ORIGINAL ARTICLE

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Distribution of ki-67, alpha smooth muscle actin and vimentin in the reticulum and omasum of Baladi goat

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SUMMARY

Seven Baladi goats of both sexes (9 to 24 months old) were used to describe the distribution pattern of ki-67, alpha smooth muscle actin (αSMA) and vimentin (VIM) in the reticulum and omasum. This study was carried out using the avidin-biotin immunoperoxidase method. Ki-67 immunostaining was restricted to the basal cells layer in the epithelia of both the reticulum and omasum, suggesting the importance of ki-67 in epithelial cells proliferation and keratin biosynthesis. Immunostaining for αSMA was detected in smooth muscle cells in reticular folds, omasal lamiae and muscularis in both the reticulum and omasum, indicating the critical role of αSMA in muscular motility. The widespread distribution of VIM immunostainings in epithelia, fibroblasts in lamina propria and submucosa, and endothelia of blood vessels supports the importance of VIM as an intermediate filament protein. Detection of VIM in glial cells of enteric plexuses indicates its supportive role in the nervous control of both reticulum and omasum. Overall, this immunohistochemical study revealed non-significant differences in the expression of ki-67, αSMA, and VIM between the reticulum and omasum. This study thus verifies the important roles of ki-67, αSMA and VIM in the structure and function of the reticulum and omasum of Baladi goats.

Key words: Reticulum – Omasum – Immunohistochemistry – Ki-67 – αSMA – Vimentin – Baladi goat

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INTRODUCTION

According to feeding habits of ruminants, goats are intermediate feeders – concentrate and roughage feeders (Hofmann, 1989). Moreover, goats are known as the best users of poor roughage among ruminants (Gihad et al., 1980), and they are highly adapted to grazing over a wide range of vegetation (El-Gendy and Derbalah, 2010).

Digestion is the key for animal production and economy. The rumen, reticulum and omasum are responsible for digestion in ruminants. Most researches about digestion in ruminants were done on the rumen, but the reticulum and omasum have little works in comparison to rumen.

Reticulum is known as the honeycomb, due to its elevated reticular folds. It has similar functions to rumen, as both are home to microorganisms that break down plant cells into carbohydrates and produce volatile fatty acids used for energy. Also, it moves smaller digesta particles into the omasum (Parish et al., 2009). However, the omasum consists of many laminae which are composed of thin muscular layers that are covered with non-glandular mucous membrane (Yamamoto et al., 1994). These laminae increase the surface area for the absorption of volatile fatty acids, water and electrolytes that were not absorbed through the rumen (Parish et al., 2009).

Recently, prenatal histological and immunohistochemical studies on the reticulum and omasum of goats have been done (Garcia et al., 2014a, 2014b, 2013a, 2013b) but, the reticulum and omasum of adult goats have little immunohistochemical studies. Therefore, the present

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study was carried out to detect immunohistochemical localization and distribution of the ki67, αSMA, and VIM in the reticulum and omasum in Baladi goat in order to evaluate their roles in cells proliferation, muscular motility, and cellular cytoskeleton respectively.

MATERIALS AND METHODS

Specimen collection and processing

The reticula and omasa of 7 Baladi goats of both sexes (4 males and 3 females, with age range from 9 to 24 months) were collected from the local abattoir in Kalubyia governorate, Egypt. The goats had been sacrificed according to the guidelines of the animal ethics committee in the Faculty of Veterinary Medicine, Benha University. Small specimens from the reticula and omasa were taken and washed with physiological saline, then fixed in 10% neutral buffered formalin for 48 h at 4°C. Tissue specimens were dehydrated in alcohol, cleared in xylene, and embedded in paraplast.

Histological examination

Sections of 5 µm thick were cut and stained with hematoxylin and eosin, and Crossmon's trichrome as outlined by Bancroft and Gamble (2007).

Immunohistochemical examination

After dewaxing and reducing of endogenous peroxidase with 3% hydrogen peroxide in methanol for 20 minutes (min), sections were treated with citrate buffer pH 6 in steamer for 40 min, to induce

