

RESEARCH ARTICLE

Prenatal development and histochemical characteristics of gastrointestinal mucins in sheep fetuses

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Abstract

The object of this study was to describe the prenatal development and histochemical properties of mucins in the sheep gastrointestinal tract. To determine changes in the mucin profile, the sections were stained with specific histochemical stains for carbohydrates. While neutral and mixed mucins were observed in the superficial epithelial cells of the abomasal pyloric region, acidic mucins were detected in the secretory ducts and corpus of the glands. Acidic mucins consisted predominantly of sialomucins. In the duodenal villi, the number of goblet cells containing neutral mucins increased toward the end of gestation, whereas Brunner's glands contained acidic mucins until the 95th day of gestation and both acidic and neutral mucins thereafter. The jejunal goblet cells contained either acidic, neutral, or mixed mucins. Goblet cells containing acidic mucins, which were mainly localized to the ileal crypts and villi, mostly contained sulfated mucins. While villi were observed in the proximal colon until the 115th day of gestation, later the typical crypt structure emerged. During the period in which the villi were found in the proximal colon, the goblet cells containing sulphomucins were predominant, whereas the goblet cells containing sialomucins were predominant after the typical crypt structure was formed. In conclusion, gastrointestinal mucins may be involved in the formation of meconium during the prenatal period, and acidic mucins may contribute to the strength of the intestinal barrier against pathogens and digestive enzymes, as the barrier is not fully functional after birth.

KEYWORDS

fetus, gastrointestinal, goblet cell, mucin, sheep

1 | INTRODUCTION

The gastrointestinal tract is the largest body surface, which is in continuous contact with the external environment. It not only contains everything we swallow and eat, but also harbors a huge colony of bacteria in the intestines. Potential hazards are not all exogenous as potentially dangerous molecules such as bile salts, digestive enzymes and hydrochloric acid are secreted into the gastrointestinal tract (Pelaseyed et al., 2014). The functional integrity of the gastrointestinal mucosa mainly depends on the regular mucus layers, intercellular tight junctions, host innate, and adaptive immune responses and epithelial cells (Deplancke & Gaskins, 2001; Kim & Ho, 2010; Pelaseyed et al., 2014). Mucus is essential to the protection of the epithelial layers from self-digestion (Graziani et al., 2016; Schade, Flemström, & Holm, 1994). Mucus also

constitutes a microenvironment for microflora, allowing a biofilm to form on the mucosal surface, which maintains intestinal homeostasis (Corfield & Shukla, 2003; Peterson & Artis, 2014; Probert & Gibson, 2002). Mucus content continuously changes with different secretions and cells sloughed from the epithelium (Atuma, Strugala, Allen, & Holm, 2001). The physiological and physical characteristics of the mucus barrier are mainly determined by highly glycosylated high-molecular-weight proteins, referred to as mucins. Histochemically, mucin can be classified as neutral or as acidic when its nonreducing terminal is modified by sialic acid or a sulfate group, which results in an overall negative charge (Brockhausen, 2003). Neutral mucins predominate in the gastric mucosa (Deplancke & Gaskins, 2001), whereas acidic mucins are present in the entire intestinal epithelium and predominate in the large intestine (Deplancke & Gaskins, 2001). To date, more than 20 mucin-encoding

TABLE 1 Proportion of staining epithelial and goblet cells in the gastrointestinal tract mucosa of the sheep fetuses during the various gestational ages obtained with traditional histochemistry

Gastrointestinal tract	Stains	Gestational ages		
		70–100	101–120	121–136
Abomasum	AB -PAS	AB ++++ PAS ++++ Mixed +	AB ++++ PAS +++ Mixed ++	AB ++++ PAS ++ Mixed ++
	HID-AB	HID + AB +++++ Mixed +	HID + AB +++++ Mixed +	HID + AB +++++ Mixed +
	α -amylase -PAS	++	++	++
Duodenum	AB -PAS	AB ++++ PAS +++ Mixed ++	AB +++ PAS +++ Mixed ++	AB +++ PAS +++ Mixed ++
	HID-AB	HID ++ AB +++ Mixed ++	HID +++ AB +++ Mixed ++	HID +++ AB +++ Mixed ++
	α -amylase -PAS	++	++	++
Jejunum	AB -PAS	AB ++ PAS ++++ Mixed ++	AB ++ PAS +++ Mixed ++	AB ++ PAS ++ Mixed ++
	HID-AB	HID ++++ AB + Mixed +	HID +++++ AB + Mixed +	HID +++++ AB + Mixed +
	α -amylase -PAS	+	+	+
Ileum	AB -PAS	AB ++ PAS ++++ Mixed +	AB +++ PAS +++ Mixed ++	AB +++ PAS +++ Mixed ++
	HID-AB	HID +++ AB +++ Mixed ++	HID +++ AB ++ Mixed ++	HID ++++ AB ++ Mixed +
	α -amylase -PAS	++	++	++
Proximal colon	AB -PAS	AB ++ PAS ++++ Mixed +	AB +++ PAS +++ Mixed +	AB ++++ PAS ++ Mixed +
	HID-AB	HID +++ AB ++ Mixed ++	HID ++ AB +++ Mixed ++	HID ++ AB +++ Mixed ++
	α -amylase -PAS	++	++	++

Proportion of staining epithelial and goblet cells was scored on a scale of – (– = no cells with staining, + = 0% to 10% of cells with staining; ++ = 11% to 25%; +++ = 26% to 50% of cells with staining; ++++ = 51% to 75% of cells with staining; +++++ = 76% to 100% of cells with staining) to +++++. AB-PAS: Alcian Blue- Periodic Acid Schiff, HID-AB: High Iron Diamine-Alcian Blue, α -amylase-PAS: Periodic Acid Schiff.

(MUC) genes have been determined in humans and a few homologous MUC genes have been identified in other animal species (Corfield, 2015). Mucins are classified as secreted gel forming mucins, secreted nongel forming mucins and membrane-bound mucins (Boonzaier, Van der Merwe, Bennett, & Kotzé, 2013). Among the secreted mucins, the main gel-forming molecules are MUC2, MUC5AC, MUC5B, and MUC6 (Lesuffleur, Zweibaum, & Real, 1994) and MUC2 is the predominant mucin in the small and large intestines (Holmén Larsson, Karlsson, Sjövall, & Hansson, 2009). Membrane-bound mucins are found in glyco-calyx in the apical surface of the gastrointestinal epithelium (Hatrup & Gendler, 2008). While intestinal mucins are mainly secreted by goblet cells, membrane-bound and secreted mucins are produced by intestinal

epithelial cells (Atuma et al., 2001). Fresh mucus is always produced by goblet cells, in particular in the crypt openings (Ermund, Schütte, Johansson, Gustafsson, & Hansson, 2013). Mucus produced in the crypts mixes with the secretion of Paneth cells, which contains antibacterial peptides, lysozyme, DMBT1, and MUC2 (Pelaseyed et al., 2014). Mucin distribution in different part of the gastrointestinal tract varies with anatomical region, cell type, pathology, and species (Scillitani, Zizza, Liquori, & Ferri, 2007). Types of mucin found in the gastrointestinal tract have been histochemically described in several mammals (Filipe, 1979; Forman, 1971; Karakoç, Sağsöz, & Ketani, 2016; Kotzé & Coetzee, 1994; Krause, 2000; Schumacher, Duku, Katoh, Jörns, & Krause, 2004; Sheahan & Jervis, 1976). Information available on the prenatal distribution of mucins in the

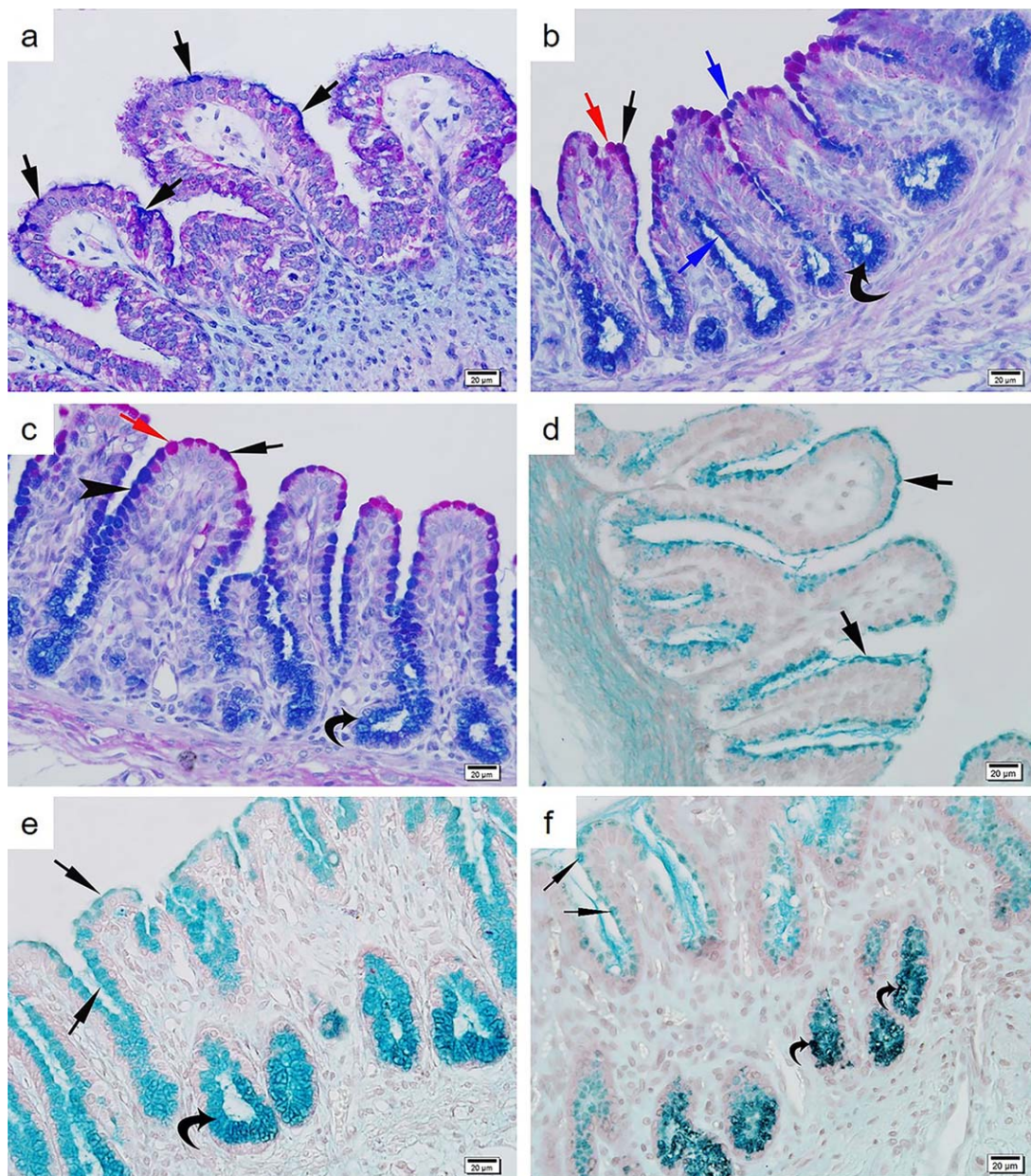


FIGURE 1 The histochemical distribution of gastric mucins in the pars pylorica of the abomasum during the prenatal development of sheep fetuses. (a) 70-day-old fetal lamb, superficial cells containing acidic mucins (arrows), AB-PAS pH 2.5 staining. Bar; 20 μ m. (b) 115-day-old fetal lamb, superficial cells containing neutral (red arrow), acidic (blue arrows) and mixed (black arrow) mucins, gastric gland cells containing acidic mucins (curved arrow), AB-PAS pH 2.5 staining. Bar; 20 μ m. (c) 136-day-old fetal lamb, superficial cells containing neutral (red arrow) and mixed (black arrow) mucins, superficial glandular epithelial cells containing acidic mucins in the opposing surfaces of the gastric foveola (arrow head) and cells of the corpus glandula containing acidic mucins (curved arrow), AB-PAS pH 2.5 staining. Bar; 20 μ m. (d) 70-day-old fetal lamb, superficial cells containing sialomucins (arrows). HID-AB pH 2.5 staining. Bar; 20 μ m. (e) 105-day-old fetal lamb, superficial cells containing sialomucins (arrows) and cells of the corpus glandula containing sialomucins (curved arrow), HID-AB pH 2.5 staining. Bar; 20 μ m. (f) 120-day-old fetal lamb, superficial cells containing sialomucins (arrows) and cells of the corpus glandula containing sulfomucins (curved arrows), HID-AB pH 2.5 staining. Bar; 20 μ m [Color figure can be viewed at wileyonlinelibrary.com]

