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Original article

Immunohistochemical study on the development of cholinergic and nitrergic nerve structures in the bovine esophageal groove

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Abstract

The proper functioning of the perinatal sucking reflex in calves is essential for the prevention of milk leakage into the rumen. The complex process behind its regulation is mediated at the gut level via multiple excitatory and inhibitory neurotransmitters, of which acetylcholine and nitric oxide are of fundamental importance. The aim of our study was to depict age-related alterations in the cholinergic and nitrergic innervation of the esophageal groove (EG) using immunohistochemistry and Real-Time PCR methods. We found out that the highest number of cholinergic nerve cells was present in the second trimester fetuses. From this developmental stage onward, their amount was gradually decreasing and reached the lowest value in 4-year-old cows. The same developmental pattern was observed for nitrergic nerve structures with the highest percentage of nitrergic neurons in the third trimester fetuses. Our observations prove that both neuronal populations are crucial for a proper closure of EG in calves. Therefore, their contribution to a general neuronal activity in the ENS diminishes with age as the high motility of a gastric groove is not necessarily required in older cattle.

Key words: esophageal groove, acetylcholine, nitric oxide, immunohistochemistry, development

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Introduction

The esophageal (gastric) groove (EG) is the shortest route between the esophagus and the pylorus consisting of three segments: the reticular, omasal, and abomasal groove (Budras 2003). In healthy, suckling calves, milk bypasses the forestomachs and enters into the abomasum where enzymatic digestion of milk proteins takes place (Kaba et al. 2018). A calf using only the abomasum to digest milk or milk replacer is considered monogastric for the first two weeks after birth (Guilhermet et al. 1975, Kaba et al. 2018). Therefore, the proper closure of the EG is essential for the prevention of milk leakage into the rumen which may induce ruminal and metabolic acidosis in a clinical case (Gentile et al. 2004, Herrli-Gygi et al. 2008, Labussiere et al. 2014). The complex process behind its regulation, referred to as perinatal sucking reflex, is mediated at the gut level via multiple excitatory and inhibitory neurotransmitters, of which acetylcholine and nitric oxide (NO) are of fundamental importance (Olsson and Holmgren 2011, Dreher et al. 2019).

The enteric nervous system (ENS) is entirely derived from the neural crest, a transient structure that arises early in development during formation of the neural tube (Goldstein et al. 2013). Emigres from the vagal region of the neural crest, adjacent to somites 1–7, provide the main contribution to the formation of the enteric ganglia and give rise to the majority of ENS cells (Weyns 1988, Furness 2006, Goldstein et al. 2013), including cholinergic and nitrergic neuronal populations.

Acetylcholine is the primary transmitter of excitatory neurons innervating the smooth muscle and main substance released from terminals at the end of the vagus nerve projections to the digestive tract components (Furness 2006, Harrington et al. 2010). Cholinergic population of nerve cells in the ENS comprises ascending excitatory motor neurons, as well as ascending and descending interneurons, whereas descending inhibitory motor neurons are considered nitrergic (Furness 2006, Arciszewski and Zacharko-Siembida 2008, Olsson and Holmgren 2011). NO is thought to be an important mediator of inhibitory neurotransmission of non-adrenergic, non-cholinergic neurons (NANC), however some of the descending interneurons may contain both NO and acetylcholine (Furness 2006, Olsson and Holmgren 2011, Groneberg et al. 2016, Sanders 2016, Sanders and Ward 2019). Therefore, the aforementioned neurotransmitters – as the key components in the gastrointestinal motility regulation – play a crucial role in the closure of the EG, in both the pathological and physiological conditions.

The aim of our study was to depict age-related alterations in the cholinergic and nitrergic innervation of the EG. We would like to focus on changes in the total number of nerve cells immunoreactive (IR) for aformentioned neuropeptides, as well as on the distribution of acetylcholine- and NO-expressing nerve fibers suppling the investigated organ. Eventually, based on our outcomes, we intended to elucidate the role of those substances in the forestomach motility at different developmental stages. Our observations may, therefore, help to better understand the physiology behind many gastrointestinal disorders in ruminants resulting from an abnormal functioning of the EG.