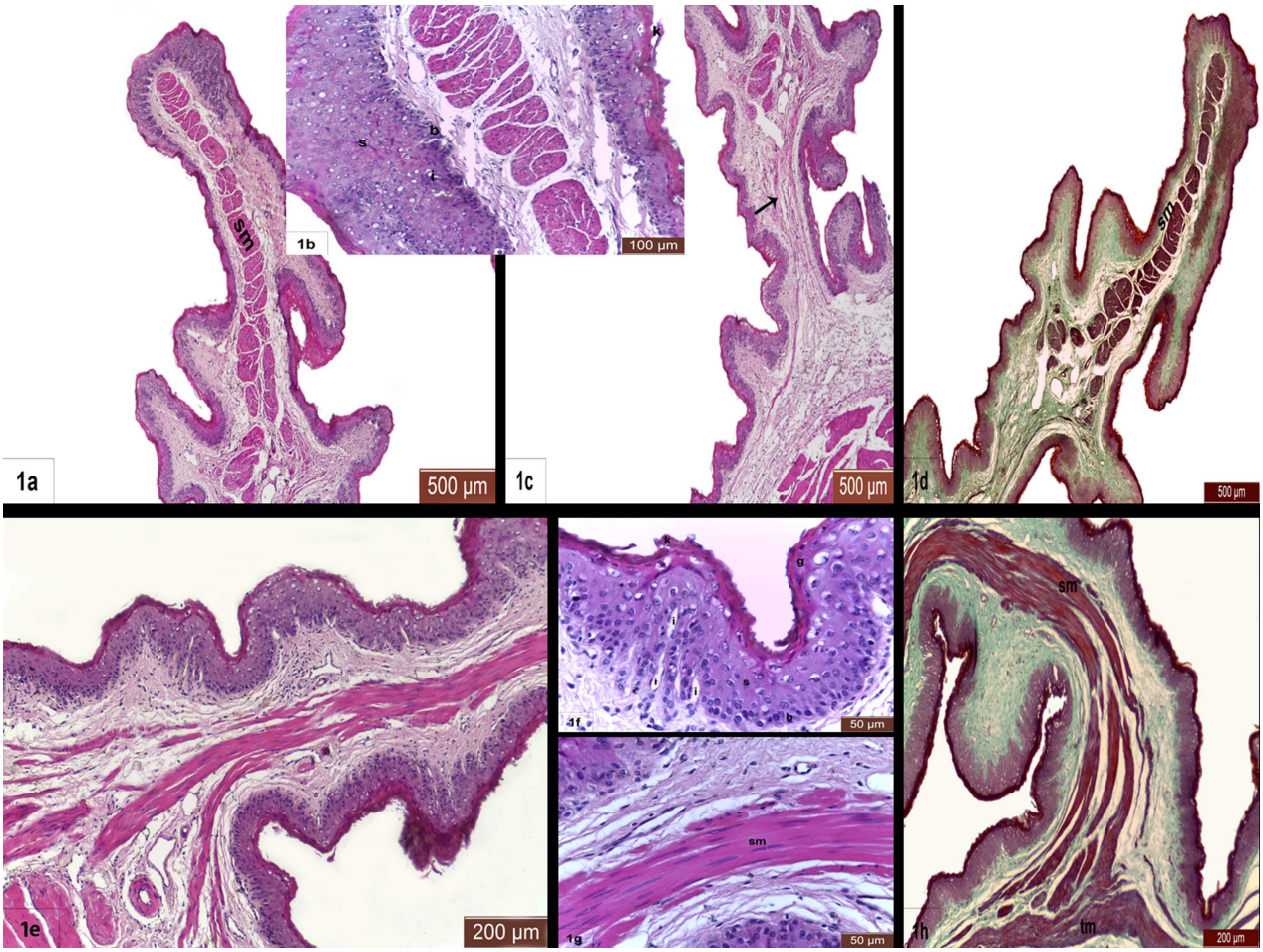

Fig. 1. Histological sections in reticulum and omasum of goat. **(a)** Showing lamina muscularis mucosae in the upper part of the reticular fold. It appeared as aggregations of smooth muscle cells (sm). H&E. **(b)** A higher magnification of (a) showing lamina epithelialis that consisted of stratum basal (b); stratum spinosum (s); intercellular space (i); stratum corneum (k). H&E. **(c)** Showing that both the base of reticular fold and tunica submucosa were free from smooth muscles (arrow). Note tunica muscularis was mainly smooth muscle. H&E. **(d)** Showing a reticular fold containing smooth muscle cells of lamina muscularis mucosae (sm) in its upper half. Note that collagen fibers of lamina propria appeared green. Crossman's trichrome stain. **(e)** Showing an omasal lamina. H&E. **(f)** A higher magnification of (e) showing stratum basal (b); stratum spinosum (s); intercellular space (i); stratum granulosum (g); stratum corneum (k) of the lamina epithelialis. H&E. **(g)** A higher magnification of (e) showing smooth muscle cells of lamina muscularis mucosae and extension from tunica muscularis along the mid of omasal lamina (sm). H&E. **(h)** Showing smooth muscle cells in along the mid of omasal lamina (sm) and tunica muscularis (tm). Note green color of collagenous connective tissue of lamina propria. Crossman's trichrome stain. Scale bars: (a,c,d) 500 µm; (e,h) 200 µm; (b) 100 µm; (f,g) 50 µm.

antigen retrieval. Sections were then incubated for 1 h at room temperature (RT) with the primary antibodies which were purchased from Santa Cruz Biotechnology, CA, USA (mouse anti- ki-67, sc-23900, at 1:200; mouse anti- smooth muscle α actin, sc-32251, at 1:250; mouse anti- vimentin, sc -6260, at 1:250). Sections were subsequently incubated with secondary antibody for 30 min at RT; then the reaction products were visualized using the ready-to-use Vectastain® Elite ABC reagent (Vector laboratories, CA, USA) for 30 min at RT. Sections were treated with a freshly prepared solution of 3.3-diaminobenzidine tetrahydrochloride (Dako Cytomation, CA, USA). Sections were counterstained with haematoxylin. For the negative controls, the primary antibodies were substituted with normal mouse IgG. The specificity of the immunoreactivities was confirmed by the absence of immunostainings.

