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Immunohistochemical Evaluation of Ruminal Lamina Propria-α Smooth Muscle Actin in Sheep Fed on Concentrate

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Abstract: The objective of this study was to characterize the immunohistochemical localization of lamina propria-α smooth muscle actin (α SMA) in the rumen of sheep after changing the diet from hay (*ad libitum*) to a mixed hay/concentrate diet. A total of 24 sheep were fed mixed hay/concentrate for different periods ranging from 0 weeks (control; hay *ad libitum*) to 12 weeks (1-1.5 kg hay plus 780 g concentrate per day in two equal portions). Using immunohistochemical technique the present study confirmed the existence of a specialized layer in the rumen mucosa of sheep, which composed of actin-immunoreactive cells. These cells were distributed as a condensed accumulation in the layer at the position equivalent to the muscularis mucosa. Intense lamina propria-αSMA expression (very thick layer) was seen in 4 weeks concentrate-fed sheep.

Key words: Actin · Sheep · Rumen · Feed · Immunohistochemistry

INTRODUCTION

The lamina muscularis mucosa is a thin layer consisting of aggregation of smooth muscle cells between the tunica mucosa and the tunica submucosa. In the omasum, it occurs as a continuous muscle layer following contours of the omasal laminae. It is also present in the reticulum but confided to the upper core of the reticular crest (Crista reticuli). On the other hand, the rumen is reported to contain a condensed fibrous layer, which mimics the muscularis mucosa [1-4]. In some studies, this condensed fibrous layer is considered to be the muscularis mucosa of the rumen. In a study on the buffalo rumen, it has been referred to as lamina muscularis mucosa [5]. However, other references have described it not as muscularis mucosa, but as a condensed fibrous layer [4, 6] connective tissue band [7] or condensation of connective tissue similar to the stratum compactum [1, 8]. On the other hand, α SMA and γ SMA are the major actin isoforms of vascular and enteric smooth muscle cells, respectively [9]. The occurrence of occasional single smooth muscle cells was found at the limit between propria and submucosa [10]. In cattle, water buffalo, sheep, goat, Barbary sheep, japanese serow, sika deer and mouse deer, the muscularis mucosa in both reticulum and omasum show immunoreactivity for both αSMA and γSMA [4]. Nevertheless, the condensed fibrous layer in the rumen of these mentioned animals is immunoreactive only for lamina propria-αSMA. Furthermore, the smooth muscle cells of the external muscle layer are immunoreactive for αSMA and γSMA whereas those of blood vessels and pericytes are only for αSMA.

MATERIALS AND METHODS

Experimental Animal: A total of 24 of German dairy sheep of different sex were used in this study. Animals were 9-10 months old at the time of the experiment and their weights ranged between 33.5-50 kg. The animals were divided into 8 groups of three animals in each group.

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Feedings: Prior to the experiment the sheep were fed only hay ad libitum for at least 8 weeks, in order to adapt them to a low-energy food. Thereafter, the sheep were either solely fed hay (1-1.5 kg hay/day) (Control-group) or received, in addition to that, 780 g concentrate (Experimental-groups). The experimental animals were assigned to 7 groups according to the duration of concentrate feeding: 2, 4 days, 1, 2, 4, 6 and 12 weeks. Introduction of concentrate diet was preceded by an adaptation period of 4 days in which the concentrate feed was increased gradually. The concentrate diet was supplied in equal portions at 07.00 am and 02.30 pm. All animals had free access to tap water and salt block.

Compositions of Diet: The nutrient content of both concentrate and hay rations were shown in Tables 1 and 2, respectively.

