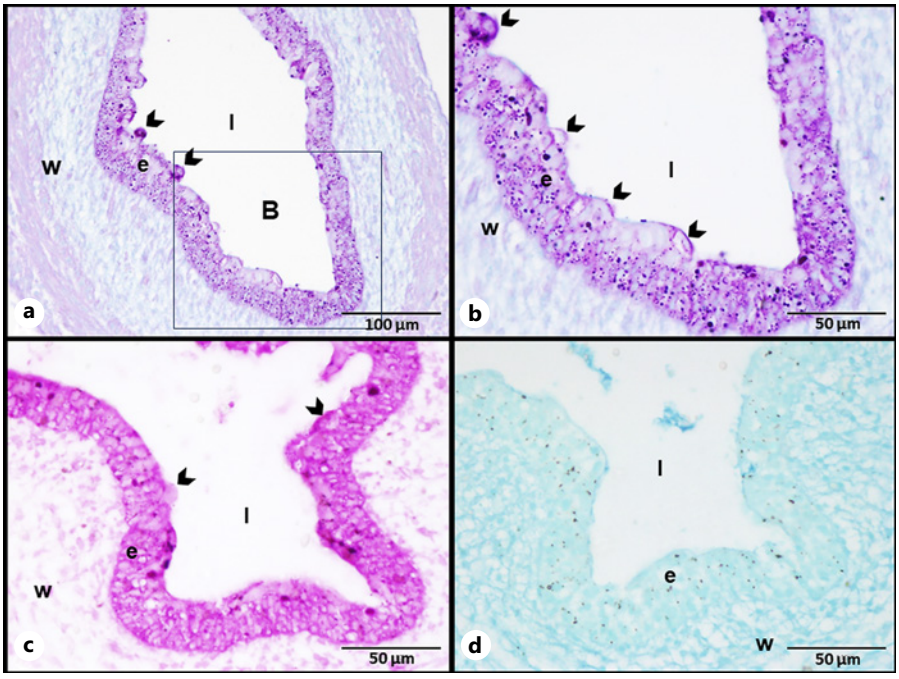

Fig. 2. Histochemical features of the rabbit esophageal wall of 22nd day of gestation stained with PAS-AB combination (**a**, **b**), PAS (**c**), or AB (**d**) showing mucins reacted intensely with all stains and distributed within the whole esophageal epithelium (**e**). Notice the PAS-AB-positive epithelial extensions or blebs (arrowheads) into the esophageal lumen. l esophageal lumen; w, esophageal wall.

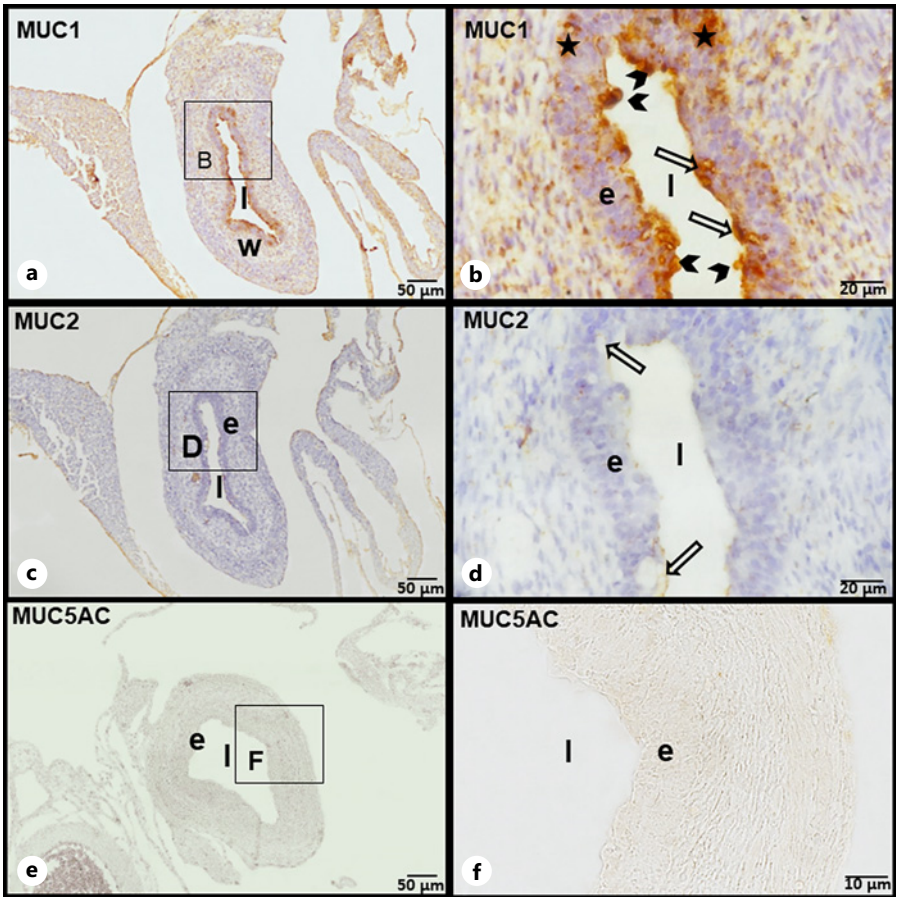

Fig. 3. The rabbit esophageal wall of 22nd day of gestation immunoassayed with MUC1 (**a**, **b**) MUC2 (**c**, **d**), or MUC5AC antibody (**e**, **f**). In (**a**, **b**), the MUC1 intensely stained intraepithelial mucous cells (arrows) were evident. Some basal epithelial cells (asterisks) were strongly labeled with MUC1. Notice the esophageal blebs (arrowheads) reacted strongly with MUC1 protein. In (**c–f**), no MUC2 or MUC5AC-immunoreactive signals could be detected in the esophageal components. e, esophageal epithelium; l, esophageal lumen; w, esophageal wall.