mammalian gastrointestinal tract is scarce (Beyaz & Liman, 2009; de la Hoz, Eyheramendy, Felipe, & Díaz, 2016; Felipe, Callejas, & Cabodevila, 1998; Tano de la Hoz, Flamini, & Díaz, 2014). To the best of knowledge, the presence and distribution of mucins in the gastrointestinal tract of sheep fetuses has not been investigated before. Therefore, the purpose of this study was to draw a map of prenatal mucin secretion by the different epithelial cell types in different

parts of the gastrointestinal tract in sheep fetuses by applying variety of histochemical techniques.

2 | MATERIALS AND METHODS

Twenty clinically healthy Akkaraman sheep fetuses of varying gestational age, from 70 to 136 days, were obtained from slaughterhouses

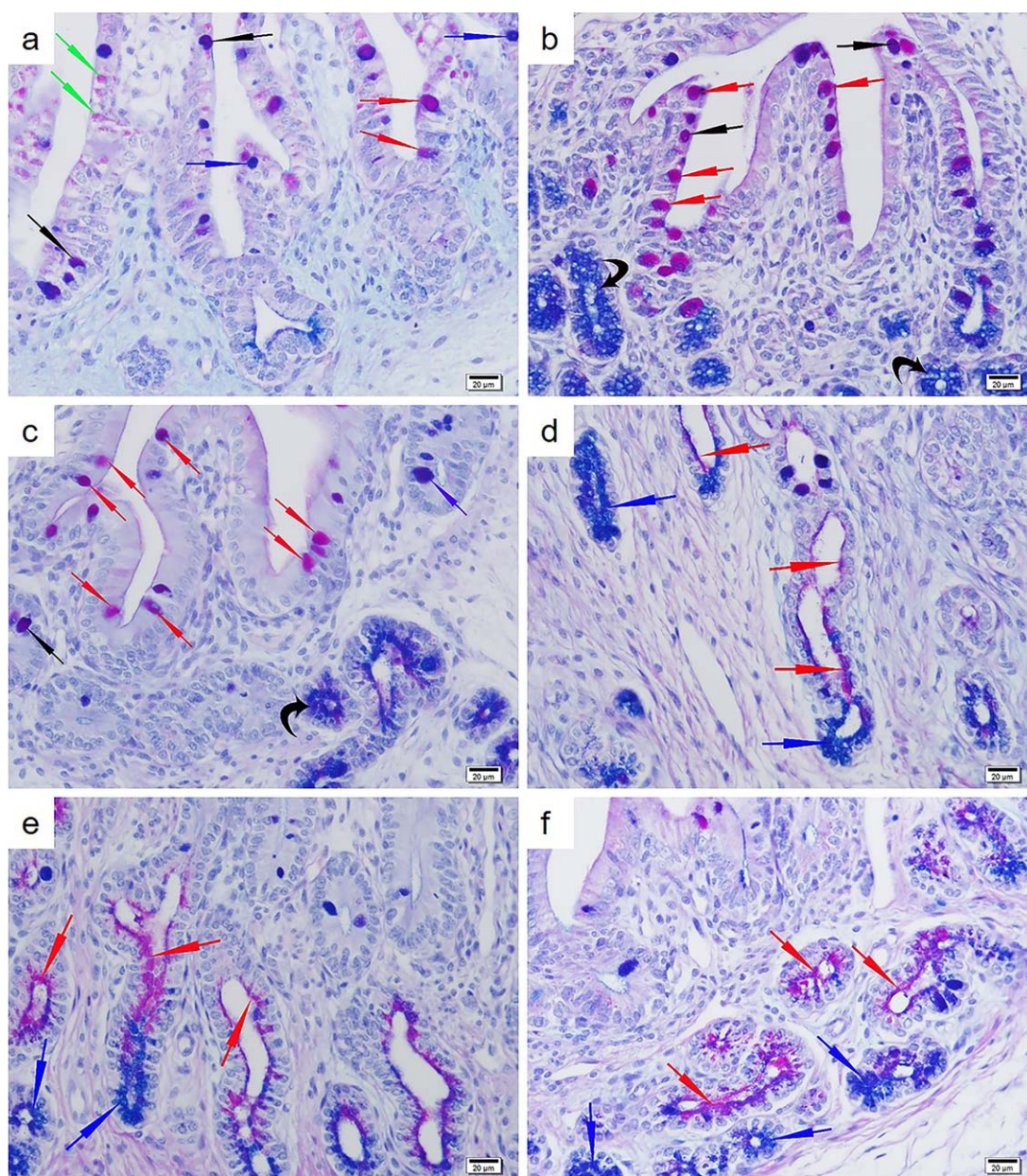


FIGURE 2 The histochemical distribution of duodenal mucins during the prenatal development of sheep fetuses. (a) 70-day-old fetal lamb, goblet cells containing neutral (red arrows), acidic (blue arrows) and mixed (black arrows) mucins in the villi and crypts, glycogen accumulation in epithelial cells in the villi (green arrows), AB-PAS pH 2.5 staining. Bar; 20 μ m. (b) 105-day-old fetal lamb, increased number of goblet cells containing neutral (red arrows) mucins in the villi, goblet cells containing acidic mixed (black arrows) mucins, Brunner's glands containing acidic mucins (curved arrows), AB-PAS pH 2.5 staining. Bar; 20 μ m. (c) 130-day-old fetal lamb, goblet cells containing neutral (red arrows), acidic (blue arrow) and mixed (black arrow) mucins, Brunner's glands containing acidic mucins (curved arrow), AB-PAS pH 2.5 staining. Bar; 20 μ m. (d) 115-day-old fetal lamb, Brunner's glands containing acidic mucins (blue arrows), Brunner's glands containing neutral mucins (red arrows), AB-PAS pH 2.5 staining. Bar; 20 μ m. (e) 120-day-old fetal lamb, Brunner's glands containing acidic mucins (blue arrows), Brunner's glands containing neutral mucins (red arrows), AB-PAS pH 2.5. Bar; 20 μ m. (f) 126-day-old fetal lamb, Brunner's glands containing acidic mucins (blue arrows), Brunner's glands containing neutral mucins (red arrows), AB-PAS pH 2.5. Bar; 20 μ m [Color figure can be viewed at wileyonlinelibrary.com]

in Ankara and its surrounding villages. Of the animals included in this study, 7 were of a gestational age ranging from 70 to 100 days, 8 from 100 to 120 days, and 5 from 120 to 136 days. The distribution of ages studied was as follows: 70 days ($n = 1$), 74 days ($n = 1$), 84 days ($n = 1$), 88 days ($n = 1$), 90 days ($n = 1$), 95 days ($n = 1$), 100 days ($n = 1$), 105 days ($n = 1$), 107 days ($n = 1$), 111 days ($n = 1$), 112 days ($n = 2$), 115

days ($n = 1$), 118 days ($n = 1$), 120 days ($n = 1$), 122 days ($n = 1$), 124 days ($n = 1$), 126 days ($n = 1$), 130 days ($n = 1$), and 136 days ($n = 1$). The age of the fetuses was calculated using Richardson's equation: $x = 2.1 * (17 + y)$, where "x" is the age of the fetus in days, and "y" is the distance between the crown and rump in centimeters (CRL) (Richardson, Hebert, & Terlecki, 1976).