Table 1: Composition of the concentrate diet

Nutrients	%	Nutrients	%
Dry matter	89.1	Organic ADF	13.28
Crude ash	6.42	Organic NDF	25.46
Crude protein	18.03	ADL	3.99
Crude fiber	9.65	Vitamin A	7200 Iu
Calcium	0.65	Vitamin D3	1800 Iu
Phosphorus	0.59	Selenium	0.5 mg
Magnesium	0.27	Copper	10 mg
Potassium	1.35	DCAB	+299 meq/kg DM
Sodium	0.42	Net energy lactation (NEL)	6.7 MJ/kg
Chloride	0.46	Metabolic energy (ME)	10.41 MJ/kg
Sulfur	0.21		

Table 2: Composition of hay diet

Nutrients	%	Nutrients	%
Dry matter	93.5	ADL	4
Crude ash	4.9	Non fibers carbohydrates (NFC)	22.5
Crude protein	8.8	Metabolizable energy (ME)	9.3 MJ/Kg
Crude fiber	29.3	Net energy lactation (NEL)	5.5 MJ/Kg
Potassium	1.44	Usable crude protein (nXP)	120.3 g/Kg
Sodium	0.032	Degradable crude protein (UDP)	17.6 g/Kg
Organic ADF	34	Ruminal nitrogen balance (RNB)	-5.2 g/Kg
Organic NDF	56.5		

Table 3: Composition of the transport buffer

Compound	Concentration (mmol / L)
Sodium	145.2
Potassium	5
Calcium	1
Magnesium	1
Bicarbonate	25
Chloride	120
Dihydrogenphosphate	0.4
Hydrogenphosphate	2.4
Glucose	5
pH value	7.4
Osmolarity	300 mosmol/L

Tissue Collection and Preparation: Following the feeding trial and at the end of each experimental period, Full thickness mucosa or rumen papillae isolated directly with scissors from identical site of the rumen (Left wall of the recessus ruminis ventral sac; adjacent to the left longitudinal groove) [11]. The ruminal mucosa was cleaned by immersion in a transportation buffer solution (See Table 3). Immunohistochemical staining was performed on both cryostat sections and on deparaffinized sections of the ruminal epithelium. Full thickness mucosa or rumen papillae were chilled with liquid nitrogen and stored at -76 °C. Cryostat sections were cut 5 µm thick and collected on microscope slides precoated with Silane solution (3-Aminopropyl) (Co. Sigma, Code No. A3648). The sections were fixed in acetone for 9 sec. and dried for at least 1h at room temperature, then refrozen and stored at -20 °C. Prior to staining the sections were dried 1h at room temperature and fixed in cold aceton for 8 min. before immunostaining. Fresh rumen- mucosal tissues were fixed in 4% formalin solution for 24 hours [12]. After rinsing with water, samples were stored in 0.1 mmol / L phosphate buffer (pH 7.2). They were then dehydrated in graded series of ethanol alcohol, cleared with xyline, saturated with and embedded in paraffin. Tissue blocks were then cut at 5-7µm thickness and collected on microscope slides precoated with Silane solution. Tissues were deparaffinized, hydrated, rinsed twice with distilled water before immunostaining.

Antibodies

Primary Antibody: Mouse monoclonal anti-human smooth muscle Actin, (Fa. Serotec, Oxford, UK) Catalog Nr. MCA 1905.

Secondary Antibody: ShpXMs (Fab`2) Ig Biotin conjugate, Cat. No. AQ300B, (Fa Chemicon, California, USA).

Negative Controls: The specificity of the staining reaction was determined in control experiments. These comprised either substitution of the primary antibody by PBS, normal mouse serum 1:200 (Negative control Ig GI Cat, No. X0931, Fa DakoCytomation, Carpinteria, USA), or omission of the primary antibody; in all cases, sections were negative.

Procedure of Immunohistochemistry: For immunolabeling of α smooth muscle actin, both frozen and deparaffinized sections were stained according to the protocol Dako Envision+System, Peroxidase (DAB)

(Fa Dako Cytomation, Carpinteria / USA). Lamina propria-α smooth muscle actin was detected in deparaffinized sections. All immunohistochemistry was repeated for material obtained three animals and examined directly under a light microscope (Leica DM LB, Leica Microsystems, Wetzlar, Germany).