Materials and Methods

Materials

The study was performed in three groups of bovine (bos taurus taurus) fetuses (from the first, second, and third trimester of pregnancy) and in three groups of the individuals collected after birth (calves at age of six weeks, two-year-old and four-year-old cows). Each experimental group consisted of 5 individuals. All of the specimens were obtained immediately after slaughter. In accordance to the Polish legislation and the Act on animal protection (Act of 21 August 1997, Dziennik Ustaw, 1997 No. 111, item 724, with later amendments, uniform text Dziennik Ustaw 2017, item 1840), experiments performed on tissues collected from animals during routine veterinary procedures, or in slaughterhouse, do not require the consent of the Institutional Animal Care and Use Committee. In order to determine the exact age of the fetuses, we measured their size from the tip of the head to the base of the tail (de Bruin and Wyman 1901), as demonstrated in Table 1 where particular data on the specimens can be found.

Immunohistochemistry

The representative samples of the cranial, middle, and caudal part of EG containing both right and left lips, as well as fundus, were removed from animals of groups I, II, III, IV, V and VI. Due to the great size of EG, the samples obtained from the animals of group V and VI were cut in half at the level of the fundus before preserving for further processing. The tissues were fixed by immersion in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.4 (PBS). After 24 hours of fixation, they were rinsed in PBS, transferred into 30% saccharose solution in PBS, and stored until they sank to the bottom of the container. Then they were immersed in the matrix for cryostat sectioning (Tissue-Tek O.C.T. Compound). Serial cryostat sections 16 µm thick were put on chrome alum-coated slides and stored in a freezer (-30˚C) until further processing. After washing with PBS (3 × 10 min), the sections were processed

Table 1. Animals used in the study. N/A – not applicable.

Group no.	Age	Length of the body (cm)	No. of specimens per group
I	2-3nd month of pregnancy	7-12	5
II	5-6th month of pregnancy	35-46	
III	7-9th month of pregnancy	65-100	
IV	6 weeks	N/A	
V	2 years		
VI	4 years		

Table 2. Antisera used in the study.

Primary antibodies				
Antigen	Species	Catalog no.	Dilution	Supplier
VAChT	rabbit polyclonal	V5387	1:5000	Sigma-Aldrich
NOS	mouse monoclonal	N2280	1:1000	Sigma-Aldrich
Secondary antibodies				
		Catalog no.	Dilution	Supplier
Alexa Fluor 555 goat anti-rabbit IgG (H+L)		A-21428	1:1000	Invitrogen
Alexa Fluor 488 goat anti-mouse IgG (H+L)		A-11001	1:1000	Invitrogen

for double-labeling immunohistochemistry using antisera against vesicular acetylcholine transporter (VAChT) and nitric oxide synthase (NOS) (Table 2). The sections were incubated in a blocking mixture containing 1% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.5% Triton X100 in PBS for 1 hour. Then, they were rinsed in PBS (3×10 min) and incubated with the primary antiserum for 24 hours at room temperature (RT). After rinsing in PBS (3×10 min), the sections were incubated with a secondary antiserum for 1 hour at RT (Table 2). They were subsequently rinsed in PBS (3 × 10 min) and mounted with 70% glycerole solution in PBS. The stained sections were studied using a confocal laser scanning microscope (Zeiss LSM 700).

Antibody specificity controls

The secondary antibodies controls were run in parallel with each experiment by processing a sample with the omission of each primary antibody and replacing it with the same amount of NGS to prove that the labeling observed was due only to binding of the secondary antibodies to the primary antibodies (Burry 2011). Additionally, control of specificity of staining was performed by preabsorbtion of a diluted antiserum with 20 µg/ml of an appropriate antigen, which abolished the specific immunoreaction completely.