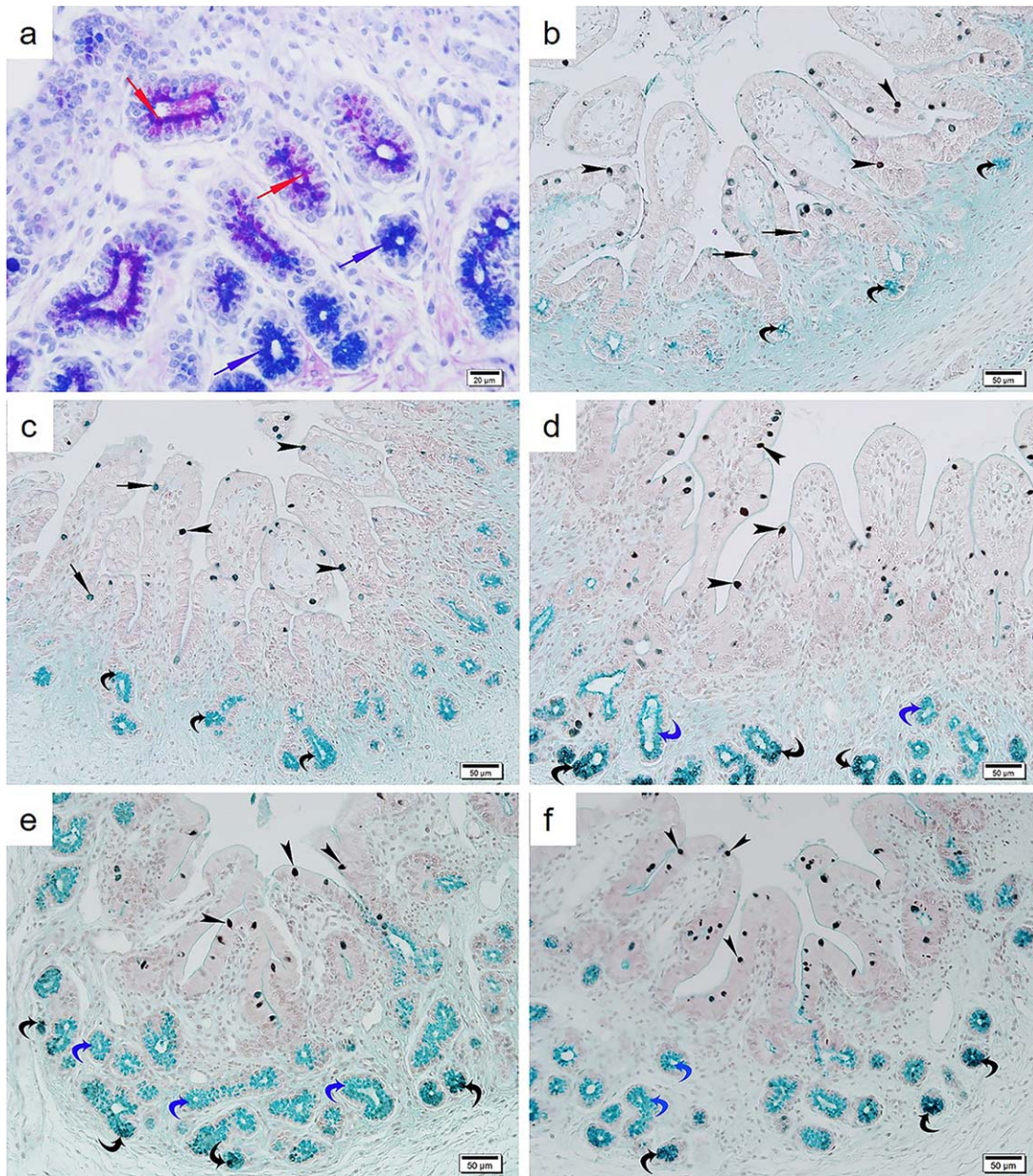


FIGURE 3 The histochemical distribution of duodenal mucins during the prenatal development of sheep fetuses. (a) 130-day-old fetal lamb, Brunner's glands containing acidic mucins (blue arrows), Brunner's glands containing neutral mucins (red arrows), AB-PAS pH 2.5. Bar; 20 μ m. (b) 70-day-old fetal lamb, goblet cells containing sialomucins (arrows) and sulfomucins (arrowheads) in the villi, epithelial cells of the Brunner's glands containing sialomucins (curved arrow), HID-AB pH 2.5 staining. Bar; 50 μ m. (c) 88-day-old fetal lamb, goblet cells containing sialomucins (arrows) and sulfomucins (arrowheads) in the villi and crypts, epithelial cells of the Brunner's glands containing sialomucins (curved arrows), HID-AB pH 2.5 staining. Bar; 50 μ m. (d) 115-day-old fetal lamb, increased number of goblet cells containing sulfomucins (arrowheads) in the villi, epithelial cells containing sulfomucins in the Brunner's glands (black curved arrows), epithelial cells containing sialomucins in the Brunner's glands (blue curved arrows), HID-AB pH 2.5 staining. Bar; 50 μ m. (e) 120-day-old fetal lamb, goblet cells containing sulfomucins (arrowheads) in the villi, epithelial cells containing sulfomucins in the Brunner's glands (black curved arrows), epithelial cells containing sialomucins in the Brunner's glands (blue curved arrows), HID-AB pH 2.5 staining. Bar; 50 μ m. (f) 130-day-old fetal lamb, goblet cells containing sulfomucins (arrowheads) in the villi, epithelial cells containing sulfomucins in the Brunner's glands (black curved arrows), epithelial cells containing sialomucins in the Brunner's glands (blue curved arrows), HID-AB pH 2.5 staining. Bar; 50 μ m [Color figure can be viewed at wileyonlinelibrary.com]

Fetal tissue samples were taken from the pyloric region of the abomasum, duodenum, jejunum, terminal ileum, and proximal colon. The samples were fixed in formol-alcohol solution for 18 hr. Fixed tissues were embedded in paraffin using routine histological procedures. Five-

μ m-thick sections were cut and mounted on microscopic slides. Various staining procedures were applied to the sections for the histochemical description of mucins. The periodic acid-Schiff (PAS) staining method was employed for the detection of glycogen, epithelial sulfomucins,

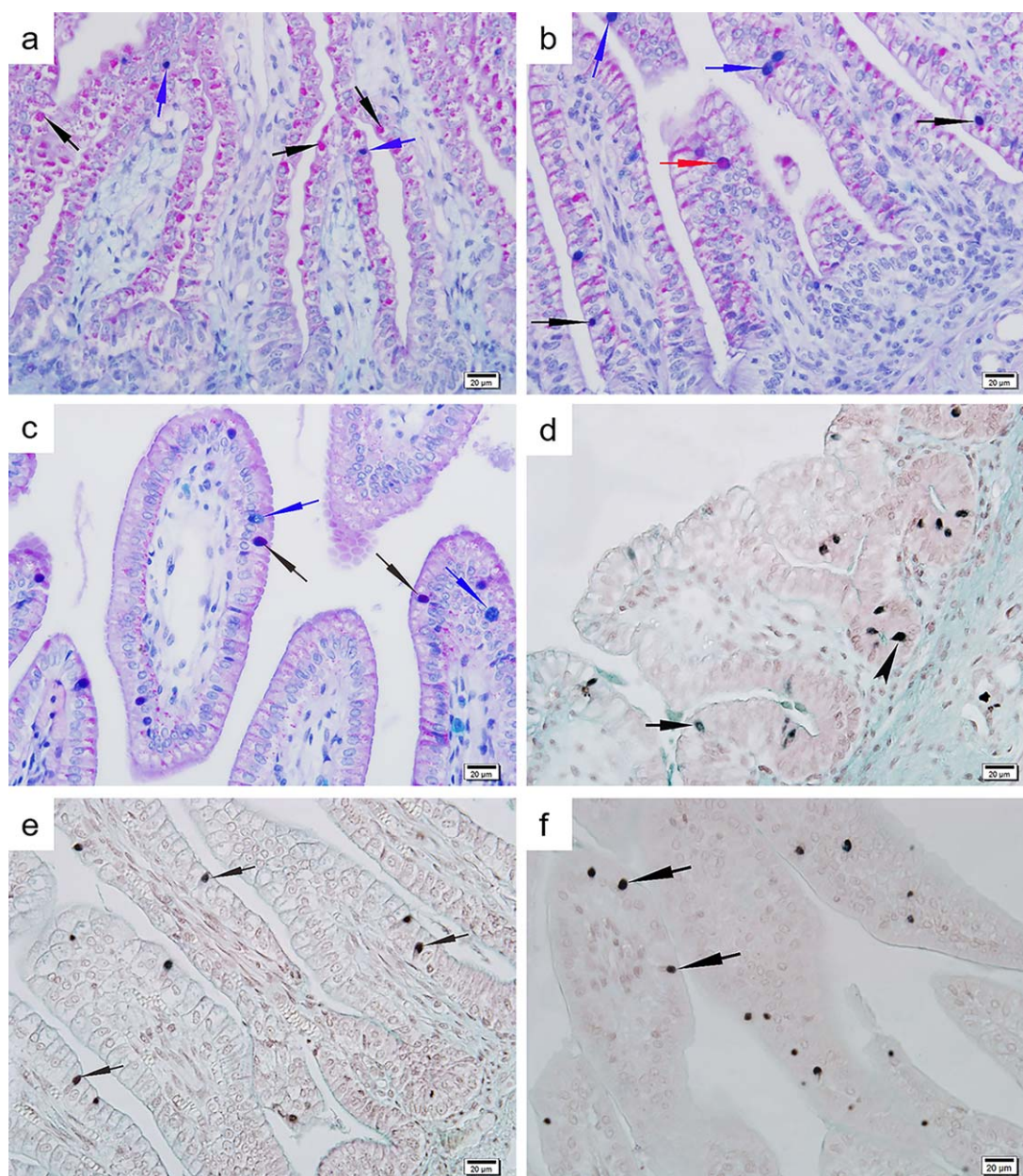


FIGURE 4 The histochemical distribution of jejunal mucins during the prenatal development of sheep fetuses. (a) 73-day-old fetal lamb, goblet cells containing acidic mucins in the villi (blue arrows), glycogen accumulation in the epithelial cells (black arrows), AB-PAS pH 2.5 staining. Bar; 20 μ m. (b) 95 day-old fetal lamb, goblet cells containing acidic (blue arrows), neutral (red arrow) and mixed (black arrows) mucins in the villi, AB-PAS pH 2.5 staining. Bar; 20- μ m. (c) 130-day-old fetal lamb, goblet cells containing acidic (blue arrows) and mixed (black arrows) mucins in the villi, AB- PAS pH 2.5 staining. Bar; 20 μ m. (d) 84-day-old fetal lamb, goblet cells containing sialomucins (arrow) in the villi, goblet cells containing sulfomucins in the crypts (arrowhead), HID-AB pH 2.5 staining. Bar; 20 μ m. (e) 115-day-old fetal lamb, goblet cells containing sulfomucins (arrows) in the villi, HID-AB pH 2.5 staining. Bar; 20 μ m. (f) 126-day-old fetal lamb, goblet cells containing sulfomucins (arrows) in the villi, HID-AB pH 2.5 staining. Bar; 20 μ m [Color figure can be viewed at wileyonlinelibrary.com]

neutral mucins, vicinal diols and N-acetyl sialomucins (Bancroft & Cook, 1994). The α -amylase-PAS staining protocol was employed to distinguish glycogen from other PAS-positive elements, such as mucins that could be present in the tissue sample (Spicer, Horn, & Leppi, 1967). Alcian Blue (AB)-PAS (pH: 2.5) combined staining (Mowry, 1956) was performed for the distinction of neutral and acidic mucins and high iron

diamine (HID)-AB (pH: 2.5) staining was performed for the detection of sulfo- and sialomucins (Spicer, 1965).

Furthermore, the presence of mucin core proteins (MUC) within the gastrointestinal tract of sheep fetuses during the prenatal period was determined by immunohistochemistry. Because specific antibodies targeted to ovine MUCs are not available, the MUC1 (Santa Cruz