Immunostaining grading scores

Nuclear immunostaining of ki-67 and cytoplasmic immunostainings of αSMA and VIM were considered as positive immunoreactivities. Different microscopic fields (n=12) of stained slides (n=3 slides), using objective ×40, were examined for each antibody in each organ. This study was single blinded as someone else who examined these sections. Immunostainings in the current study were scored according to the method of Vermeirsch et al. (2002). The cells were given a proportional score (PS) from 0 to 5 for no positive cells to >65% positive cells respectively. Also the cells were given an intensity score (IS) from 0 to 4 for no staining to very strong staining respectively. The total score (TS) was obtained by the addition of PS to IS. The TS of each immunostaining was scored at 1-3, 4-6, and 7-9 representing weak, moderate, and strong grades, respectively.

Statistical analysis

Student's t test was used to compare the TS of ki -67, αSMA and VIM in the reticulum to that of the omasum. $P < 0.05$ was considered statistically significant.

RESULTS

Histological observations

Mucosa of the reticulum was characterized by reticular folds (Figs. 1a-d), while mucosa of the omasum was characterized by omasal laminae (Figs. 1e,h). The lamina epithelialis of both reticulum and omasum was a keratinized stratified squamous epithelium, which consisted of 4 strata; basalis, spinosum, granulosum, and corneum (Figs. 1b,f). Intercellular spaces in the spinous and basal layers were seen in epithelia of both reticulum and omasum, but they were larger in the omasum than in the reticulum (Figs. 1b,f). Lamina propria of both reticulum and omasum consisted of non-glandular connective tissue (Figs. 1b,d,g,h). Lamina muscularis mucosae of reticulum appeared as aggregations of longitudinal smooth muscle cells that were present only in the upper half of the reticular fold (Figs. 1b,d), but were absent in tunica submucosa that filled the area between the base of the fold and tunica muscularis (Fig. 1c).

Lamina muscularis mucosa of the omasum with an extension from tunica muscularis appeared as a sheet of circular smooth muscles that ran along the mid of omasal lamina (Figs. 1e,g,h). Tunica muscularis of both reticulum and omasum consist-

Table 1. Immunostaining scores for ki-67, αSMA and VIM ± standard deviation in reticulum and omasum of Baladi goat. Student's t test was used to compare the TS.

	Reticulum			Omasum		
	PS	**IS**	**TS**	**PS**	**IS**	**TS**
Ki-67						
Epithelium (BC)	4.8±0.5	3.6±0.6	8.4±0.5	4.8±0.4	3.8±0.5	8.6±0.5
Stromal cells	0.00	0.00	0.00	0.00	0.00	0.00
Endothelium	0.00	0.00	0.00	0.00	0.00	0.00
Pericytes	0.00	0.00	0.00	0.00	0.00	0.00
Glial cells	0.00	0.00	0.00	0.00	0.00	0.00
Smooth muscle cells	0.00	0.00	0.00	0.00	0.00	0.00
αSMA						
Epithelium	0.00	0.00	0.00	0.00	0.00	0.00
Stromal cells	0.00	0.00	0.00	0.00	0.00	0.00
Endothelium	0.00	0.00	0.00	0.00	0.00	0.00
Pericytes	4.8±0.4	2.6±0.6	7.4±0.6	4.8±0.4	2.4±0.5	7.2±0.5
Glial cells	0.00	0.00	0.00	0.00	0.00	0.00
Smooth muscle cells	4.8±0.4	3.2±0.6	8.0±0.6	4.8±0.4	2.8±0.5	7.6±0.5
VIM						
Epithelium (BC, ICS)	1.4±0.5	2.8±0.5	4.2±0.5	1.4±0.5	2.8±0.5	4.2±0.5
Stromal cells	3.8±0.4	3.4±0.4	7.2±0.4	3.8±0.4	3.2±0.5	7.0±0.7
Endothelium	4.8±0.4	2.8±0.6	7.6±0.6	4.8±0.4	2.6±0.6	7.4±0.6
Pericytes	0.00	0.00	0.00	0.00	0.00	0.00
Glial cells	4.8±0.4	3.2±0.6	8.0±0.6	4.8±0.5	3.4±0.5	8.2±0.5
Smooth muscle cells	1.2±0.5	0.8±0.5	2.0±0.5	1.2±0.4	1.2±0.6	2.4±0.6

BC, basal cells; ICS, intercellular spaces.

ed of smooth muscle layers (Figs. 1c,h).