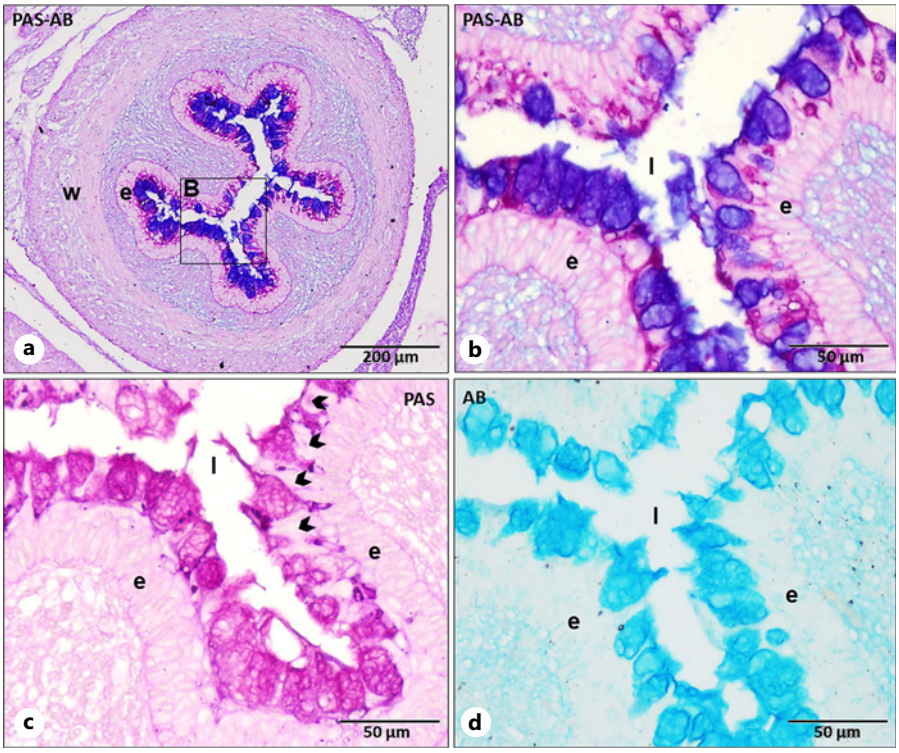

Fig. 4. Histochemical features of the rabbit esophageal wall of 24th day of gestation stained with PAS-AB combination (**a**, **b**), PAS (**c**), or AB (**d**) showing surface mucous cells with their mucins reacted intensely with all stains. The staining reaction became localized to the upper layers of the epithelium. Notice the PAS-negative/AB-positive mucous cells (arrowheads). e, esophageal epithelium; l, esophageal lumen.

and some other cell types (Hattrup et al., 2008). Mucin 1 is proposed to safeguard the epithelia from enzymatic digestion, microbial infection, and other irritants. During embryogenesis, MUC1 acts as a barrier to embryo implantation [5].

Mucin 2 (MUC2), a mucin glycoprotein, is located in the cytoplasm of goblet cells including small intestine, colon, bronchus, salivary gland, conjunctiva of the eye, but not in normal gastric mucosa [6], while mucin 5AC (MUC5AC) is a glycoprotein having numerous cysteine-rich domains in N- and C-terminal regions which is the key former of polymers, the essential feature for gel forming [7].

A protecting surface mucous gel barrier has been proposed in the normal rat and human esophagi [8, 9]. Small quantities of secretory mucin, with a diverse composition from that of salivary or gastric mucins, could be detected in normal human, pig, and rat esophagi [10].

Expression of mucins within the developing normal human fetal gastroesophageal junction has been demonstrated (gestational age 5–38 weeks) using periodic acid Schiff (PAS), alcian blue (AB) pH 2.5, combined AB-PAS, and combined AB-aldehyde fuchsin. Mucins could be detected in the esophageal epitheliocytes at 5–38 weeks of gestation. Both acidic and neutral mucins could be identified, though the neutral mucins were more frequent [11]. To study human gastric epithelial development, expression of MUC1, MUC2, MUC5AC, and MUC6 in the stomachs of 22 fetuses at 9–30 weeks' gestation has been recorded. The primitive gastric epithelial cells expressed MUC1 and later MUC5AC [12].

Various functional roles have been attributed to mucins in adult humans and other mammals [8–10]. However, little is known about the distribution and possible functional roles of mucins during development. Our previous investigation revealed the presence of numerous intraepithelial mucous cells in the developing rabbit esophagus [13, 14]; however, the nature and composition of these mucins need to be investigated. In the present work, the histochemical and immunohistochemical features of the surface mucous cells during prenatal life are to be clarified to provide information regarding cell-specific expression of mucin proteins and their relation to developmental patterns of esophageal epithelial cytodifferentiation.