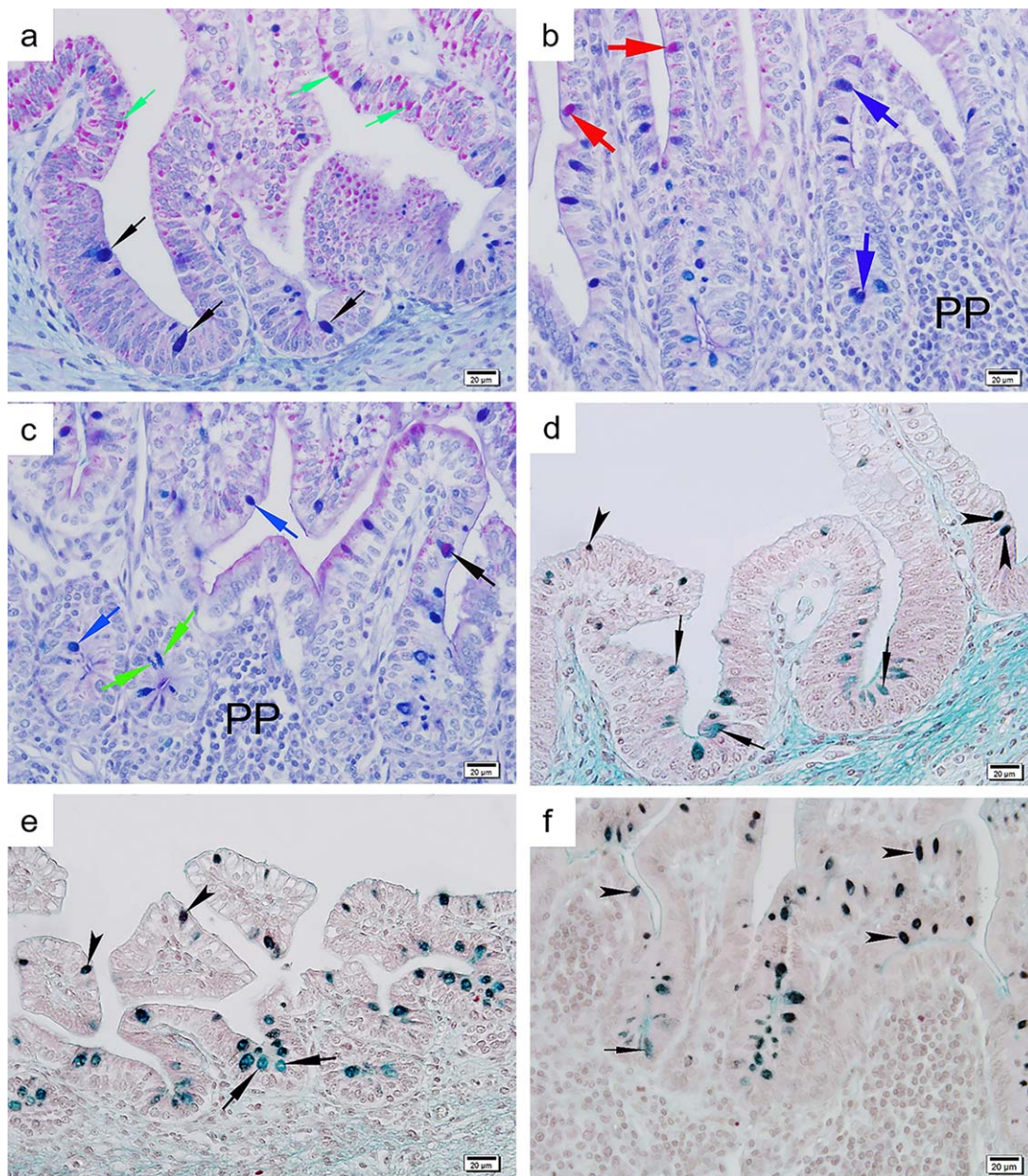


FIGURE 5 The histochemical distribution of ileal mucins during the prenatal development of sheep fetuses. (a) 70-day-old fetal lamb, goblet cells containing acidic (blue arrows) mucins in the villi and crypts, glycogen accumulation in the epithelial cells (green arrows) AB-PAS pH 2.5 staining. Bar; 20 μ m. (b) 126-day-old fetal lamb, goblet cells containing neutral (red arrows) and acidic (blue arrows) mucins, PP (Peyer's patches), AB-PAS pH 2.5 staining. Bar; 20 μ m. (c) 130-day-old fetal lamb, goblet cells containing acidic (blue arrows) and mixed (black arrow) mucins, Paneth cells containing granules (green arrows) positive for Alcian blue, PP (Peyer's patches), AB-PAS pH 2.5 staining. Bar; 20 μ m. (d) 70-day-old fetal lamb, goblet cells containing sialomucins (arrows) and sulfomucins (arrowheads) in the villi and crypts, HID-AB pH 2.5 staining. Bar; 20 μ m. (e) 105-day-old fetal lamb, goblet cells containing sialomucins (arrows) and sulfomucins (arrowheads) in the villi and crypts, HID-AB pH 2.5 staining. Bar; 20 μ m. (f) 130-day-old fetal lamb, goblet cells containing sialomucins (arrows) and sulfomucins (arrowheads) in the villi and crypts, HID-AB pH 2.5 staining. Bar; 20 μ m [Color figure can be viewed at wileyonlinelibrary.com]

Biotechnology, sc-53381), MUC2 (Santa Cruz Biotechnology, sc-73146), MUC5AC (Santa Cruz Biotechnology, sc-33667), and MUC6 (Santa Cruz Biotechnology, sc-33668) antibodies recommended for detection of Mucin 1, Mucin 2, Mucin 5AC, and Mucin 6 of mouse, rat and human origin were used in this study. They are unknown if they are reactive for sheep tissues. Four slides were prepared from each sample, and each slide contained a minimum of three sections cut at 5

μ m thickness. After the processes of blocking endogenous peroxidase activity and antigen retrieval, the sections were washed in PBS (pH 7.4), incubated in blocking serum (Ultra V Block; TA-125UB; Thermo Fisher Scientific Lab Vision Corporation) for 5 min at room temperature to block nonspecific binding, and then left in the corresponding primary antibodies overnight at 4°C. Next, the slides were washed in PBS, and then treated with biotinylated anti-mouse secondary antibodies

(Thermo Fisher Scientific Fremont, USA) for 20 min. Next, the slides were incubated with enzyme-conjugated streptavidin (Thermo Fisher Scientific Fremont, USA) for 20 min. Immunoreactions were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Thermo Fisher

Scientific Fremont, USA) and Gill's hematoxylin was used to counter-stain the sections. The negative control sections were stained with the same procedures except the replacement of the primary antibodies with nonspecific mouse IgG (sc-2025, Santa Cruz Biotechnology, Santa

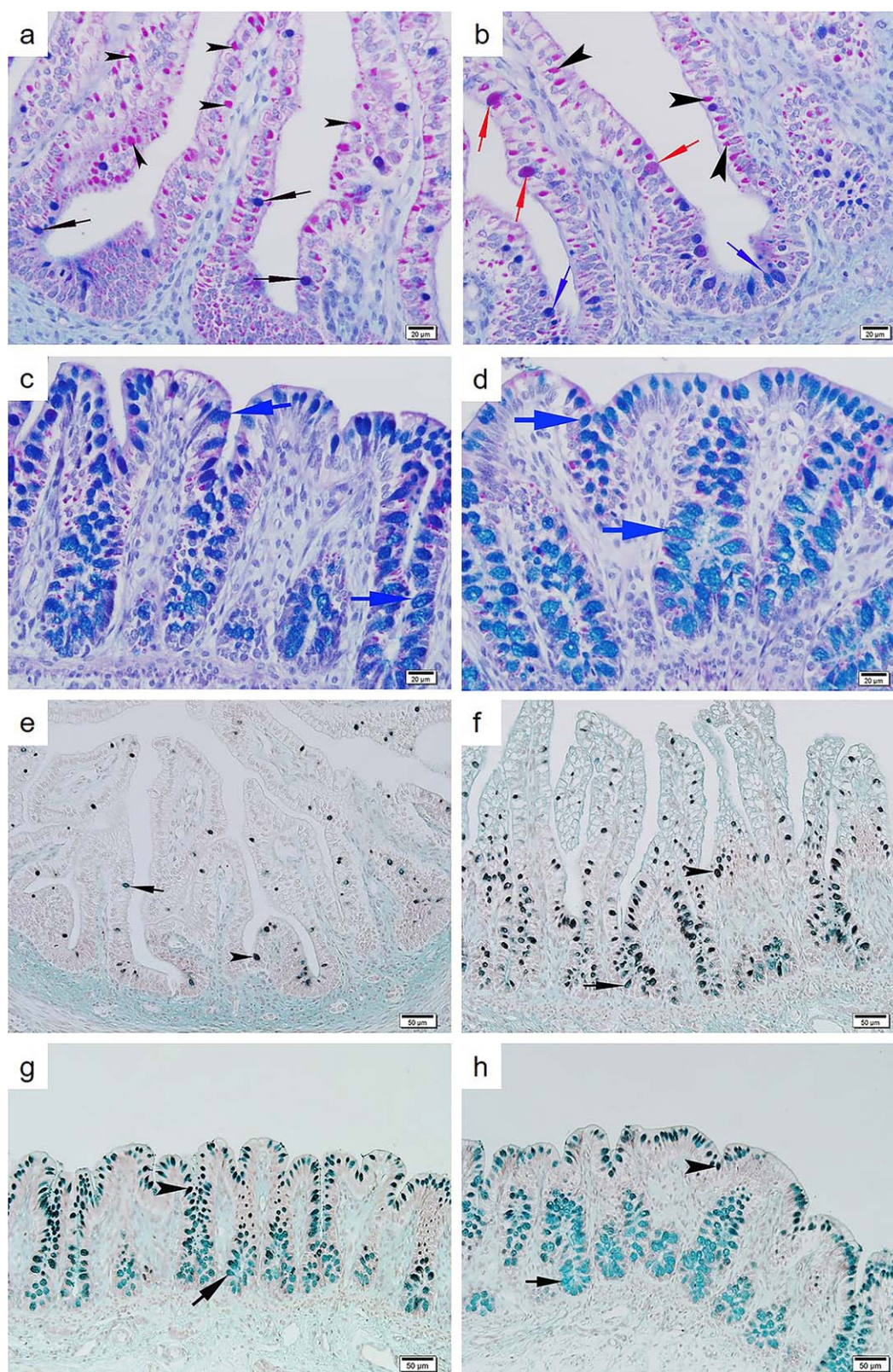


FIGURE 6.

Cruz, CA). Human colon and rabbit ileum slides served as positive controls for MUC-2, while the sections obtained from archival blocks of mammary gland and human colon used as positive controls for MUC6, MUC5AC, and MUC-1.

Tissue sections were investigated by light microscopy and assessed for both histochemical and immunohistochemical staining. The slides were photographed using a digital camera (Olympus DP72) attached to an Olympus BX51 microscope. This study was approved by the Committee for Animal Care and Use of Ankara University (2014-04-07).

In this study, the histochemical staining was semiquantitatively evaluated using a score (proportional score, PS) based on the percentage of positively stained cells as previously reported by Alan and Liman (Alan & Liman, 2010). Two independent observers performed all histochemical scoring. Proportion of staining epithelial and goblet cells was scored on a scale of - to +++++ (- = no cells with staining; + = 0% to 10% of cells with staining; ++ = 11% to 25%; +++ = 26% to 50% of cells with staining; ++++ = 51% to 75% of cells with staining; +++++ = 76% to 100% of cells with staining) (Table 1).

3 | RESULTS

3.1 | Histochemistry

The results of histochemical staining reactions are summarized in Table 1. In the present study, in slides that were employed combined staining with AB-PAS (pH 2.5), blue regions demonstrated the presence of acidic mucins, a magenta color indicated the presence of neutral mucins and purple colored regions showed the existence of neutral and acidic mucins together. To investigate the presence of sialomucins and sulfomucins, the sections were double stained with HID-AB (pH 2.5), where black or brown regions demonstrated the presence of sulfomucins and green or blue areas indicated the presence of sialomucins. Furthermore, α -amylase-PAS staining was used to differentiate glycogen from other PAS-positive elements such as mucins.

3.1.1 | AB-PAS (pH 2.5) and HID-AB (pH 2.5) stainings

The pyloric region of the abomasum

On the 70th day, combined AB-PAS (pH 2.5) staining revealed that while acidic mucins were found only in the apical cytoplasm of the superficial glandular epithelial cells, since the glandular corpora have

not formed yet (Figure 1a). As of the 115th day, while some of the superficial cells contained neutral mucins and some mixed mucins, the secretory ducts of the glands and the glandular corpus contained only acidic mucins (Figure 1b). On the 122nd day, it was observed that the superficial cells contained mainly neutral and mixed mucins. On the 130th day, the superficial glandular epithelial cells of the proximal foveola gastrica contained mostly neutral mucins while those in the opposing foveolar surfaces and those lining the glandular corpus contained acidic mucins (Figure 1c). Throughout the prenatal period, the cells lining the glandular corpus contained only acidic mucins (Figure 1b,c).

Combined HID-AB pH 2.5 staining revealed that, between days 70 and 100, the superficial epithelial cells and the cells lining the glandular corpus contained only sialomucins (Figure 1d,e). Following this period, while some of the cells lining the glandular corpus contained sulfated mucins, the majority contained primarily sialomucins (Figure 1f).