Immunohistochemical observations

Immunostainings for ki-67, αSMA, and VIM were detected in the different compartments of both reticulum and omasum. There were no immunostainings for ki-67, αSMA, and VIM in the negative control sections (Fig. 5).

Immunostaining for ki-67

As shown in Table 1, the immunostaining for ki-67 was restricted to the epithelia of reticulum and omasum (Fig. 2); however, there was non-significant difference in immunoreactivities of reticulum and omasum for ki-67. Fibroblasts in propria and submucosa, and smooth muscle cells of both reticulum and omasum showed no immunostaining for ki-67, while nuclear ki-67 was distributed only in the basal cells layers in the epithelia of reticulum and omasum (Figs. 2b, d).

Immunostaining for αSMA

Immunostaining for αSMA was localized to cytoplasm of the positive cells. It was distributed in the aggregation of smooth muscle cells in reticular folds (Figs. 3a,b) and the smooth muscle cells sheet in omasal laminae (Figs. 3d,e). Moreover, αSMA immunostainings were seen in smooth muscle cells in media of blood vessels (Figs. 3c,f,h), muscularis (Figs. 3c,f,g), and pericytes of blood capillaries (Figs. 3e,f) in both reticulum and omasum. Epithelial cells and fibroblasts of propria and submucosa showed no immunostaining for αSMA in reticulum and omasum (Fig. 3 and Table 1). There were non-significant differences between immunoreactivities of reticulum and omasum for αSMA (Table 1).

Immunostaining for VIM

Immunostaining for VIM was localized to cytoplasm of the positive cells. It was distributed throughout the different compartments of the reticulum and omasum (Fig. 4 and Table 1). VIM immunostaining was seen in boundaries of the intercellular spaces and in some spinous cells in the epithelia of both reticulum and omasum (Figs. 4b,f). Moreover, VIM immunostainings were detected in fibroblasts of propria and submucosa and endothelia of blood capillaries and vessels in the reticulum (Figs. 4b-d) and omasum (Figs. 4f,h,i). Also, glial cells in submucosal and myentric plexuses were VIM positive (Figs. 4g,h,j). Smooth muscle cells of blood vessels and muscularis in both reticulum and omasum showed weak VIM immunostaining (Figs. 4c,d,h,i and Table 1). There were non-significant differences between immunoreactivities of reticulum and omasum for VIM (Table 1).