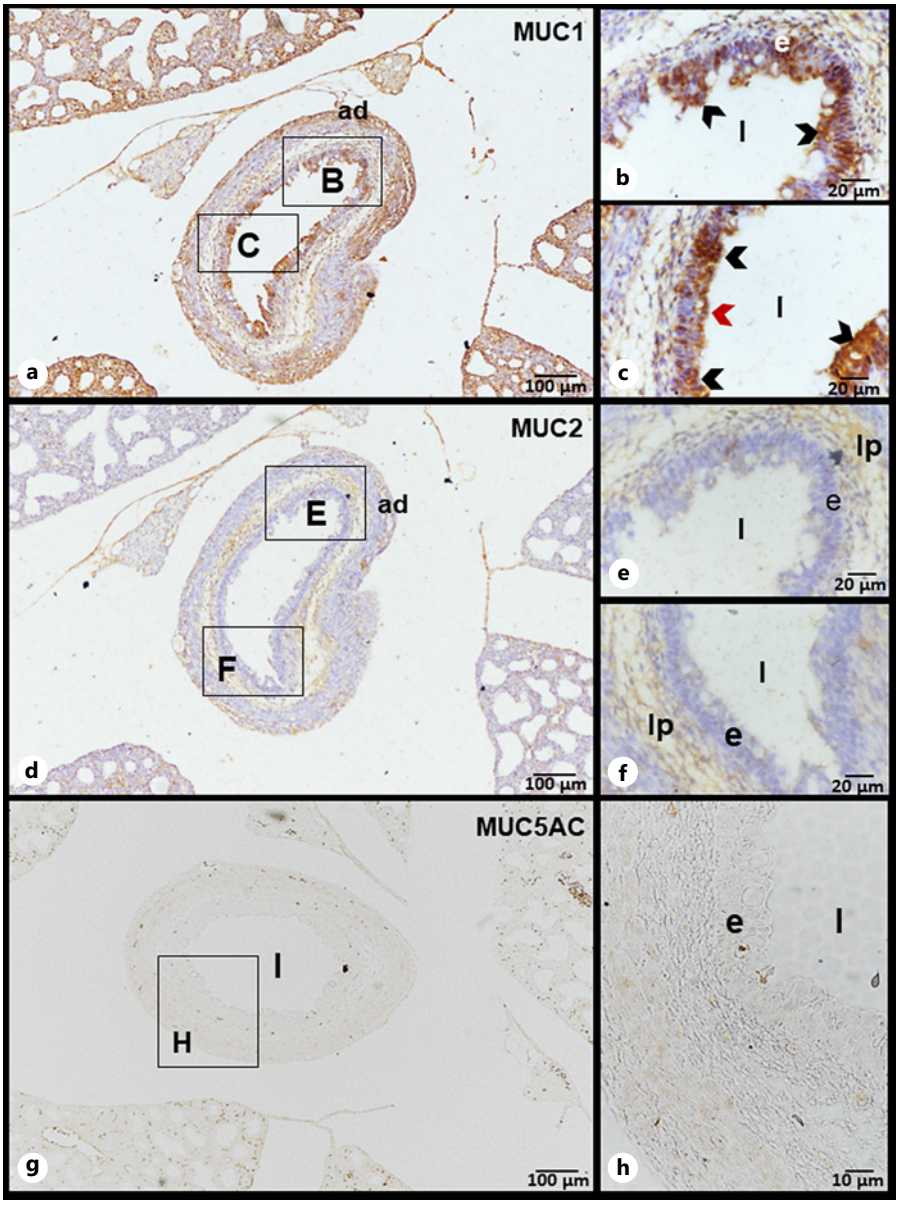

Fig. 5. The rabbit esophageal epithelium of 24th day of gestation immunoassayed with MUC1 (**a–c**), MUC2 (**d**–**f**), or MUC5AC (**g**, **h**). In (**a–c**), MUC1 antibody intensely reacted to intraepithelial mucous cells. Notice the patches of surface mucous cells and basal cells reacted strongly with MUC1 protein (black arrowheads). Red arrowhead indicates MUC1-positive surface cells and negative underlying basal cells. No immunoreactive signals for MUC2 or MUC5AC could be detected in the esophageal components. ad, adventitia; e, esophageal epithelium; l, esophageal lumen; lp, lamina propria.

Materials and Methods

Sample Collection

Healthy New Zealand white rabbit embryos and fetuses ($n = 21$) were used. They were collected shortly after evisceration the following gestational days: 16th, 18th, 22nd, 24th, 26th, 28th, and 30th, from the Research Farm of Faculty of Agriculture, South Valley University, Egypt.

Histochemical Analysis

Esophagi of the collected embryos and fetuses were fixed in 10% neutral buffered formalin for 24 h. Then they were dehydrated using different concentrations of ethyl alcohol (50%, 70%, 80%, 90%, 95%, and 100%). Samples were cleared using xylene and embedded in paraffin. Paraffin sections of 5–6 µm from the cervical and abdominal regions were stained with PAS to elucidate the neutral mucopolysaccharide content of the surface mucous cells [15]. AB pH (2.5) was used to identify the acid mucopolysaccharide content [16]. Additionally, PAS-AB combination was performed [17].

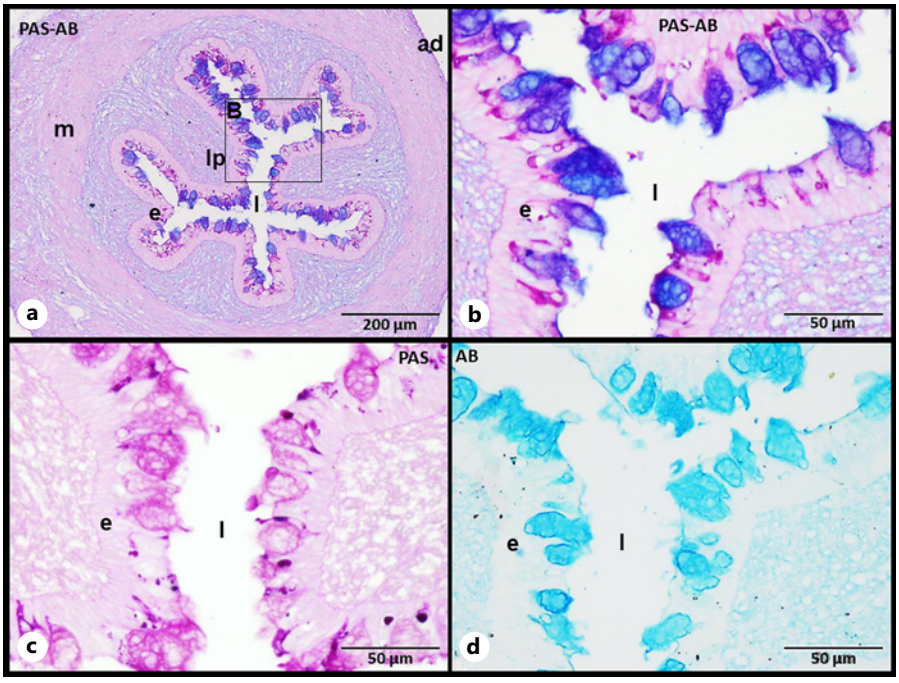

Fig. 6. Histochemical features of the rabbit esophageal wall of 26th day of gestation stained with PAS-AB combination (**a**, **b**), PAS (**c**), or AB (**d**) showing fewer surface mucous cells with their mucins reacted intensely with all stains. The staining reaction became more localized to the upper layers of the epithelium. ad, adventitia; e, esophageal epithelium; l, esophageal lumen; lp, lamina propria; m, muscularis.