Quantification strategy

In order to determine the percentages of the VAChT-, NOS-, and VAChT/NOS-IR perikarya, at least 300 nerve cell bodies from each neuronal group in each experimental group were analysed. The results were presented as average percentages ± SEM. Statistical analysis was performed using one-way ANOVA, followed by Tukey's honest significant difference test. Differences were considered significant when $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.

To visualize the progressive alterations in the total number of nerve fibers in EG during its development, an arbitrary evaluation has been performed according to the method described in our previous paper (Majewski et al. 1995).

Real-Time PCR

The representative samples of the cranial, middle, and caudal part of EG containing both right and left lips, as well as fundus, were removed from animals of group I with a sterile single-use scalpel and from animals of groups II, III, IV, V, and VI with a disposable punch (diameter of 2 mm, 3 mm, or 4 mm, depending on a organ size). Sampled tissues were transferred into RNAlater™ Stabilization Solution (Thermo Fisher Scientific, catalog # AM7020) for 24 hours at 8°C and were subsequently stored at -80°C.

Three hudred µg of every collected tissue sample was homogenized with 600 µl of fenozolone. Then, the appropriate volume of the liquid homogenate containing 50 µg of the tissue sample was used to isolate total RNA with a Total RNA Mini Plus kit (A&A Biotechnology, catalog # 036-25). This has provided certainty that the samples were unified. Reverse transcription was performed with 1.5 µg of total RNA and

Table 3. Sequences of primers used in Real-Time PCR. *GAPDH primers used in this study have been designed based on bovine GAPDH gene.

Gene	Sequences of reverse primers	Sequences of forward primers	Sequence of origin (in Gene Bank)
ChAT	TCTACAGGCTCCACGGGAAA	TCGAATGTTGTCCACCCGTC	XM_015461094.2
NOS	ATACAGCAGGGCTGGAAACC	CTCAAACTTGGGGTGCCTGA	XM_024977753.1
GAPDH	GGCGTGGACAGTGGTCATAA	GGCGTGAACCACGAGAAGTA	NC_037332.1*

Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific, catalog # K1672). Then, from each cDNA sample Real-Time PCRs were performed for choline acetyltransferase (ChAT) and NOS genes, both in triplicate. Composition of PCR mix was as follows: 10 µl of SYBR® Select Master Mix (Thermo Fisher Scientific, catalog # 4472903), 8 µl of ultra-pure DNase/RNase-free distilled water, 1 µl of cDNA preparation and 1 µl of 5 mM primer mix (reverse and forward, Table 3). The PCR reaction was performed in 7500 fast Real-Time PCR system (Applied Biosystems) with the thermal profile consisting of initial denaturation (10 min) at 95°C, then denaturation (15 s) at 95°C, and annealing (1 min) at 60°C for 40 cycles. Obtained results were presented as relative quantities (RQs) of mRNA in each experimental group and then analysed using a comparative Ct method. The data for both genes were normalised against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 3) using software 7500 v. 2.0.2 (Applied Biosystems, USA). Statistical analysis was performed using one-way ANOVA, followed by Tukey's honest significant difference test. Differences were considered significant when $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.

Results

Immunohistochemistry

Immunohistochemical analysis of the EG samples from fetuses of group I revealed that most of cholinergic and nitrergic nerve cells were gathered within the myenteric plexus (Fig. 1A). Contrary to the common organisation of instrinsic innervation of the gut, the ENS at the level of EG does not contain submucous ganglia. Observed perikarya remained mostly oval in shape and were packed tightly in ganglia (Fig. 1A). They were greater in number in the region of the lips than in the fundus. Those cells had conspicuous nuclei surrounded by slight strands of cytoplasm, therefore the exact borders between them were hard to determine. However, careful investigation showed that 36±2.03% of myenteric neurons were VAChT-positive, whereas the population of NOS-IR somata constituted 26,33±1,85% of myenteric nerve cells. 13.89±1.41% of neurons exhibited immunoreactivity for both, VAChT and NOS.