DISCUSSION

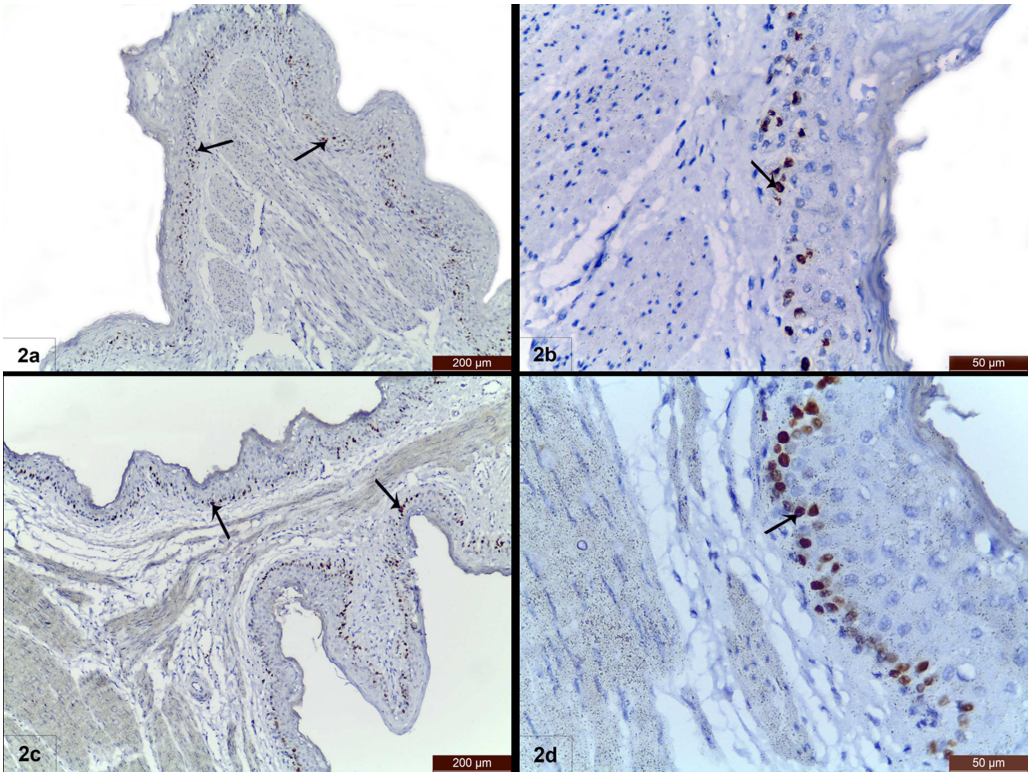

Fig. 2. Immunohistochemical staining for ki-67 in reticulum and omasum of goat. **(a)** General view of immunostaining for ki-67 in reticular fold. (arrows) refer to positive cells in epithelium only. **(b)** A higher magnification of (a) showing immunostaining for ki-67 in basal cells layer of the reticular epithelium. **(c)** General view of immunostaining for ki-67 in omasal fold. (arrows) refer to positive cells in epithelium only. **(d)** A higher magnification of (c) showing immunostaining for ki-67 in basal cells layer of the omasal epithelium. Scale bars: (a,c) 200 µm; (b,d) 50 µm.

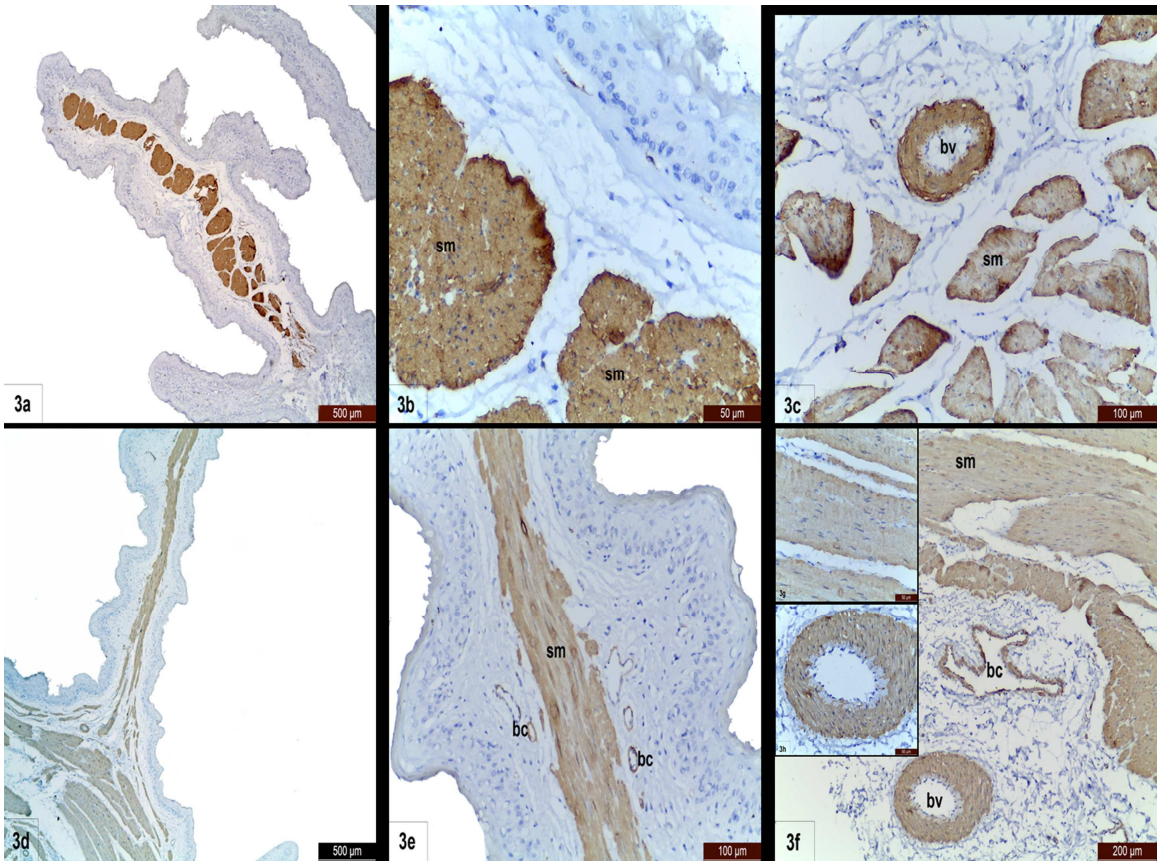

Fig. 3. Immunohistochemical staining for αSMA in reticulum and omasum of goat. **(a)** General view of immunostaining for αSMA in reticular fold. **(b)** A higher magnification of (a) showing immunostaining for αSMA in the aggregated smooth muscle cells in the reticular fold. **(c)** Immunostaining for αSMA in smooth muscle cells of blood vessels (bv) and tunica muscularis (sm) of reticulum. **(d)** General view of immunostaining for αSMA in omasum. **(e)** A higher magnification of (d) showing immunostaining for αSMA in pericytes of blood capillaries (bc) and smooth muscle cells (sm) in the mid of omasal lamina. **(f)** Immunostaining for SMA in pericytes of of blood capillaries (bc), smooth muscle cells of blood vessels (bv) and tuinca muscularis (sm) of omasum. **(g)** A higher magnification of (f) showing immunostaining for SMA in smooth muscle cells of tunica muscularis. **(h)** A higher magnification of (f) showing immunostaining for SMA in smooth muscle cells in media of blood vessels. Scale bars (d) 500 µm; (a,f) 200 µm; (c,e) 100 µm; (b,g,h) 50 µm.