MUC1, MUC2, and MUC5AC Immunostaining

Some formalin-fixed, paraffin-embedded sections (4–6 µm thick) were selected for immunohistochemical analysis from the thoracic esophagus of 22nd, 24th, 26th, 28th,' and 30th gestational days according to [18]. In brief, heat-mediated antigen retrieval was performed by the treatment of the tissue sections with 10 mM sodium citrate, 0.05% Tween 20 (pH 9.0) for 10 min at 121°C. Inhibition of endogenous peroxidase was performed by immersing the sections in 3% H_2O_2/methanol. The sections were incubated overnight at 4°C with rabbit MUC1 monoclonal antibody (ABclonal, A19081) or rabbit MUC2 monoclonal antibody (A4767) or polyclonal rabbit MUC5AC (Sigma-Aldrich, HPA040615) according to the avidin-biotin peroxidase complex method. Counterstaining was done with Mayer's hematoxylin. Later, the stained tissues were examined, and images were recorded under a Leica microscope (Germany).

Results

Starting at the middle gestation period, the epithelium lining the embryonic esophagus of the rabbit comprised four to 5 cell layers. Histochemical analysis of the esophageal epithelium of New Zealand white rabbit at the 16th and 18th gestational days revealed the existence of both neutral and acidic mucopolysaccharides. Strong PAS-AB staining reaction spots were distributed among the whole lining epithelium and not restricted to the surface cells (Fig. 1). The intensity of staining was higher at the 18th day than that of the 16th day of gestation. The basement membrane of the esophageal epithelium strongly reacted with AB (Fig. 1c, d). In some sections of the 18th day of pregnancy, two to four PAS-positive vesicular outgrowths of a plasma membrane of the surface epithelial cells (blebs) could be identified (Fig. 1f).

On the 22nd day of gestation, the esophageal epithelium developed to ciliated stratified columnar type where some of the basal cells show basally located nuclei and some of the luminal cells become ciliated columnar. At this age, the mucosal folds could be observed for the first time. They were four in number, with wide dorsal and ventral folds as well as narrow right and left ones. Using PAS-AB combination revealed the PAS-AB darkly stained spots became noticeable and distributed in the different epithelial cells. The PAS-AB-positive blebs became more prominent (Fig. 2). Immunohistochemically, MUC1 protein was strongly detected in some singly positioned surface cells together with the luminal surface and the epithelial blebs of the esophageal epithelium (Fig. 3a, b). Interestingly, some basal cells were intensely labeled with MUC1 antibody. On the other hand, MUC2 (Fig. 3c, d) and MUC5AC immunostaining did not yield any immunoreactivities in the esophageal epithelium.