The general organisation of ENS in organ from experimental group II was very similar to that observed in younger fetuses. Myenteric neurons were grouped in ganglia uniformly distributed along the entire EG (Fig. 1B). Among them, 44.33 ± 2.99 % were immunoreactive to VAChT and 32.78 ± 2.94 % contained NOS. 28.22 ± 2.48 % of myenteric nerve cells were simultaneously positive for both substances.

In the fetuses from group III well-developed myenteric ganglia comprised easily distinguishable neurons (Fig. 1C). Cholinergic and nitrergic nerve cells forming ganglia were more numerous in the region of lips than in the fundus of the EG. 36.33±1.85% of them were VAChT-positive, whereas 35.22±1.76% contained NOS. 25.67±1.12% of myenteric perikarya were VAChT- and NOS-IR.

The EG of calves from group IV contained 29.07±1.44% of VAChT-IR and 27.47±0.80% of NOS-positive nerve cells forming single ganglia between the circular and longitudinal muscle layers (Fig. 1D). Similarly, to the previous age groups, the ganglia were more numerous in the region of the lips of EG than in the fundus. Among all neurons, only 5.4±0.71% exhibited immunoreactivity for both chemical compounds.

In tissue samples of animals from group V single myenteric ganglia at the level of EG lips and fundus were encountered (Fig. 1E). They contained neurons, among which 25.56±2.31% was cholinergic, whereas 19.11±1.81% were NOS-positive. The percentage of nerve cells simultaneously immunoreactive for VAChT and NOS was equal to 11.67±1.80 %.

Only single nerve cells were observed within the myenteric plexus in the region of EG in animals from group VI (Fig. 1F). They were uniformly distributed in both, the lips and fundus. 24.89±2.98% of them were VAChT-IR and 18.78±1.46% contained NOS. 10.33±2.71% of all neurons displayed immunoreactivity for both substances.

A moderate amount of nerve fibers immunoreactive for VAChT was observed in all muscular layers of the EG region in fetuses from groups I, II, and III. Some of them were also present inside the myenteric plexus. Large number of VAChT-containing nerve fibres was encountered within the lips and fundus of calves from