Reticular folds and omasal laminae of Baladi goats were covered with keratinized, stratified, squamous epithelium, which consisted of 4 strata: basalis, spinosum, granuluosum, and corneum, which was in consonance with El-Gendy and Derbalah (2010); Garcia et al. (2013a, 2013b) in goats, and Dilda et al. (2012) in cows. This multi-cellular and multi-layer structure of the reticular and omasal epithelia, under normal conditions, prevents the translocation of toxic compounds into blood (Plaizier et al., 2012). Intercellular spaces in the spinous and basal layers were seen in the epithelia of both reticulum and omasum, but they were larger in the omasum than in the reticulum, a fact that was in agreement with Scala et al. (2011). The other histological structures of reticulum and omasum of the Baladi goat (lamina propria, muscularis mucosae, submucosa and muscularis) were in accordance with previous reports of Garcia et al. (2014b).

Our immunohistochemical results detected ki-67 immunostaining only in the epithelia of both reticulum and omasum of Baladi goats, which was in accordance with Blättler et al. (2001) in calves. Ki-67 immunostaining was localized mainly in the basal cells layer of the epithelia that refer to starting of keratin biosynthesis in the basal cells. In addition, ki-67 in basal cells helps in permanent renewal of the epithelial cells that was supported by Bjerknes and Cheng (2005) and Conto et al. (2010). Knowledge of the proliferation pattern is important for understanding of the normal function, and may contribute to the understanding of reticulum and omasum diseases.

The aggregation of smooth muscle cells in reticular folds, the sheet of smooth muscle cells in omasal laminae, smooth muscle cells of tunica muscularis and those of blood vessels were immunoreactive for αSMA, facts that coincided with Kitamura et al. (2003) on the forestomach of goats and other ruminants. Such findings about the distribution profile of αSMA provide additional