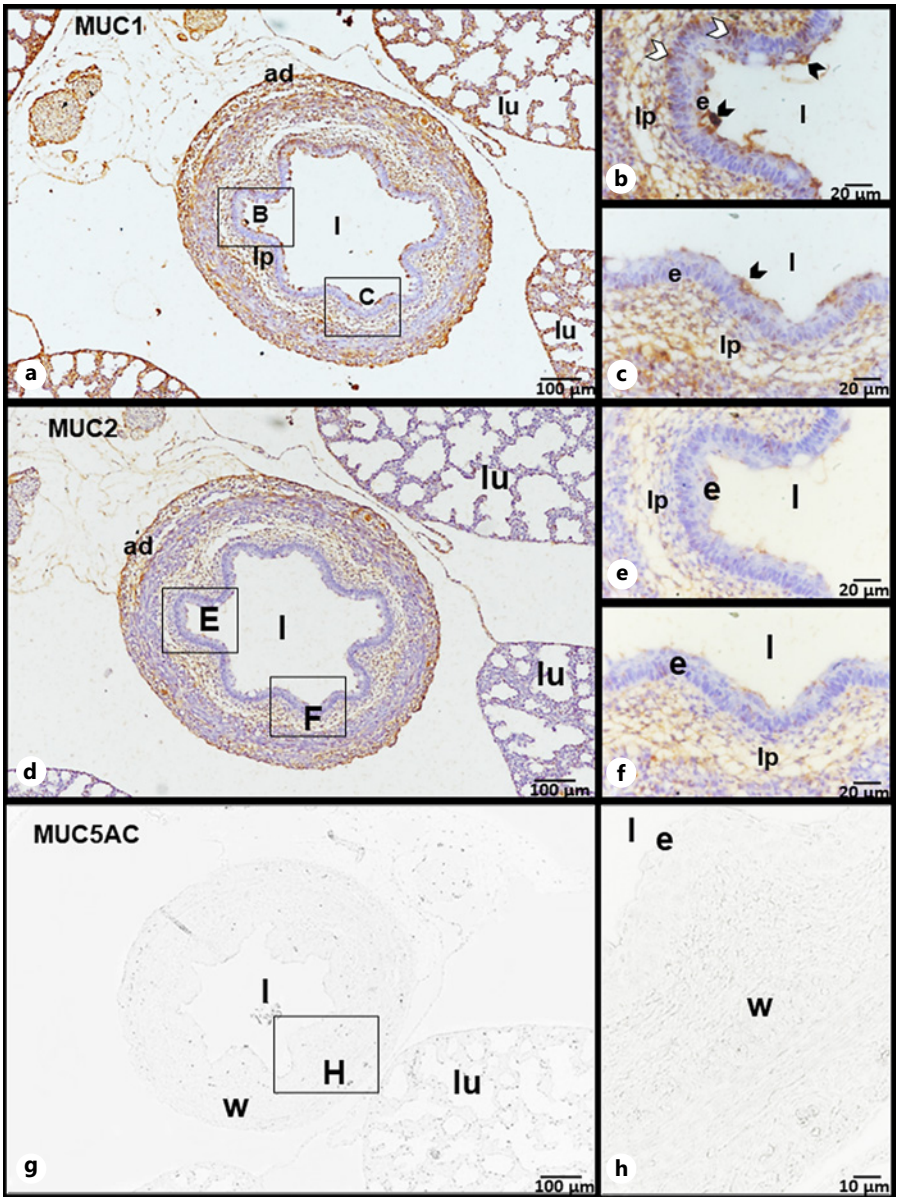

Fig. 7. The rabbit esophageal wall of 26th day of gestation immunoassayed with MUC1 (**a–c**), MUC2 (**d–f**), or MUC5AC (**g**, **h**). In (**a–c**), MUC1 antibody reacted to intraepithelial mucous cells (black arrowheads). Few basal cells in (**b**) reacted with MUC1 protein (white arrowheads). No immunoreactive signals for MUC2 or MUC5AC antibody could be detected in the esophageal epithelium. ad, adventitia; e, esophageal epithelium; l, esophageal lumen; lp, lamina propria; lu, lung; w, esophageal wall.

On the 24th gestational day, most of the luminal cells of the epithelium became mucus-secreting cells with columnar cells in between. The mucosal folds increased in number to five. In the thoracic part, they were similar in length and width, resulting in a wide star-shaped lumen, while in the abdominal part the folds were dissimilar; some folds increased in length and width, resulting in a narrow irregular-shaped lumen. Numerous PAS-AB intensely stained surface cells could be easily identified. The positive surface cells formed a continuous layer surrounding the esophageal lumen. The staining became restricted to the upper row of the lining epithelial cells. Some PAS-negative/AB-positive mucous cells could be demonstrated (Fig. 4). Immunostaining of MUC1 antibody verified intense immunoreactive signals distributed in all layers of epithelial cells lining the esophageal lumen (Fig. 5a–c). While MUC2 and MUC5AC proteins could not be detected in the esophageal epithelium, MUC2-positive signals could be detected in the lamina propria and adventitia of the rabbit esophagus (Fig. 5d–h).

On the 26th day of pregnancy, the luminal folds increased in number to about six and the distribution of the positive cells in both histochemical and immunohistochemical examinations was like the previous age, although the staining intensity was much lower than that age. The staining reaction for PAS-AB, PAS, or AB

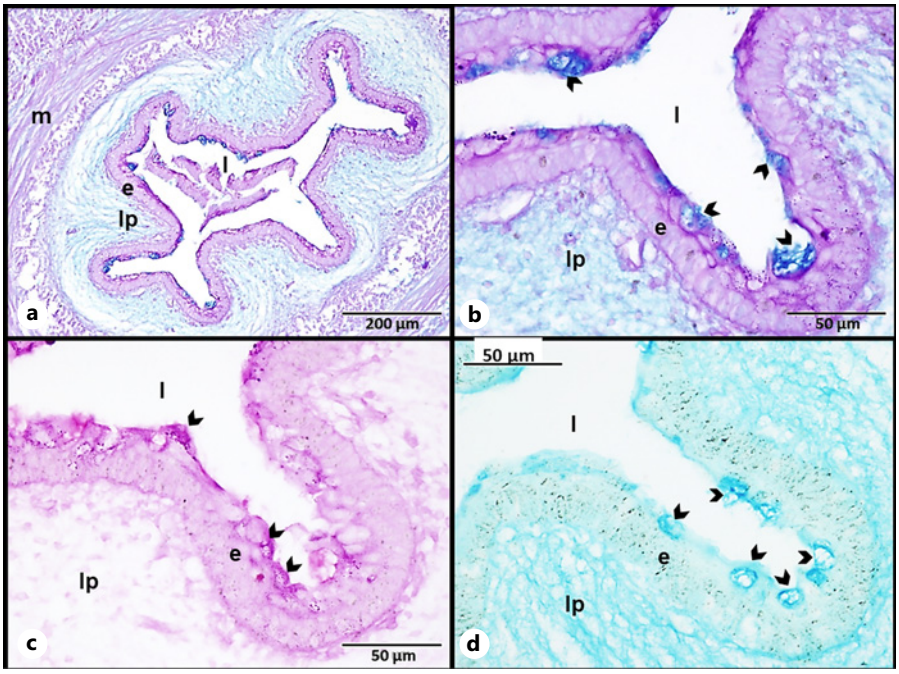

Fig. 8. Histochemical features of the rabbit esophageal wall of 28th day of gestation stained with PAS-AB combination (**a**, **b**), PAS (**c**), or AB (**d**) showing fewer surface mucous cells with their mucins reacted intensely with all stains (arrowheads). The staining reaction became more localized to the surface epithelium. e, esophageal epithelium; l, esophageal lumen; lp, lamina propria; m, muscularis.

became localized to the upper layers of the esophageal epithelium (Fig. 6). Fewer MUC1 immunoreactive signals could be detected in the surface mucous cells and some basal cells. Stronger MUC2 immunoreactive signals could be identified in the lamina propria, muscularis, and adventitia. MUC5AC staining did not yield any positive signals in the esophageal wall (Fig. 7). On the 28th day, the intensity of staining for the histochemical and immunohistochemical detections was similar to the previous age (Fig. 8, 9).