Duodenum

Combined AB-PAS (pH 2.5) staining revealed that neutral, acidic and mixed mucin-secreting goblet cells were present in both the duodenal crypts and villi on the 70th day of gestation (Figure 2a). The distribution of neutral mucin-secreting goblet cells gradually increased from the 105th day of gestation until parturition (Figure 2b). Following this period, goblet cells secreting neutral mucins predominated in the duodenal villi and crypts (Figure 2c). In the Brunner's glands, only acidic mucins were present until the 115th day of gestation, whereas neutral mucins also appeared thereafter (Figures 2d-f & 3a).

HID-AB (pH 2.5) staining demonstrated that, from the 70th day of gestation until birth, the goblet cells in the duodenal crypts and villi contained mostly sulfomucins. Brunner's glands contained only sialomucins until the 115th day of gestation, while some glands were determined to contain sulfomucins as well as sialomucins thereafter (Figure 3b-f).

Jejunum

Combined AB-PAS (pH 2.5) staining showed that the number of goblet cells in the jejunum was relatively lower than that in the other intestinal segments. Nevertheless, goblet cells secreting neutral, acidic and mixed mucins were present in the jejunal crypts and villi throughout the prenatal period (Figure 4a-c).

FIGURE 6 The histochemical distribution of colonic mucins during the prenatal development of sheep fetuses. (a) 70-day-old fetal lamb, goblet cells containing acidic (blue arrows) mucins in the colon with villi, glycogen accumulation in the epithelial cells (arrowheads), AB-PAS pH 2.5 staining. Bar; 20 μ m. (b) 84-day-old fetal lamb, goblet cells containing neutral (red arrows) and acidic (blue arrows) mucins in the colon with villi, glycogen accumulation in the epithelial cells (arrowheads), AB-PAS pH 2.5 staining. Bar; 20 μ m. (c) 120-day-old fetal lamb, goblet cells containing acidic (blue arrows) mucins in the colon with well-defined crypts, AB-PAS pH 2.5 staining. Bar; 20 μ m. (d) 130-day-old fetal lamb, goblet cells containing acidic (blue arrows) mucins in the colon with well-defined crypts, AB-PAS pH 2.5 staining. Bar; 20 μ m. (e) 70-day-old fetal lamb, large number of goblet cells containing sulfomucins (arrowhead) and small number of goblet cells containing sialomucins (arrow) in the colon with villi, HID-AB pH 2.5 staining. Bar; 50 μ m. (f) 115-day-old fetal lamb, large number of goblet cells containing sulfomucins (arrowhead) and small number of goblet cells containing sialomucins (arrow) in the colon with villi, HID-AB pH 2.5 staining. Bar; 50 μ m. (g) 120-day-old fetal lamb, increased number of goblet cells (arrow) containing sialomucins in the colon with well-defined crypts, goblet cells containing sulfomucins (arrowhead), HID-AB pH 2.5 staining. Bar; 50 μ m. (h) 126-day-old fetal lamb, colon with well-defined crypts, goblet cells containing sialomucins (arrow), goblet cells containing sulfomucins (arrowhead), HID-AB pH 2.5 staining. Bar; 50 μ m [Color figure can be viewed at wileyonlinelibrary.com]

Combined HID-AB (pH 2.5) staining showed that the vast majority of goblet cells in the villi and crypts contained sulfated mucins (Figure 4d-f).

Ileum

Combined AB-PAS pH 2.5 staining demonstrated the presence of acidic mucin-containing goblet cells in the ileal villi and crypts

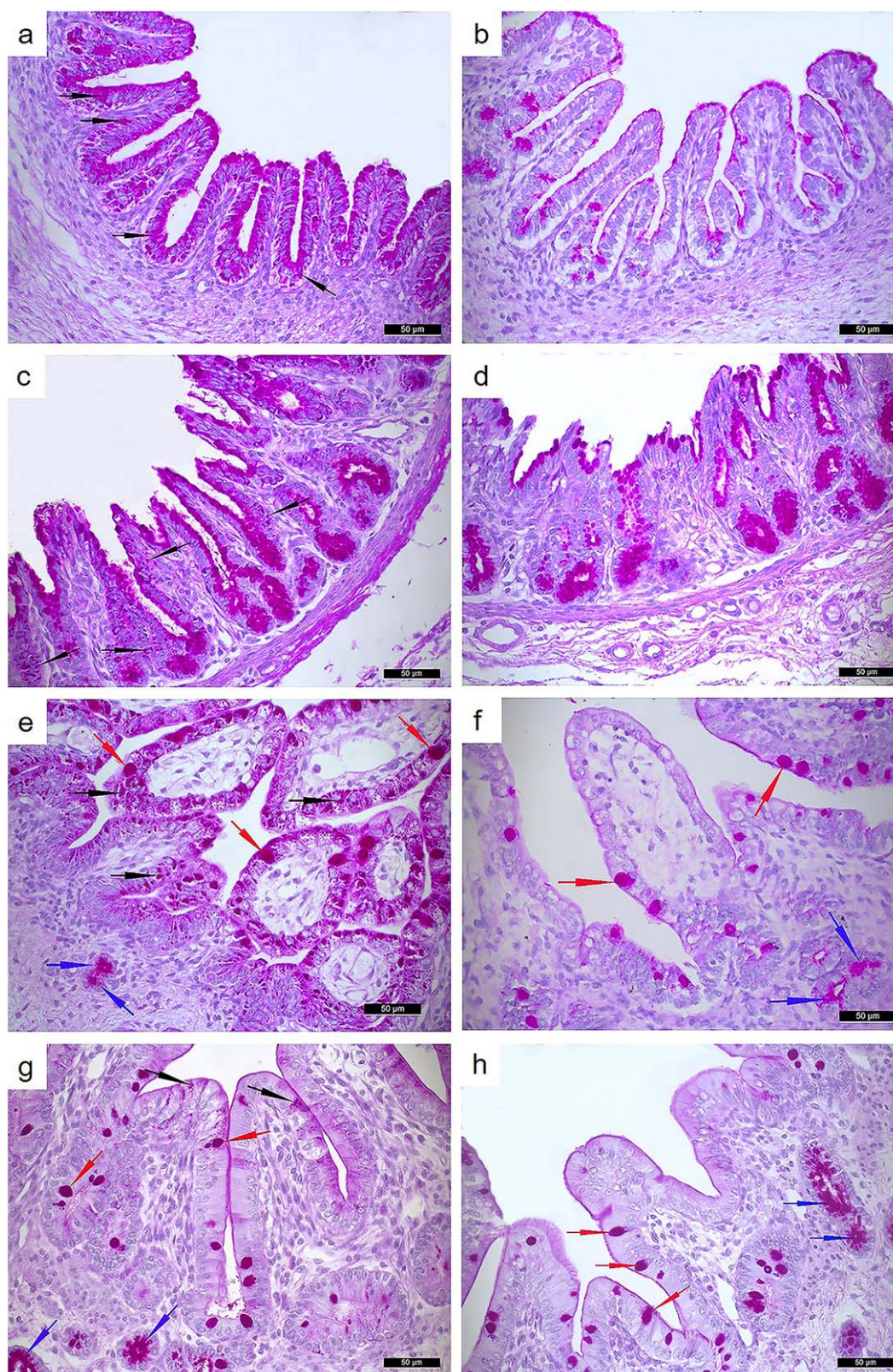


FIGURE 7.

during the early prenatal period, and mixed and neutral mucin-containing goblet cells as well in the later phases of the prenatal period. However, acidic mucin-containing goblet cells predominated in the ileal crypts and villi throughout the prenatal period (Figure 5a–c).

HID–AB (pH 2.5) staining demonstrated that while goblet cells containing both sialomucins and sulfomucins were observed in the ileal crypts and villi in early and mid-gestation, goblet cells containing sulfated mucins were dominant in late pregnancy (Figure 5d–f).

Colon

While the mucosa of the proximal colon harbored villi until the 115th day of gestation, as of the 120th day the villi were observed to have completely regressed and well-defined crypts emerged. The vast majority of the goblet cells in the villi contained acidic mucins, while very few contained neutral mucins. Once the well-defined crypts formed, only acidic mucin-secreting goblet cells existed in the crypts (Figure 6a–d).

HID–AB (pH 2.5) combined staining showed that, goblet cells containing sulfomucins were more common in the villi, and the number of goblet cells containing sialomucins gradually increased after the emergence of the crypts. Goblet cells containing sulfated mucins were mostly localized to the neck of the crypts and the surface of the crypts facing the lumen, whereas sialomucin-secreting goblet cells were observed at the base of the crypts (Figure 6e–h).

3.1.2 | PAS & α -amylase–PAS stainings

In the abomasum, throughout the prenatal period, while moderate PAS reactions were observed in the cells lining the glandular corpus, strong PAS reactions were detected in the superficial glandular epithelial cells and glandular ducts. α -amylase–PAS staining showed that glycogen granules stored in the epithelial cells gradually decreased toward the end of gestation. In the intestinal sections examined, α -amylase–PAS staining demonstrated that the number of glycogen granules stored in the epithelial cells lining the villi and crypts decreased toward the end of gestation. No significant alteration was detected in the Brunner's glands and goblet cells strongly positive for PAS after being employed α -amylase (Figures 7–9).

In addition to all these findings, Paneth cells with supranuclear acidophilic granules were also present in the duodenal, jejunal, and ileal

crypts as demonstrated by combined AB–PAS pH 2.5 staining (Figure 10a–d).

3.1.3 | Immunohistochemistry

In the present study, immunohistochemical staining was performed with the Strept-ABC method using monoclonal antibodies raised against MUC1, MUC2, MUC5AC, and MUC6. Rabbit ileum and human colon were used as positive controls. While only some cells in the villi and crypts of the rabbit ileum reacted to the MUC2 protein (Figure 10e), the majority of the crypts reacted in the human colon (Figure 10f). Conversely, no immune reaction was observed in fetal and adult ovine tissues (Figure 10g,h).

4 | DISCUSSION

We have studied the histochemical staining pattern of the epithelial mucins in sheep fetuses at various gestational ages, to determine their developmental changes and the localizations in the gastrointestinal tract. To our knowledge, although mucin types found in the gastrointestinal tract have been described in several mammals (Filipe, 1979; Forman, 1971; Karakoç et al., 2016; Kotzé & Coetzee, 1994; Krause, 2000; Schumacher et al., 2004; Sheahan & Jervis, 1976), up to now, the presence and distribution of mucins in the gastrointestinal tract of sheep fetuses has not been investigated. Furthermore, we checked whether the different MUCs (MUC1, MUC2, MUC5AC, and MUC6) were expressed in sheep fetal abomasums, small intestine and colon using conventional primary antibodies.