Tissues were deparaffinized, hydrated, washed in PBS (PBS-Puffer to Dulbecco Cat. No.47302, Fa. Serva, Heidelberg, Deutschland) for 5 min. Tissues were immersed in Methanol-hydrogen peroxide-solution for 20 min. Then, the sections were washed in PBS for 10 min and blocked with Protein block for 30 min. The sections were incubated either with the primary antibody at dilutions of 1:200 in PBS buffer (Overnight at 4 °C) or with control serum at dilutions of 1:200 (Control experiments) for 30 min in a humidified chamber. The sections were washed in PBS for 10 min. Then, sections were incubated with the secondary antibody for 30 min at room temperature. Sections were rinsed in washing PBS buffer for 10 min. Then, sections were incubated in Detection system (Streptavidin-Biotin-Peroxidase for 30 min at room temperature. Sections were rinsed in washing PBS buffer for 10 min. Sections were immersed in POD-nachweis with b3.3`-Diaminobenzidine Tablets D-5905 (Fa. Sigma-Aldrich, Taufkirchen, Deutschland) at room temperature in darkness for 5-20 min, followed by Washing with running tap water for 10 min. Then, sections were counterstained with Mayer's Haematoxylin for 10 seconds, rinsed in distilled water and washed with running tap water for 5 min. Then sections were dehydrated through 70% ethanol alcohol for 5 min and 100% ethanol alcohol three times for 5 min each, cleared three times in xyline for 5 min each and mounted with Canada balsam permanent mounting media (Sigma-Aldrich).

RESULTS

The present study showed immunoreactivity of condensed fibrous layer, the vascular wall and the muscular layer for α SMA (Fig. 1). Muscularis mucosa was not detected in the rumen of all sheep groups. Instead, numerous cells showing constantly intense immunoreactivity for α SMA were observed as a continuous muscle layer following contours of the ruminal papillae and in the interpapillary mucosa near the epithelium (Fig. 1). Additionally, more abundant actin-immunoreactive cells were detected in the ruminal papillae than the interpapillary mucosae (Fig. 1). The relative intensities of immunohistochemical expression of lamina propria-α SMA in the rumen were assessed in hay-fed sheep (Control group) and in all concentrate-fed groups. Generally, α SMA-immunostaining was found to be more intense in concentrate-fed groups compare to that of hay-fed sheep (Fig. 2; Table 4). On the basis of visual examination, the relative intensities of α SMA immunoexpressions were designated as very weak (+), weak (2+), moderate (3+), strong (4+) and very strong (5+) (Table 4). Immunohistochemical staining for lamina propria- α SMA in rumen showed different degrees of staining among sheep groups. The degree of antibody reaction was very weak (Thin layer) in hay-fed group, weak in 2 days and 12 weeks, moderate in 4 days and 1week concentrate-fed group, strong in 2 and 6 weeks concentrate-fed groups and very strong (Thick layer) in 4 weeks concentrate-fed group (Fig. 2; Table 4). On the other hand, In 4 weeks concentrate-fed group, large density of blood vessels was observed comparing to hay-fed group (Fig. 3).

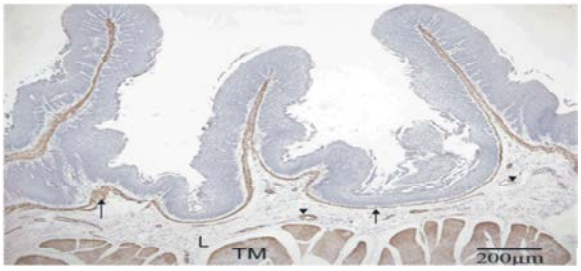

Fig. 1: Micrograph of the rumen of the sheep fed concentrate for one week. Tunica muscularis (TM), vascular wall (Arrow heads) and condensed fibrous layer (Arrows) showed immunoreactivity for α-smooth muscle actin. L: lamina propria. (Scale 200µm)

Table 4: The effect of type and duration of concentrate feeding on the expression of α-smooth muscle actin in condensed fibrous layer. (N = 3 animals for each treatment)

Animals	condensed fibrous layer
CF0W	+
CF2 D	++
CF4 D	+++
CF1W	+++
CF2W	++++
CF4W	+++++
CF6W	++++
CF12W	++