On the 30th gestational day, the PAS-positive mucins formed a continuous sheath surrounding the esophageal lumen; however, the AB-stained mucins were sporadic. The subepithelial continuous PAS-positive membrane was prominent (Fig. 10). The lumen of the esophagus became wide, irregular, and free from any content. It had about six folds of varying thickness and length. At the end of gestation period, the esophagus was lined by non-keratinized stratified squamous epithelium throughout its course. The basal layer was columnar with basally located nuclei, followed by 2–4 layers of polyhedral cells. The superficial layer was composed of a single layer of squamous cells. The mucus-secreting cells were rarely observed within the epithelium. For MUC1 immunodetection, the number of MUC1-positive cells greatly diminished. The immunoreactive signals disappeared from the surface cells; however, few basal cells reported expressing MUC1-positive signals. MUC2 and MUc5AC did not yield any positive signals in the esophageal epithelium; however, both markers revealed positive signals in the other layers of the esophageal wall. For the first time during development, MUC5AC strongly immunoassayed the lamina propria, muscularis, and adventitia of the rabbit esophageal wall (Fig. 11). MUC1, MUC2, and MUC5AC staining intensities in the developing esophageal wall are summarized in Table 1.

Discussion

The nature, type, and origin of the mucins at the surface intraepithelial non-goblet mucous cells surrounding the esophageal lumen of the white New Zealand rabbits during their prenatal life could not be identified so far. In this study, we have studied the expression of neutral and acidic mucins together with the protein expression of MUC1, MUC2, and MUC5AC in 21 rabbit fetuses at mid to late gestational stages, in order to establish their developmental pattern of distribution and staining affinity and to compare this with adult esophagus.

Previously, we reported the distribution of intraepithelial mucous-secreting cells in the uppermost layer of the esophageal epithelium of the prenatal developing rabbit. The existence of these cells and their secretory activity had been confirmed by transmission electron

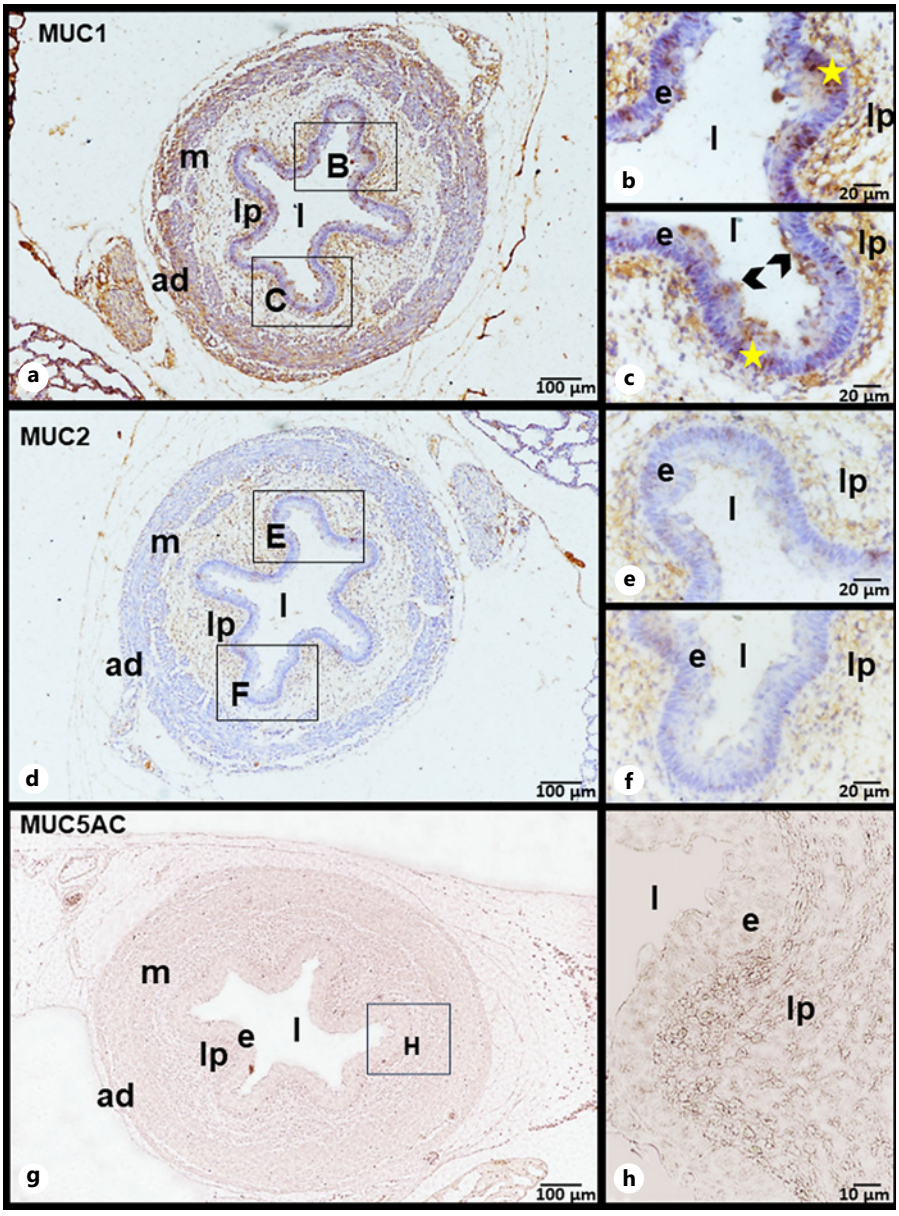

Fig. 9. The rabbit esophageal wall of 28th day of gestation immunoassayed with MUC1 (**a–c**), MUC2 (**d–f**), or MUC5AC (**g**, **h**). In (**a–c**), MUC1 antibody reacted to intraepithelial mucous cells (black arrowheads). Few basal cells in (**b**, **c**) reacted with MUC1 protein (asterisks). In (**d–f**), no immunoreactive signals for MUC2 or MUC5AC antibody could be detected in the esophageal epithelium. ad, adventitia; e, esophageal epithelium; l, esophageal lumen; lp, lamina propria; m, muscularis.

microscopy. Morphometric analysis revealed that the highest number of these cells was detected on the 24th gestational day [13]. On the 16th and 18th gestational days, PAS-AB positive spots were identified distributed in all epithelial cells of the developing esophageal mucosa, indicating the neutral and acidic nature of the secreted mucins.