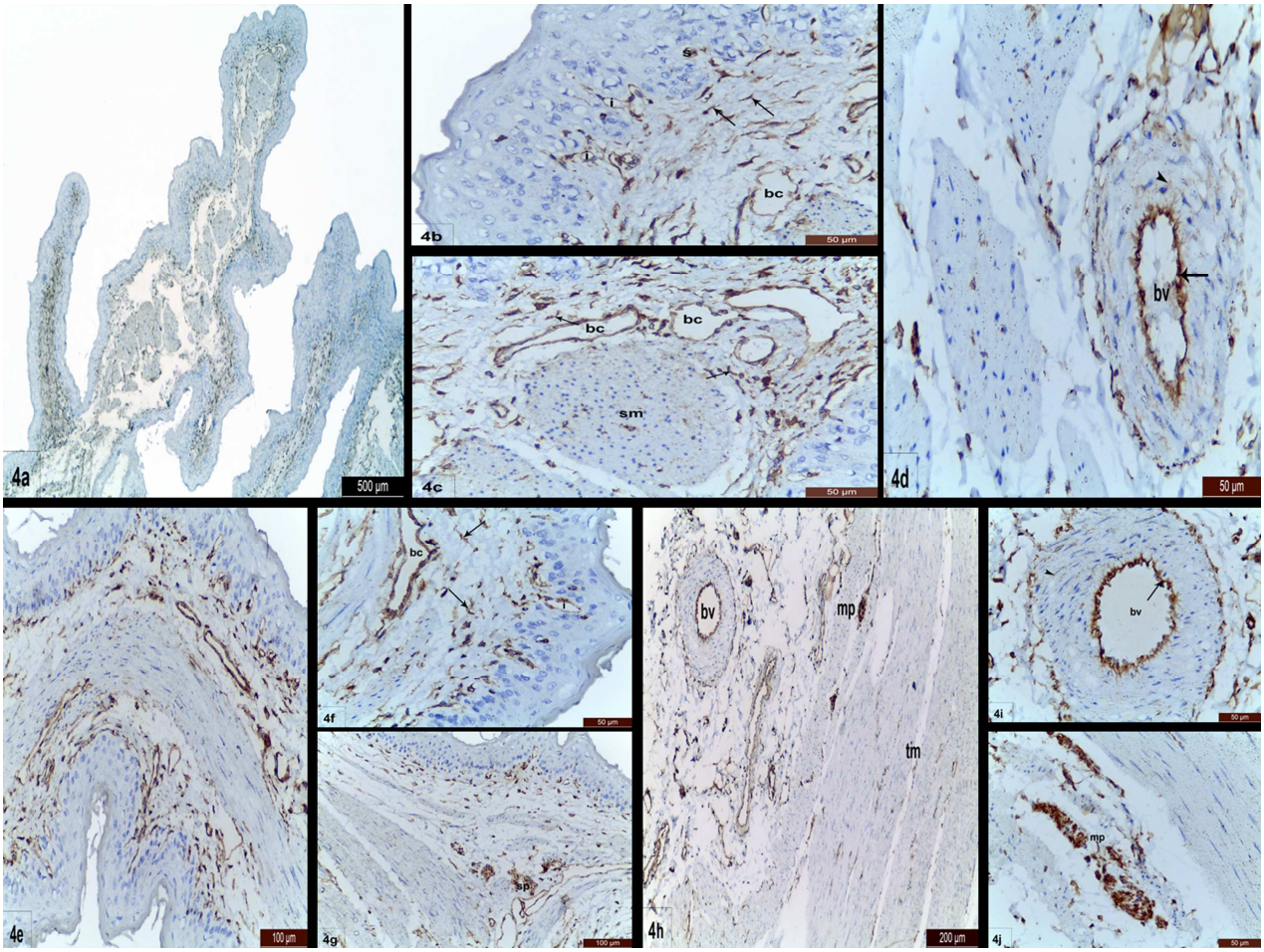

Fig. 4. Immunohistochemical staining for VIM in reticulum and omasum of goat. **(a)** General view of immunostaining for VIM in reticular fold. **(b)** A higher magnification of (a) showing immunostaining for VIM in intercellular spaces (i) in spinous layers of epithelium; stromal cells (arrows); endothelium of blood capillaries (bc) in reticulum. **(c)** A higher magnification of (a) showing immunostaining for VIM in endothelium of blood capillaries (bc); stromal cells (arrows); somewhat in the aggregation of smooth muscle cells (sm). **(d)** Immunostaining for VIM in endothelium (arrow) and smooth muscle cells (arrowhead) of blood vessels (bv). Note weak VIM in smooth muscle cells of tunica tunica muscularis. **(e)** General view of immunostaining for VIM in omasal lamina. **(f)** A higher magnification of (e) showing immunostaining for VIM in intercellular spaces (i) in spinous layers of epithelium; stromal cells (arrows); endothelium of blood capillaries (bc) in omasum. **(g)** A higher magnification of (e) showing immunostaining for VIM in glial cells of submucosal plexus (sp). **(h)** Immunostaining for VIM in myentric plexus (mp) and blood vessels (bv). Note weak VIM in smooth muscle cells of tunica tunica muscularis (tm). **(i)** A higher magnification of fig. (h) showing VIM immunostaining in endothelium (arrow) and smooth muscle cells (arrowhead) of blood vessels (bv). **(j)** A higher magnification of fig. 4h showing VIM immunostaining in glial cells of myentric plexus (mp). Scale bars: (a) 500 µm; (h) 200 µm; (e) 100 µm; (b,c,d,f,g,I,j) 50 µm.

knowledge to the understanding of the physiology of contractility of the reticulum and omasum in the Baladi goat. Also, the expression of pericytes to αSMA supports the assumption of their ability to contract (Skalli et al., 1989).

In the present study, immunostaining for VIM was demonstrated in boundaries of the intercellular spaces and some spinous cells in epithelia of reticulum and omasum. Conversely, VIM was not seen during prenatal life in the reticulum and omasum of goats (Garcia et al., 2014a, 2013a, 2013b) or in red deer (Masot et al., 2007; Redondo et al., 2005). Moreover, our study demonstrated VIM in the endothelia of blood capillaries and vessels in the reticulum and omasum. This finding has nor been reported in previous studies on goats and red deer. Such findings indicate that the action of vimentin as cytoskeleton protein (intermediate filaments) may be associated with postnatal life to support the cells of the reticulum and omasum of goats during postnatal life, where vimentin plays an important role in supporting the organelles in the cytosol of cells (Katsumoto et al., 1990).

Our findings about demonstration of VIM in epithelia and endothelial cells supports the findings of El-Gendy and Derbalah (2010), which ultrastructurally identified many intermediate filaments in cells of stratum spinosum in the omasum of Baladi goats. Moreover, fibroblasts in lamina propria and submucosa of the reticulum and omasum showed VIM immunoreactivity that was similar to the findings of Ikemizu et al. (1994) in the bovine rumen.