+= Very weak reaction ++= weak +++= moderate ++++= strong +++++= very strong

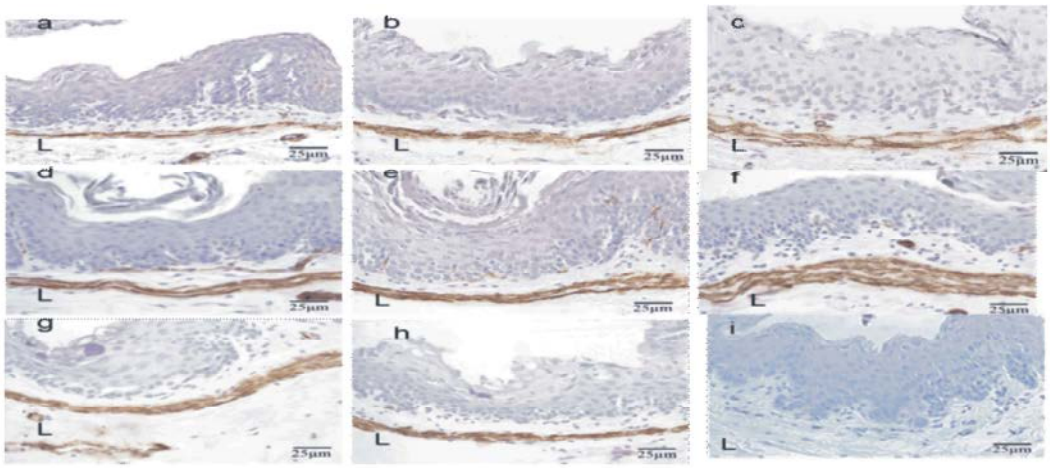

Fig. 2: Micrographs of the rumen of a. Hay-fed group, b. 2 days concentrate-fed group, c. 4 days concentrate-fed group, d. one week concentrate-fed group, e. 2 weeks concentrate-fed group, f. 4 weeks concentrate-fed group, g. 6 weeks concentrate-fed group and h. 12 weeks concentrate-fed group. i. Negative control. Notice the different thickness of the condensed fibrous layer (Immunostained for α-smooth muscle actin). (Scale 25µm)

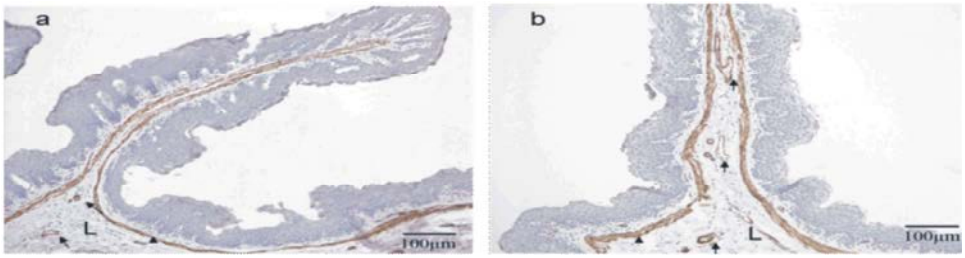

Fig. 3: Micrographs of the ruminal papilla from a. hay-fed group (Control), b. 4 weeks concentrate-fed group, showed immunoreactivity of both condensed fibrous layer (Arrow heads) and vascular wall (Arrows) for α-smooth muscle actin. Notice the large numbers of the blood vessels located at the papillary core of 4 weeks concentrate-fed group. L: lamina propria. (Scale 100µm)

DISCUSSION

Different studies reported the absence of muscularis mucosa in the rumen of domestic ruminants; however, they described the presence of smooth muscle cells and the presence of condensed fibrous layer [1, 6, 10, 13, 14]. Nevertheless, the presence of distinct layer containing smooth muscle cells, which has been referred to as lamina muscularis mucosa has been reported in the ruminal mucosa of the Buffalo [5]. However, the condensed fibrous layer in the bovine ruminal mucosa has been detected immunohistochemically and ultrastructurally [2, 4].

Using immunohistochemical technique, the present study confirmed the existence of a specialized layer in the rumen mucosa of sheep, which composed of α SMA-immunoreactive cells. On the other hand, a strong and very strong antibody reaction (Thick and very thick layers) was found in 2, 6 and 4 weeks concentrate-fed sheep, respectively. These cells were distributed as a condensed accumulation in the layer at the position equivalent to the muscularis mucosae.