On the 22nd embryonic day, distribution of the acidic and neutral mucopolysaccharides was the same as the previous age. Immunohistochemically, MUC1 immunoreactive signals were distributed similarly in the mucin-secreting cells and some basal cells at the 22nd gestational day. The staining intensity increased at the 24th day and then diminished from the 26th day till the end of gestation. Moreover, basal cells and blebs of the free surface epithelial cells showed MUC1 immunopositivity. This suggests a functional role of MUC1 protein in the development and differentiation of the developing esophageal epithelial cells during various developmental ages. This confirms the belonging of these mucous secretions to the MAMs and the non-goblet nature of these secreting cells. The stained basal cells could be the origin of these mucin-secreting cells.

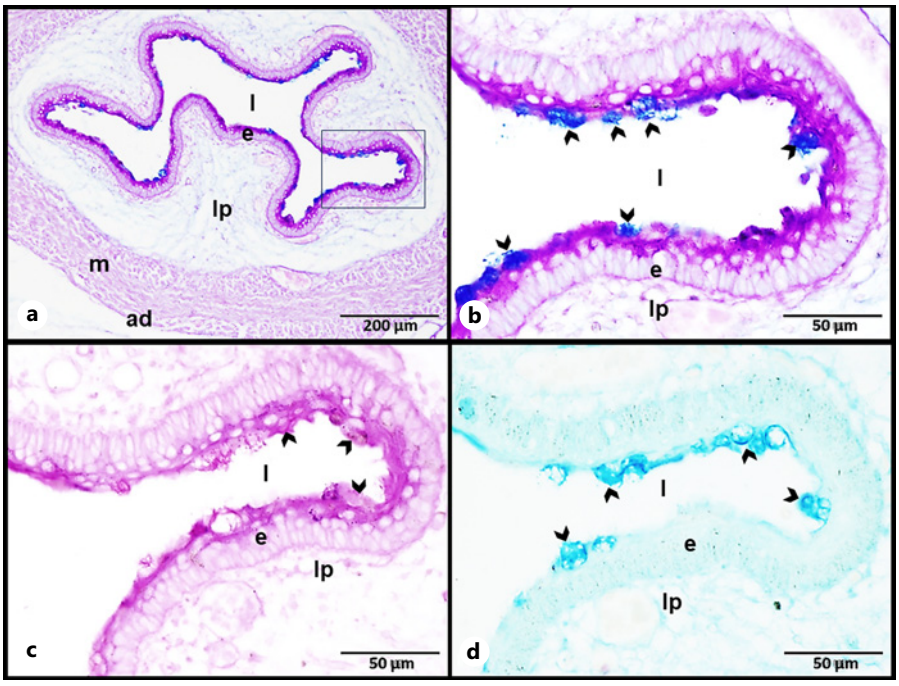

Fig. 10. Histochemical features of the rabbit esophageal epithelium of 30th day of gestation stained with PAS-AB combination (**a**, **b**), PAS (**c**), or AB (**d**) showing fewer surface mucous cells with their mucins reacted intensely with all stains (arrowheads). The staining reaction became more localized to the surface epithelium. ad, adventitia; e, esophageal epithelium; l, esophageal lumen; lp, lamina propria; m, muscularis.

On the other hand, absence of any immunoreactive signals for MUC2 and MUC5AC verifies the lack of any functional role of these proteins in the esophageal epithelial development [6, 7]. Evidence for MAMs' participation in epithelial mucosal development at the ocular surface and apical epithelial cell architecture was provided by [18]. In the human fetus, MUC1 revealed weak homogenous signals from 8 weeks gestation in the gall bladder, and from 18 weeks gestation in the colon, while it was not detected in the developing esophagus.

On the 24th day of gestation, most of the luminal cells of the epithelium are mucus-secreting cells releasing both acidic and neutral mucopolysaccharides. These cells are modified columnar cells containing neutral and acid mucopolysaccharides as it gave a strong positive reaction with PAS and AB stains.

Blebs are membrane structures created when the plasma membrane detaches from the underlying actin cytoskeleton. The blebs could be verified for the first time at the 18th day of gestation, and they reacted intensely with PAS-AB stain and MUC1 antibody. Their number and size increased greatly on the 22nd and 24th gestational days, but it decreased gradually from the 26th day till it disappeared by the end of gestation. This may suggest the amoeboid migration of the mucous-secreting cells. The migrating amoeboid cells generally form extensions termed blebs in conjunction with development, tissue stability, and immunity monitoring. Defective migration may lead to pathological conditions, including cancer metastasis and chronic inflammation. Inflation of these spherical protrusions and pushing their membranes forward depend on the actomyosin contraction [20].

MUC1 could be detected earlier in the lamina propria of the 22nd gestational day and continued till the end of gestation than MUC2 protein which was detected from the 24th gestational day onward. In the muscularis, MUC1 was identified from the 22nd gestational day and continued till the end of gestation. On the other hand, MUC2 could be faintly identified at the 26th and 30th gestational days. Earlier expression of MUC1 in the lamina propria may indicate a role for MUC1 in the early cellular differentiation of the lamina propria and muscularis during pregnancy.

Structurally the adult rabbit esophageal wall is lined by stratified squamous with a tendency of keratinization, lamina propria, muscularis mucosae, submucosa which is devoid of glands, muscularis, and adventitia. PAS stain revealed strong reaction with the inner layer of mucosa and moderate reaction with the lamina propria [21, 22]. However, surface mucins have not been recorded. Absence of these surface mucins in adult rabbit esophagus indicates their involvement into the esophageal development and cell differentiation during gestation.