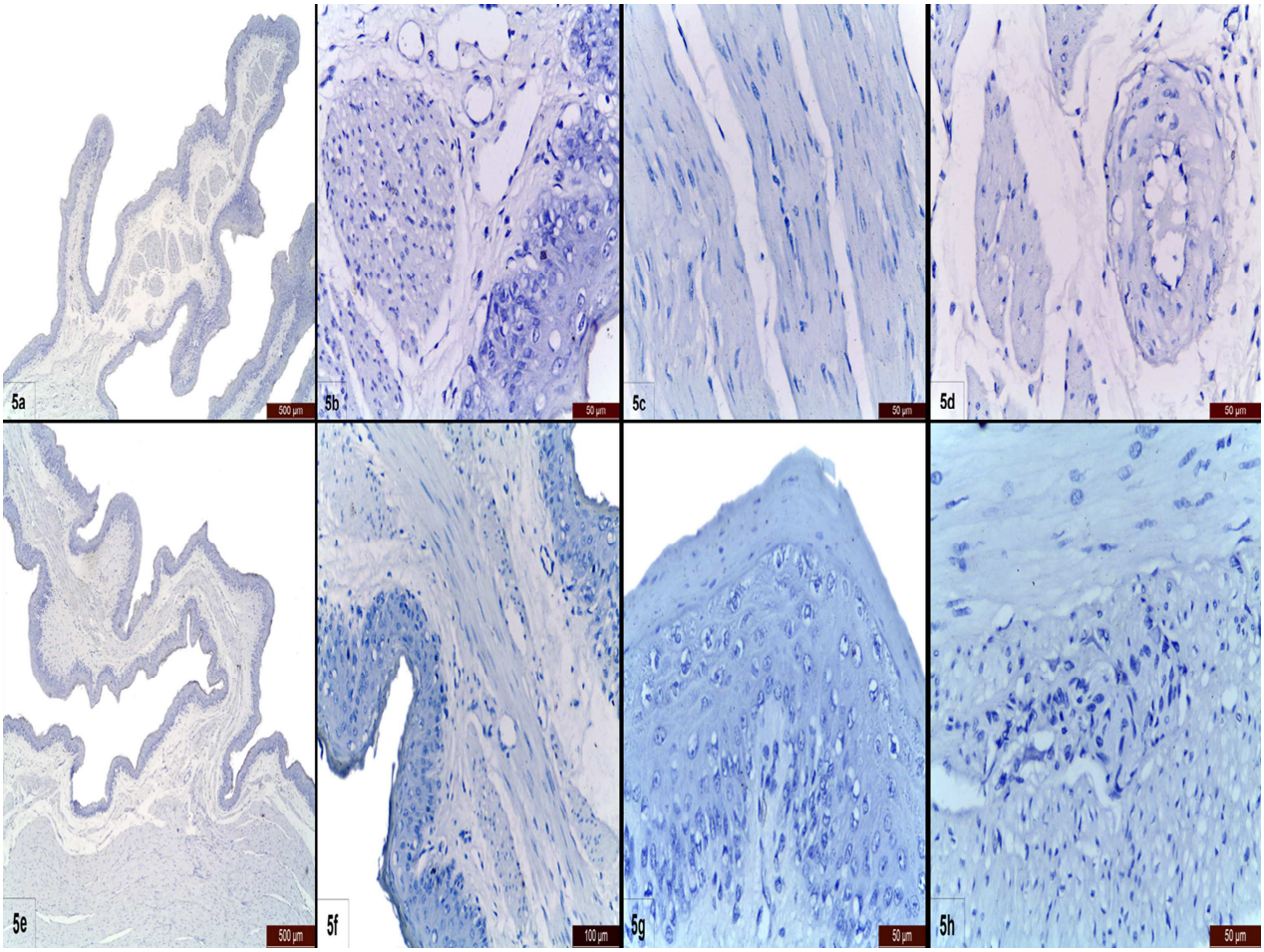

Fig. 5. Negative control sections for ki-67, αSMA and VIM in reticulum **(a-d)** and omasum **(e-h)** of goat.

Our results revealed that visible VIM immunostaining in glial cells of submucosal and myentric plexuses in both reticulum and omasum that was in accordance with the findings of Garcia et al. (2014a, 2013a, 2013b) in goats, and Franco et al. (2004), Masot et al. (2007), Redondo et al. (2005) in red deer during the prenatal period. Also, Teixeira et al. (1998), in the bovine reticulum, described immunoreactivity for glial cells in the reticular folds. The identification of glial cells in the reticulum and omasum of Baladi goat supports the critical role of glial cells as non-neuronal elements of the enteric plexuses in controlling gastrointestinal functions and protecting enteric neurons (Abdo et al., 2010).

Conclusion

In the present study, ki-67 was localized to the basal cell layer of the epithelia, αSMA was localized to smooth muscle cells and pericytes, and VIM was widely distributed in epithelia, fibroblast and glia cells. Such findings indicate the role of ki-67 in epithelial cell proliferation, αSMA in muscular motility, and VIM as a cellular cytoskeleton in the reticulum and omasum of Baladi goats. Non-significant differences were detected in immunoreactivities of reticulum and omasum for ki-67, αSMA and VIM.

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