Recently it was reported that, both type and duration of concentrate feeding affected size and shape of the ruminal papillae and among all concentrate-fed groups, 4 weeks concentrate-fed group showed the maximum increase in the papillae-dimensions (Length and width) [15]. Actin is known for its contractile and cytoskeletal functions in various types of cells [9, 16]. Thus, the condensed layer comprising these cells may play an important functional role in some physiological conditions.

Numerous blood vessels, which showed intensive immunoreactivity for α SMA, were observed in 4 weeks concentrate-fed sheep compared to hay-fed as well as other groups of concentrate-fed animals. Increase number of blood capillaries could also aid absorption [17].

CONCLUSION

Using immunohistochemical technique the present study confirmed a significant effect on the immunoreactivity of lamina propria-α smooth muscle actin (α SMA) in the rumen of sheep associated to the diet change. At 4 weeks concentrate-fed sheep the rumen mucosa showed intense lamina propria α-SMA staining.

ACKNOWLEDGEMENTS

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REFERENCES

1. Banks, W.J., 1993. Applied Veterinary Histology. 3rd ED. St louis: mosby.
2. Ikemizu, T., N. Kitamura, J. Yamada and T. Yamashita, 1994. Is lamina muscularis mucosae present in the ruminal mucosa of cattle? Immunohistochemical and ultrastructural approaches. Anat Histol Embryol., 23: 177-186.
3. Mosimann, W. and T. Kohler, 1990. Zytologie, Histologie und mikroskopische Anatomie der Haussäugertiere. Paul Parey. Berlin und Hamburg. Verdauungsorgane, pp: 166-168.
4. Kitamura, N., A. Yoshiki, M. Sasaki, E.T. Baltazar, E. Hondo, Y. Yamamoto, S. Agungpriyono and J. Yamada, 2003. Immunohistochemical evaluation of the muscularis mucosae in the ruminant forestomach. Anat Histol Embryol., 32: 175-178.
5. Taluja, J.S. and R.P. Saigal, 1987. Evidence of lamina muscularis mucosae in rumen of buffalo. Anat Histol Embryol., 16: 150-152.
6. Banks, W.J., 1981. Applied Veterinary Histology, first ED. Baltimore: The Williams and Wilkins Co.
7. Bacha, W.J. Jr and L.M. Wood, 1990. Color Atlas of Veterinary Histology. Philadelphia: Lea and Febiger.
8. Liebich, H.G., 1999. Funktionelle Histologie der Haussäugertiere. Lehrbuch und Farbatlas für Studium und Praxis. 3. Auflage. Schattauer. Stuttgart, New York, pp: 193-196.
9. Vandekerckhove, J. and K. Weber, 1978. At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence on the amino-terminal tryptic peptide. J. Mol Biol., 126: 783-802.
10. Dellmann, H.D., 1971. Veterinary Histology. An Outline Text-Atlas Lea and Febiger. Philadelphia. In Digestive System, pp: 155-164.
11. Gäbel, G., H. Martens, M. Suendermann and P. Galfi, 1987. The effect of diet, intraruminal pH and osmolarity on sodium, chloride and magnesium absorption from the temporarily isolated and washed reticulo-rumen of sheep. Q J. Exp Physiol., 72: 501-511.
12. Romeis, B., 1989. Mikroskopische Technik. Verlag Urban und Schwarzenberg, München, Wien und Baltimore, 17. Auflage.
13. Krölling, O. and H. Grau, 1960. Lehrbuch der Histologie und verglichenden mikroskopischen Anatomie der Haustiere. Paul Parey, Berlin.
14. Dellmann, H.D. and E.M. Brown, 1987. Textbook of Veterinary Histology. 3rd ED, Lea and Febiger, Philadelphia.
15. Rasha, S. Ahmed, H. Martens and C. Muelling, 2013. Scanning Electron Microscopical and Morphometrical Studies on Ruminal Papillae of Sheep Fed Concentrates. J. Anim Res., 3: 111-123.
16. Joyce, N.C., F.H. Marcy and G.E. Palade, 1987. Morphologic and biochemical evidence for a contractile cell network within the rat intestinal mucosa. Gastroenterology., 92: 68-81.
17. McGavin, M.D. and J.L. Morrill, 1976. Scanning electron microscopy of ruminal papillae in calves fed various amounts and forms of roughage. Am J Vet Res., 37: 497-508.