In our study, the secretion of neutral and acid mucopolysaccharides suggests a secretory role of the fetal esophageal epithelium. The reason for appearance and

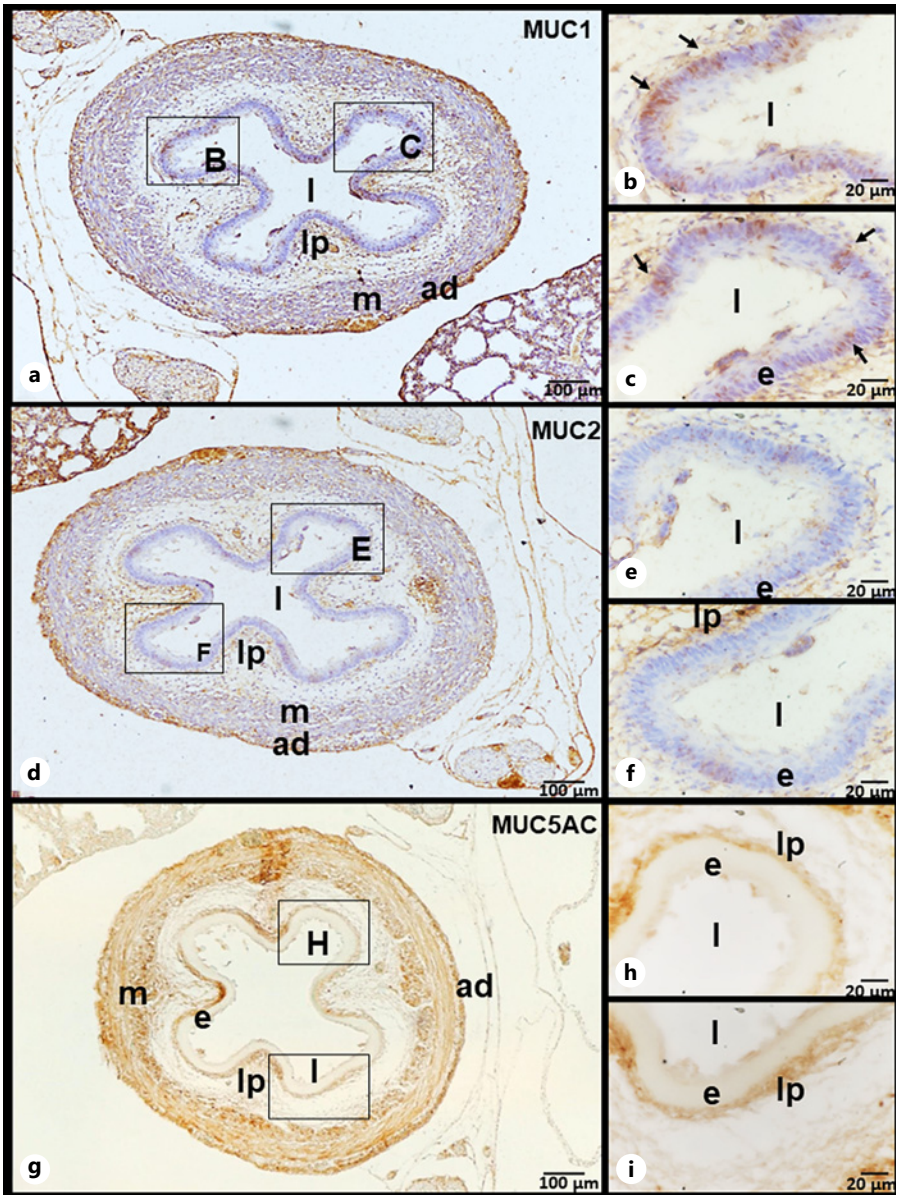

Fig. 11. The rabbit esophageal wall of 30th day of gestation immunoassayed with MUC1 (**a**–**c**), MUC2 (**d**–**f**), or MUC5AC (**g**–**i**). In (**a**–**c**), MUC1 antibody reacted to intraepithelial mucous cells (black arrowheads). Few basal cells in (**b**, **c**) reacted with MUC1 protein (asterisks). No immunoreactive signals for MUC2 or MUC5AC antibody could be detected in the esophageal epithelium. ad, adventitia; e, esophageal epithelium; l, esophageal lumen; lp, lamina propria; m, muscularis.

Table 1. Expression of MUC1, MUC2, and MUC5AC in the developing rabbit esophagus

	Epithelium			Lamina propria			Muscularis			Adventitia		
	M1	M2	M5AC	M1	M2	M5AC	M1	M2	M5AC	M1	M2	M5AC
22nd day	++	–	–	+	–	–	+	–	–	+	–	–
24th day	++	–	–	+	+	–	+	–	–	++	+	–
26th day	+	–	–	++	+	–	++	+/–	–	++	+	–
28th day	++ (loc.)	–	–	++	+	–	++	–	–	++	–	–
30th day	++ (loc.)	–	–	+	+	++	+	+/–	++	++	++	++

–, negative staining; +/–, faint staining; +, moderate staining; ++, intense staining; loc., the expression was localized in some cells.

disappearance of the mucus-secreting cells in the embryonic esophagus is unknown. We suggest that these cells are ancestral of those present in lower vertebrates like fish [23], amphibians [24], and some reptiles such as American alligator [25], Nile monitor [26], and snakes [27].

In conclusion, a possible regulatory role for MUC1 protein in esophageal epithelial cell differentiation, mesenchymal cell organization, and late organogenesis is suggested. However, future functional studies are recommended. The present data are limited by the small number of fetuses utilized in developmental studies and shortage of early embryos, and the apparent inconsistent nature of mucin protein expression within fetuses of similar gestation. Further work will be required to clearly define the changes in mucin protein expression during embryonic and fetal development. In particular, data relating to mucin product present within developing fetal upper GIT is absent.

Acknowledgment

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Statement of Ethics

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

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Author Contributions

Dalia Mohamedien: methodology and writing – original draft preparation. Wafaa Gaber: conceptualization and methodology. Makoto Hirayama: writing – review and editing. Mahmoud Awad: writing, visualization, data presentation, and review.

Data Availability Statement

The data presented in this study are available within the article. Further inquiries can be directed to the corresponding author.

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