The gastrointestinal mucosal epithelium has various functions in sustaining the health of the digestive system. A mucus layer composed of ions, water, lipids, proteins, and glycoproteins covers the mucosal epithelium and provides protection against both mechanical, chemical and enzymatic damage and pathogenic microorganisms (Karakoç et al., 2016; Rose & Voynow, 2006). Gastric mucins are secreted by the luminal epithelial cells and gastric glands in some animal species and humans (Lacunza, Bara, Segal-Eiras, & Croce, 2009a, 2009b; Liman, Alan, and Küçük Bayram, 2010; Terada, 2013; Vernygorodskyi, 2013). In humans and the opossum, superficial cells of the gastric mucosa and glandula pylorica secrete only neutral mucins, while in the vole, rat, bison, rabbit, Japanese macaque, Rhesus monkey, raccoon dog, white-tailed deer, guinea pig, and cat, they produce both acidic and neutral mucins (Karakoç et al., 2016; Schumacher et al., 2004). Ganesh,

FIGURE 7 Developmental changes of glycogen and mucin in abomasum and duodenum of sheep fetuses. (a) 74-day-old fetal lamb, glycogen accumulation in the epithelial cells (arrows). Abomasum, PAS staining. Bar; 50 μ m. (b) 74-day-old fetal lamb, decreased number of glycogen granules in the epithelial cells. Abomasum, α -amylase–PAS staining. Bar; 50 μ m. (c) 126-day-old fetal lamb, glycogen accumulation in the epithelial cells (arrows). Abomasum, PAS staining. Bar; 50 μ m. (d) 126-day-old fetal lamb, decreased number of glycogen granules in the epithelial cells. Abomasum, α -amylase–PAS staining. Bar; 50 μ m. (e) 70-day-old fetal lamb, glycogen accumulation in the epithelial cells (black arrows), goblet cells (red arrows), Brunner's glands (blue arrows). Duodenum, PAS staining. Bar; 50 μ m. (f) 70-day-old fetal lamb, decreased number of glycogen granules in the epithelial cells but no significant changes in Brunner's glands (blue arrows) and goblet cells (red arrows). Duodenum, α -amylase–PAS staining. Bar; 50 μ m. (g) 130-day-old fetal lamb, glycogen accumulation in the epithelial cells (black arrows), goblet cells (red arrows), Brunner's glands (blue arrows). Duodenum, PAS staining. Bar; 50 μ m. (h) 130-day-old fetal lamb, decreased number of glycogen granules in the epithelial cells but no significant changes in Brunner's glands (blue arrows) and goblet cells (red arrows). Duodenum, α -amylase–PAS staining. Bar; 50 μ m [Color figure can be viewed at wileyonlinelibrary.com]

Subramani, and Halagowder (2007) reported that in humans, while the gastric mucosa secretes a low level of sialomucins, neutral mucins are localized to the mucosal glands and foveolar epithelial cells in the antrum, the latter of which also contain sulfomucins. Karakoç et al. (2016) demonstrated that, in rams and bulls, the abomasum contains acidic (sulfated and carboxylated) and neutral mucins, as well as glycogen. However, these researchers also reported that the two species

differ for mucin localization in the abomasum. While the superficial epithelial cells of the cardia and fundus contain neutral mucins, the deeper glandular epithelial cells mainly contain sulfomucins. Conversely, the cardia and fundus contain less carboxylated mucins, which are found at high levels in the epithelial cells of the pyloric glands. On the contrary to the case in bulls, a large amount of carboxylated mucins was detected in the deeper layers of the pars cardiaca and the superficial

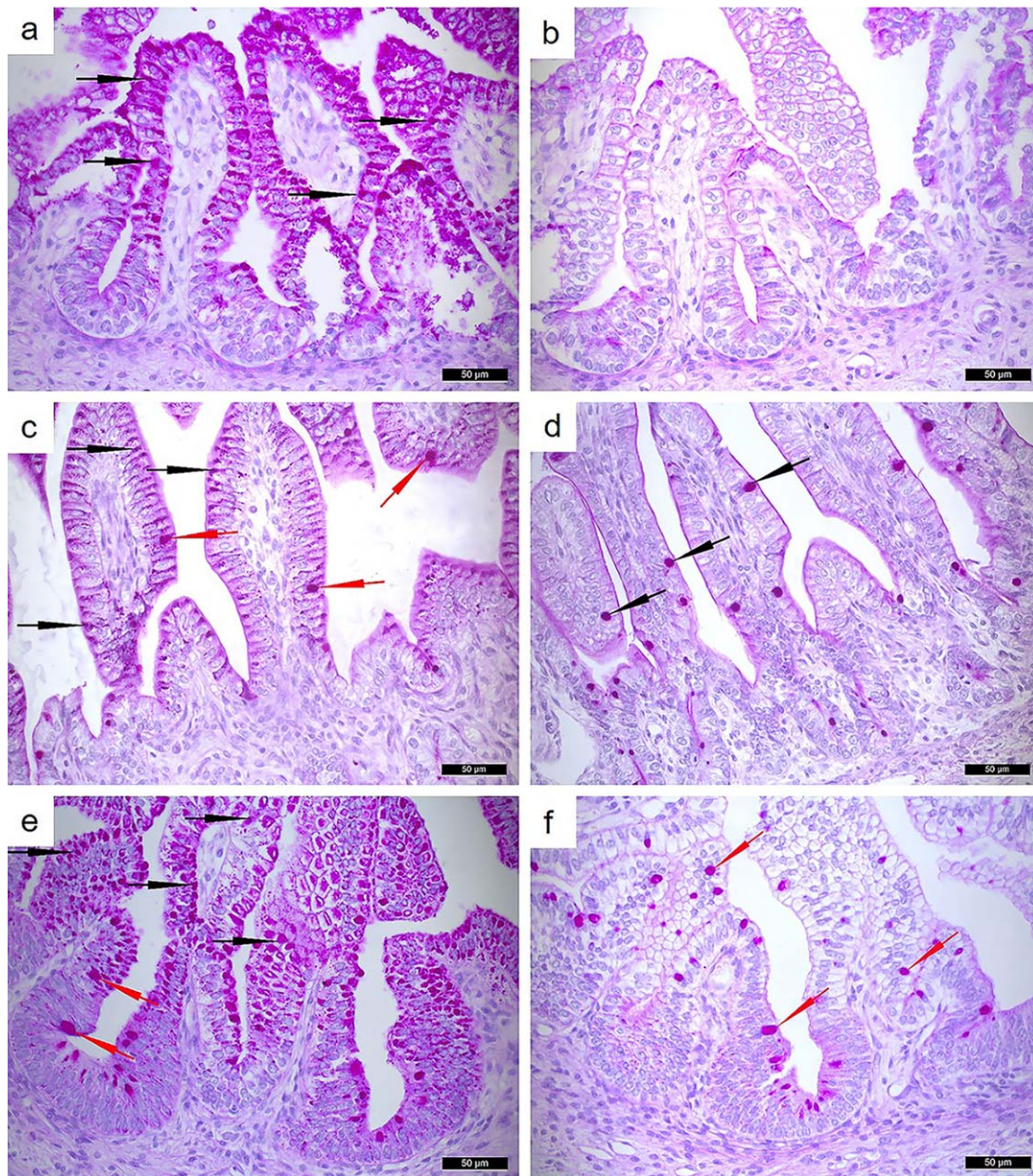


FIGURE 8 Developmental changes of glycogen and mucin in jejunum and ileum of sheep fetuses. (a) 70-day-old fetal lamb, glycogen accumulation in the epithelial cells (arrows). Jejunum, PAS staining. Bar; 50 μ m. (b) 70-day-old fetal lamb, decreased number of glycogen granules in the epithelial cells. Jejunum, α -amylase-PAS staining. Bar; 50 μ m. (c) 130-day-old fetal lamb, glycogen accumulation in the epithelial cells (black arrows), goblet cells (red arrows). Jejunum, PAS staining. Bar; 50 μ m. (d) 130-day-old fetal lamb, decreased number of glycogen granules in the epithelial cells but no significant changes in goblet cells (arrows). Jejunum, α -amylase-PAS staining. Bar; 50 μ m. (e) 70-day-old fetal lamb, glycogen accumulation in the epithelial cells (black arrows), goblet cells (red arrows). Ileum, PAS staining. Bar; 50 μ m. (f) 70-day-old fetal lamb, decreased number of glycogen granules in the epithelial cells but no significant changes in goblet cells (arrows). Ileum, α -amylase-PAS staining. Bar; 50 μ m [Color figure can be viewed at wileyonlinelibrary.com]

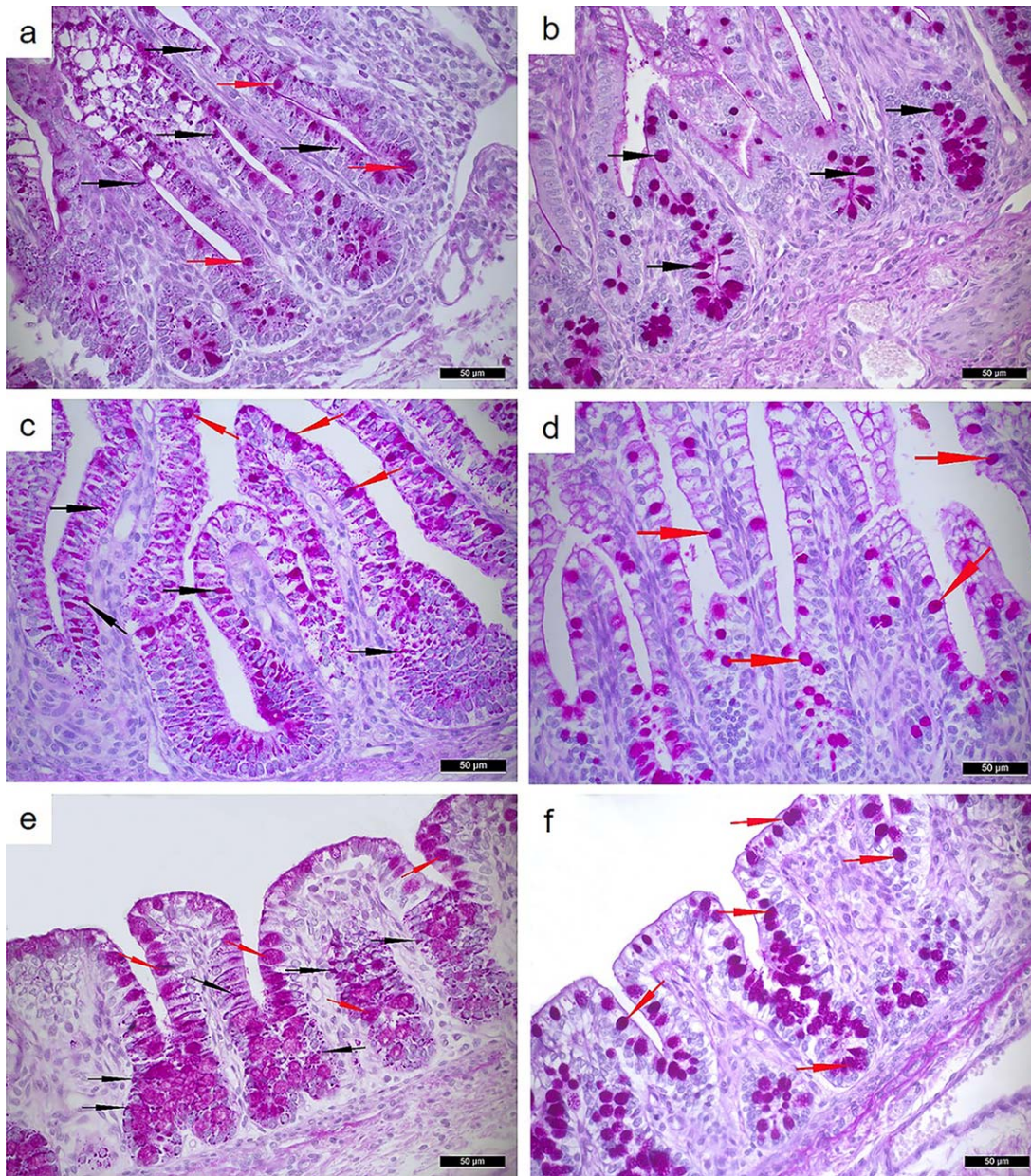


FIGURE 9 Developmental changes of glycogen and mucin in ileum and colon of sheep fetuses. (a) 126-day-old fetal lamb, glycogen accumulation in the epithelial cells (black arrows), goblet cells (red arrows). Ileum, PAS staining. Bar; 50 μ m. (b) 126-day-old fetal lamb, decreased number of glycogen granules in the epithelial cells but no significant changes in goblet cells (arrows). Ileum, α -amylase-PAS staining. Bar; 50 μ m. (c) 70-day-old fetal lamb, glycogen accumulation in the epithelial cells (black arrows), goblet cells (red arrows). Colon with villi, PAS staining. Bar; 50 μ m. (d) 70-day-old fetal lamb, decreased glycogen granules in the epithelial cells (black arrows), goblet cells (red arrows). Colon with villi, α -amylase-PAS staining. Bar; 50 μ m. (e) 130-day-old fetal lamb, glycogen accumulation in the epithelial cells (black arrows), goblet cells (red arrows). Colon with well-defined crypts, PAS staining. Bar; 50 μ m. (f) 130-day-old fetal lamb, decreased number of glycogen granules in the epithelial cells but no significant changes in goblet cells (arrows). Colon with well-defined crypts, α -amylase-PAS staining. Bar; 50 μ m [Color figure can be viewed at wileyonlinelibrary.com]