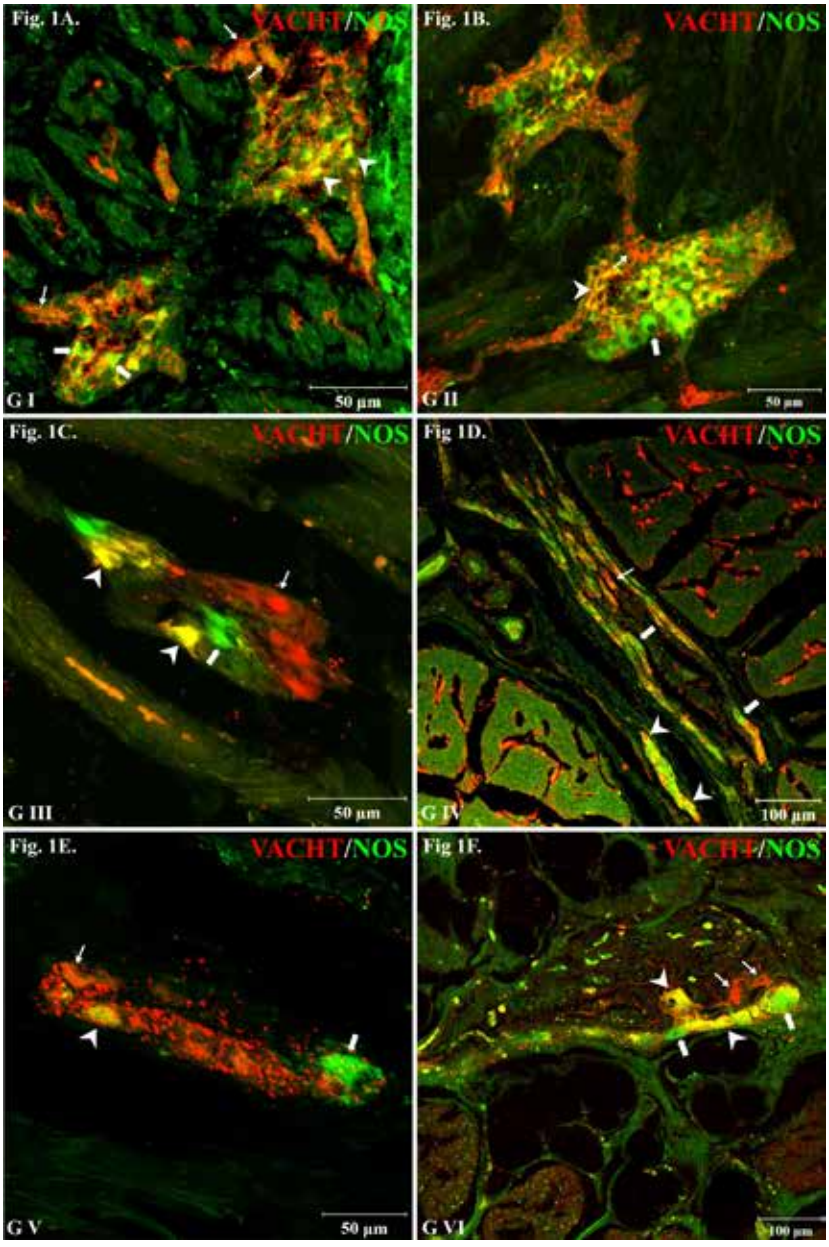

Fig. 1. Staining of the verve structures in esophageal groove (EG) of the cow with antisera containing anti-VAChT and anti-NOS antibodies. Cholinergic nerve structures are red, whereas nitrergic nerve elements are green

A. 2-month-old fetus. The reticular groove in the region of junction between the lips and fundus. Note the presence of numerous nerve cells immunoreactive for VAChT (thin arrows), NOS (thick arrows), and some neurons simultaneously positive for both substances (arrowheads). A moderate number of intraplexial VAChT-positive nerve fibers forming basket-like formations around cells within the ganglia are visualised.

B. 5-month-old fetus. Two easily distinguished myenteric ganglia in the region of the EG lips are pictured. Large number of VAChT-IR (thin arrow), NOS-IR (thick arrow), and VAChT/NOS-IR (arrowheads) nerve cells are present.

C. 8-month-old fetus. The region of the junction between the EG lips and fundus. Single myenteric ganglion containing VAChT-IR (thin arrow) and NOS-IR (thick arrow) perikarya. Large number of nerve cells display immunoreactivity for both, VAChT and NOS (arrowheads).

D. 6-week-old calf. Region of the reticular groove lip. Note the presence of elongated myenteric plexus located between the longitudinal and circular muscular layers. Numerous VAChT-positive (thin arrow) and NOS-IR (thick arrows) nerve cells can be observed. Single neurons simultaneously immunoreactive for both substances are also present (arrowheads). Large number of VAChT-containing axons are seen within the circular muscular layer and inside the ganglia.

E. 2-year-old cow. Myenteric ganglion in the region of the EG lips. A moderate number of VAChT-IR (thin arrow), NOS-IR (thick arrow), and double immunolabeled (arrowheads) nerve cells can be noticed.

F. 4-year-old cow. Region of the EG lips. Note the presence of VAChT-positive (thin arrows) and NOS-positive (thick arrows) nerve cells. Neurons containing both, VAChT and NOS (arrowheads), can also be observed. Single intraplexial VAChT-IR nerve fibres forming basket-like formations around nerve cells within the Auerbach's plexus.