glandular epithelial cells of the pars pylorica in rams (Karakoç et al., 2016). The prenatal abomasal mucin profile of sheep has not been investigated before. In the present study, alterations were detected in the mucin content of the pyloric abomasum. While neutral mucin was absent in the pyloric epithelial cells and gastric gland cells, acidic mucin was detected in the superficial cells until the 115th day of gestation. In agreement with the results of previous studies performed in adult

animals (Karakoç et al., 2016; Schumacher et al., 2004) and humans (Ganesh et al., 2007), thereafter both acidic and neutral mucins were detected in the abomasal pylorus. Neutral mucins were found in the superficial epithelial cells of the proximal foveola gastrica, whereas acidic mucins were detected in the opposing foveolar surfaces and the corpus glandula. Sulfated mucins were detected in some of the gastric glands only after the 115th day of gestation. Throughout gestation, a

high level of sialomucins was present in both the mucous epithelial cells and the gastric gland cells. The findings obtained in the present study are largely concordant with previous results obtained for the mucin profile of adult ruminants (Karakoç et al., 2016).

The continuous mechanical effect of ingested food results in the abrasion of the duodenal mucosa. Furthermore, the secretion of bile acids and hydrochloric acid into the digestive tract also damages the epithelium (Ermund et al., 2013). Different from the other intestinal

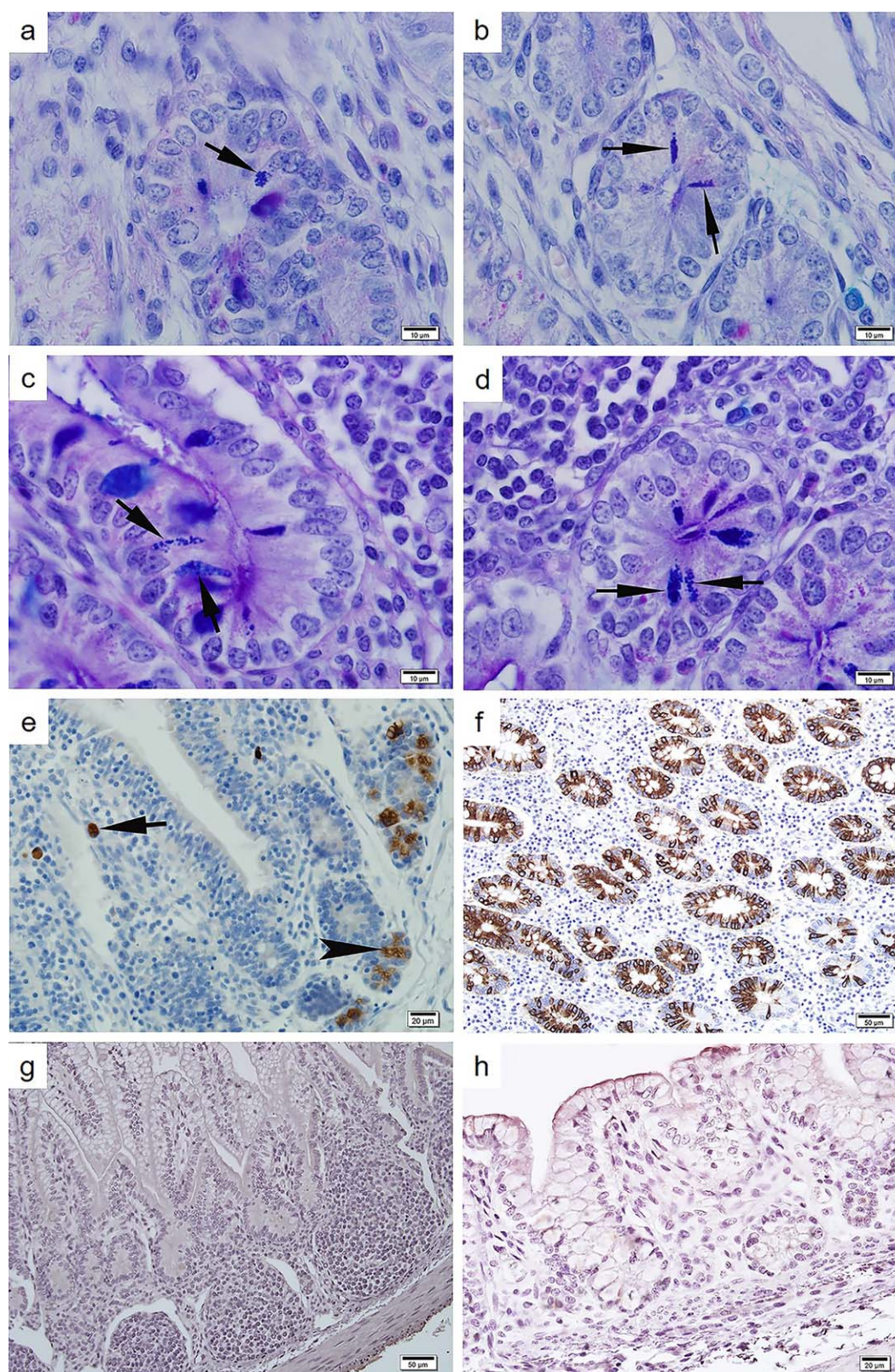


FIGURE 10.

segments, the duodenum releases mucin from both goblet cells and the Brunner's glands in the submucosa into the lumen, which enables its protection from damage. Thus, in mammals, the proximal duodenum has a complex cellular structure composed of several types of secretory cells (Collaco et al., 2013). Previous research in mammals has demonstrated that duodenal glands predominantly produce neutral mucins, and a lower level of acidic mucins (Krause, 2000). However, the glycosylation pattern of duodenal glands varies greatly among animal species (Ergün, Ergün, Kürüm, Özen, & Bayraktaroglu, 2010). In bison, voles, guinea pigs, deer, and domestic and cotton-tailed rabbits, the glandular secretion is composed of acidic mucins (sulfated and carboxylated), whereas in Rhesus macaques, Japanese macaques rats, cats, and raccoons the glandular secretion is comprised of neutral mucins (Schumacher et al., 2004). Furthermore, in research conducted in the coypu (*M. coypus bonariensis*) (de la Hoz et al., 2016), cattle (Krause, 2000; Takehana, Mitsuo, Iwasa, Hiraga, & Miyata, 1991), sheep (Krause, 2000), goats (Krause, 2000), and fallow deer (Scocco, Pedini, Fagioli, & Ceccarelli, 2001) duodenal glands were found to contain both acidic and neutral mucins. The differences observed in the feature of the duodenal secretion are not considered to be related to the phylogenetic relationship between the mammalian species or to their diet. For instance, although the rat and vole are both rodents, the Brunner's glands produce only acidic mucins in the vole, and neutral mucins in the rat. The results of the present study are in agreement with the results of a previous study performed to investigate the Brunner's glands of 27 bat species with different diets (fish-, insect-, plant-, nectar-, blood-), which demonstrated the presence of only neutral mucins in the glandular secretion of all of the species (Forman, 1971). A study on the alterations in the fetal mucin profile of the fallow deer demonstrated that, as labor approached, not only did neutral mucins significantly increase in both the goblet cells and duodenal glands, but also carboxylated mucins predominated over sulfomucins (Scocco et al., 2001). In consistent with the results of Scocco et al. (2001), our results indicated that, toward the end of gestation, neutral mucin-producing goblet cells predominated in the crypts and villi. In the present study, we noted that Brunner's glands produced only acidic mucins until the 95th day of gestation and thereafter, neutral mucins as well. Furthermore, we observed that goblet cells contained both sialomucins, although few, and sulfomucins in the second trimester of gestation and those containing sulfomucins outnumbered the others in the third trimester of gestation. Brunner's glands mainly produced acidic mucins throughout pregnancy and some started to produce sulfomucins as of the 115th day. In this study, while the mucin profile of the Brunner's

glands of sheep fetuses resembled that of adult sheep, acidic mucins consisted of both sialomucins and sulfated mucins, and postnatal acidic mucins were comprised of sialomucins (Krause, 2000). Based on the increase observed in neutral mucin-producing goblet cells in the villi toward the end of gestation, it could be suggested that neutral mucins help protect the epithelial layer from corrosion by offsetting the acidic content of the abomasum.

In newborn small ruminants, the jejunum has an essential role in the first line of defense as the cells in this segment are involved in immunoglobulin absorption in the first few hours after birth, when neonates are highly vulnerable to the outer world (Clarke & Hardy, 1971; Machado-Neto, Grigolo, Moretti, Kindlein, & Pauletti, 2011; Nordi et al., 2012). The presence of colostrum-containing vacuoles in the jejunal enterocytes of goat kids during the first hours postpartum suggests the possible occurrence of absorption at macromolecular level (Clarke & Hardy, 1971; Machado-Neto et al., 2011; Moretti et al., 2012; Nordi et al., 2012). Twenty-four to 36 hr after birth, the membrane of enterocytes undergoes drastic changes resulting in the loss of absorptive capacity and intestinal closure, which ends macromolecule transfer across the intestine within a few hours or days after delivery in certain species (e.g., pigs, sheep, cows, rabbits, and guinea pigs). Machado-Neto, Pontin, Nordi, Lima, and Moretti (2013) reported that the number of jejunal goblet cells significantly altered between the 16th and 96th hours after birth in goat kids. Kleessen, Hartmann, and Blaut (2003) and Machado-Neto et al. (2013) suggested that alterations in the composition or mucin content of the jejunal mucosa could significantly alter absorption in the small intestine. In our study, the jejunum contained a relatively small number of goblet cells when compared to the other intestinal segments. Goblet cells, which contained mixed, neutral and acidic mucins, were localized to the villi and crypts and acidic mucins mostly consisted of sulfomucins. The relatively small number of goblet cells in the jejunum could be related to the high number of columnar epithelial cells required for the maximal absorption of colostrum after birth.

The ileum, which is the final section of the small intestine, is responsible not only for nutrient absorption, but also the protection of the intestines against microbial colonization and overgrowth owing to the Peyer's patches it contains. In a previous study on the fetal bovine ileum, goblet cells containing neutral mucins were reported to gradually decrease, while those containing acidic and mixed mucins were determined to increase toward the end of gestation. Moreover, research has demonstrated that as from the 6th month of pregnancy, in parallel with the development of Peyer's patches in the ileum, sulfomucin-

FIGURE 10 AB-positive Paneth cells in the small intestines (a–d). Positive controls for MUC2 (e, f) and negative control MUC (g, h). (a) Duodenum of a 130-day-old fetal sheep, a Paneth cell with granules containing acidic mucins (arrow) in the crypts, AB–PAS pH 2.5 staining. Bar; 10 μ m. (b) Jejunum of a 130-day-old fetal sheep, a Paneth cell with granules containing acidic mucins (arrows) in the crypts, AB–PAS pH 2.5 staining. Bar; 10 μ m. (c) Ileum of a 126-day-old fetal sheep, a Paneth cell with granules containing acidic mucins (arrows) in the crypts, AB–PAS pH 2.5 staining. Bar; 10 μ m. (d) Ileum of a 130-day-old fetal sheep, a Paneth cell with granules containing acidic mucins (arrows) in the crypts, AB–PAS pH 2.5 staining. Bar; 10 μ m. (e) Rabbit ileum, MUC2-positive immunoreactivity in the villi (arrow) and crypts (arrowhead), Immunohistochemical staining, DAB. Bar; 20 μ m. (f) Human colon, MUC2-positive immunoreactivity in the crypts, Immunohistochemical staining, DAB. Bar; 50 μ m. (g) Ileum of a 115-day-old fetal sheep, no immunoreactivity for MUC2 in the villi and crypts, Immunohistochemical staining, DAB. Bar; 50 μ m. (h) Colon of a 130-day-old fetal sheep, no immunoreactivity for MUC2 in the crypts, Immunohistochemical staining, DAB. Bar; 20 μ m [Color figure can be viewed at wileyonlinelibrary.com]

containing goblet cells show a gradual increase (Beyaz & Liman, 2009). In the present study, goblet cells containing acidic mucins predominated in the crypts and villi throughout the second and third trimester of gestation and were associated by cells containing mixed and neutral mucins. Toward the end of gestation, acidic mucins were mainly comprised of sulfomucins. These findings are in agreement with literature reports on ileal mucins in bovine fetuses (Beyaz & Liman, 2009). However, while Beyaz and Liman (2009) reported that ileal Peyer's patches are involved in mucin heterogeneity in bovine fetuses, we observed no significant effect of the ileal Peyer's patches on the mucin profile in ovine fetuses.

The development of the digestive tract differs between species. In animals with a short gestation period, such as mice and rats, intestinal development is nearly completed at the time of nursing. However, in humans and guinea pigs, which both have relatively long gestation periods, intestinal maturation occurs to a great extent during the prenatal period (Smith, Christianson, Moss, & Bailey, 1985). The colon of the embryo and fetus differs both structurally and functionally from that of the adult. While villi are not observed in the adult colon, primordial villi exist in birds on the 11th day of embryonic development (Gheri & Gheri, 1988). Furthermore, the development of the proximal colon differs from that of the distal colon. In the fetus, while the proximal colon bears villi, the distal colon does not (Helander, 1973; Lacy & Colony, 1985; Ménard, Dagenais, & Calvert, 1994; Smith et al., 1985). To the authors' knowledge, the prenatal development of the ovine proximal colon has not been investigated before. In the present study, villi existed in the proximal colon until the 115th day of gestation and later disappeared. By the 120th day, villi were observed to have completely regressed and well-defined crypts appeared. In human fetuses, while sulfomucins first appear in the colon after the emergence of goblet cells (Lev, 1968) at the 14th week of gestation, O-acetylated sialomucins are observed as of the 23rd week. In humans, the colonic mucin profile of the newborn resembles that of the adult, in that it is comprised mostly of acidic mucins (Filipe, Sandey, & Carapeti, 1988). While sulfomucins were detected in all goblet cells in the colon of newborn mice (Hill, Cowley, & Andremon, 1990), the mucins in the colon of newborn piglets were determined to consist of both sulfomucins and sialomucins (Turck, Feste, & Lifschitz, 1993). In the present study, it was observed that, nearly the entire goblet cell population in the crypts and villi of the colon contained acidic mucins. During the period in which the villi were found in the proximal colon, the goblet cells containing sulphomucins were predominant, whereas the goblet cells containing sialomucins were predominant after the typical crypt structure was formed.

Paneth cells, which are secretory cells in the epithelium lining the small intestine; exist in the form of small clusters at the base of the crypts of Lieberkühn (Bevins & Salzman, 2011). Research has shown that Paneth cells secrete MUC2 and antimicrobial agents (Pelaseyed et al., 2014). The histochemical data obtained in the present study suggest that the secretion of Paneth cells, which contains acidic mucin, may protect the neonatal mucosa against microbial colonization.

The physiological relation between different types of mucins has not been completely elucidated (Deplancke et al., 2000). Two

hypotheses are suggested to explain the role of sialo- and sulphomucins during the fetal period. According to the first, sulfate groups and sialic acid are primarily involved in determining the viscoelastic characteristics of mucins (Corfield, 2007; Deplancke & Gaskins, 2001; Jentoft, 1990; Van der Reijden, Veerman, & Amerongen, 1992), as well as in electrolyte and water transport in the adult distal colon. Although the intestines are functional in the fetus, there is no need for digestive function as nutrient passage occurs transplacentally. After being swallowed, the amniotic fluid is transported along the esophagus, stomach and large intestine, and once it reaches the large intestine, most of the fluid is absorbed alongside glucose, hormones, electrolytes, and urea. This process, which nutritionally supports the fetus, is a digestive pre-function. The second hypothesis suggests a protective role of sialic acid and sulfated mucins against protease-induced degradation and bacterial attack (Corfield, Wagner, Clamp, Kriaris, & Hoskins, 1992). Viral agents, which damage the mucus layer in the gastrointestinal tract, such as reoviruses, adenoviruses, enteroviruses and influenza viruses, are eliminated from the body by binding to the sialic acid residues of mucins (Linden, Sutton, Karlsson, Korolik, & McGuckin, 2008). Given that they are less degraded by bacterial glycosidases and the proteases of the organism itself, acidic mucins, and in particular sulfomucins, are considered to have a protective effect against bacterial translocation (Fontaine, Meslin, Lory, & Andrieux, 1996; Robertson & Wright, 1997). This opinion is supported by the higher number of acidic mucin-producing goblet cells in the intestinal sections harboring large bacterial populations (Amerongen, Bolscher, Bloemena, & Veerman, 1998; Deplancke et al., 2000; Robertson & Wright, 1997). The gastrointestinal tract, which is sterile during the prenatal period, is dominated by cells containing acidic mucins. In the postpartum period, the digestive tract of the newborn is rapidly colonized by environmental and maternal microorganisms (Deplancke et al., 2000; Mackie, Sghir, & Gaskins, 1999). In our study, similar to other species, such as ostriches (Duritis, Mugurevics, & Mancevica, 2013), cattle (Beyaz & Liman, 2009) and broiler chickens (Uni, Smirnov, & Sklan, 2003), acidic mucins were also determined to predominate in the proximal colon and terminal ileum of sheep fetuses. The high concentration of acidic mucins in the colon and ileum, which are characterized by strong microbial colonization, could be related to the protective effect of acidic mucins against intestinal pathogenic invasion in the neonatal period.

In the human fetus, swallowing is observed as of the 16th week (Montgomery, Mulberg, & Grand, 1999). Owing to the swallowing of the amniotic fluid by the fetus, the intestinal epithelium is exposed to bioactive substances of very different functions starting from the fetal period. The terminal differentiation and also morphogenesis of the intestinal epithelium depends on the regular activity of several substances including cytokines, peptides, and growth factors (Kanai, Rosenberg, & Podolsky, 1997; Ng et al., 2002). These bioactive substances, which regulate both functional development and mucosal morphogenesis, are found in the amniotic fluid during the prenatal period (Lebenthal & Lebenthal, 1999). Reports also indicate that the amniotic fluid meets 10%-15% of the energy and nitrogen requirements of the fetus during the prenatal period (Pitkin & Reynolds, 1975). The meconium, which forms during the fetal period, contains swallowed amniotic fluid,

secretions such as bile, sloughed epithelial cells and pancreatic secretion, and maternal and fetal metabolites excreted into the intestines (Heitlinger, 1989). In humans, mucus secretion in the fetus is reported to start in the 10th week of pregnancy. It is believed that this mucus secretion contributes to the content of the meconium and also protects the mucosa against biliary secretion and swallowed amniotic fluid (Hounsell et al., 1985; Stauffer, Lallemand, & Gaillard, 1989).

Glycogen is the main source of energy in developing organisms. While glycogen is mainly stored in the liver in adults, during the prenatal period, hepatic glycogen storage is possible only at the terminal stage. Furthermore, while glycogen storage in the digestive epithelium is less in adults, it is common in the early period of fetal life (Lev, 1968). During embryonic life, glycogen stores are believed to be used for hormone-regulated rapid differentiation (Black, 1988). Research conducted on the prenatal period in ostriches (Duritis et al., 2013), humans (Hashimoto, Tamura, Otani, & Tanaka, 1995; Lev & Weisberg, 1969), cattle (Beyaz & Liman, 2009) and rodents (de la Hoz et al., 2016; Tano de la Hoz et al., 2014) has shown that the amount of glycogen stored in epithelial cells gradually decreases as labor approaches. In the present study, the gradual decrease observed in intracytoplasmic glycogen in the intestinal epithelial cells toward the end of the fetal period is in consistent with the findings of these previous studies. No notable change was observed after α -amylase application in the goblet cells and Brunner's glands strongly reacting to PAS. Our data suggest that glycogen stored in the intestinal epithelium in the prenatal period is the main energy source used for metabolic functions, including mucin synthesis.

The 20 human genes, which encode mucins (MUC), can be classified under secreted and membrane-associated forms. Secreted mucins can be subclassified into nongel-forming mucins and gel-forming mucins (Corfield, 2015). To date, the investigation of the bovine genome has yielded the identification of 6 secreted mucins (MUC19, MUC7, MUC6, MUC5B, MUC5AC, and MUC2) and 9 membrane-bound mucins (MUC21, MUC20, MUC16, MUC15, MUC13, MUC12, MUC4, MUC3A, and MUC1) (Hoorens et al., 2011). Currently, there are no commercially available specific MUC primary antibodies for sheep tissues. Therefore, some anti-human MUC primary antibodies, previously reported to show cross reactivity in the sheep abomasum on the basis of immunohistochemical staining results (Karakoç et al., 2016), were used in this study. However, no immune reaction was detected in the gastrointestinal tissues of the sheep fetuses for the MUC6, MUC5AC, MUC2, and MUC1 antibodies.

In conclusion, the present study is the first description of the histochemical properties of mucins found in the gastrointestinal tract of sheep fetuses. The different types of mucins present in the gastrointestinal tract of sheep fetuses might protect the gastrointestinal epithelium from metabolites in the amniotic fluid swallowed by the fetus as well as from biliary acid secretion in the prenatal period. Furthermore, the intestinal mucins of sheep fetuses may be involved in the formation of meconium, and acidic mucins can contribute to the establishment of a strong intestinal barrier against digestive enzymes and potential pathogens in the gastrointestinal tract, which is not fully functional after birth.

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