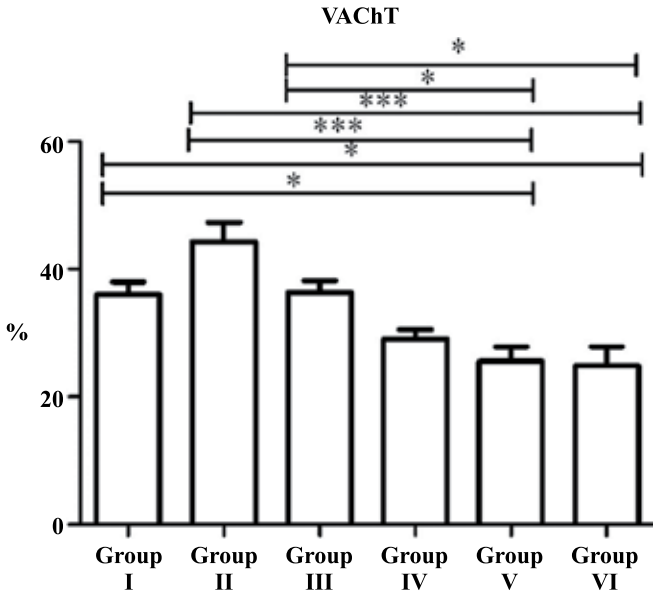

Fig. 2. Percentages of nerve cells expressing VAChT in the EG region. Differences were considered significant when p≤0.05 (*), p≤0.001 (***).

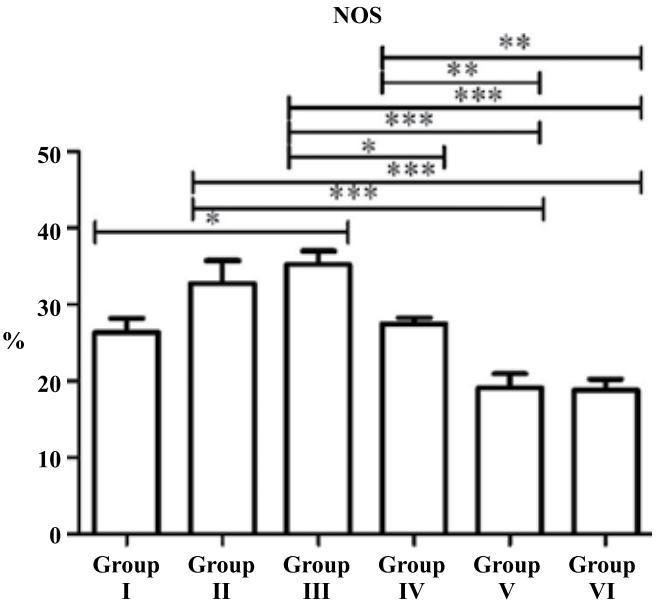

Fig. 3. Percentages of nerve cells expressing NOS in the EG region. Differences were considered significant when p≤0.05 (*), p≤0.01 (**), p≤0.001 (***).

group IV. Some of them were intraplexial and many penetrated into the mucosa. In cowes of group V and VI, single cholinergic nerve fibers were observed among the nerve cells in ganglia, as well as within the longitudinal and circular muscular layers.

The differences in the total numbers of VAChT-, NOS-, and VAChT/NOS-IR nerve cells were statistically significant. However, there were no statistically significant differences in the amounts of neurons containing investigated substances between the cranial, middle, and caudal portions of the EG.

Single NOS-positive axons were present within the longitudinal and circular muscular layers along the entire EG in specimens from all age groups. Solitary nerve fibers were also observed inside the submucosa and mucosa, as well as inside the Auerbach's plexus.

All data on the percentages of VAChT-, NOS-, and VAChT/NOS-IR nerve cells in particular groups are presented in Figs. 2, 3, and 4, whereas arbitrary evaluation of amount of nerve fibers supplying the region of the EG was shown in Table 4.

Real-Time PCR

With a quantitative RT-PCR method it was revealed that the expression of mRNA encoding ChAT was high-

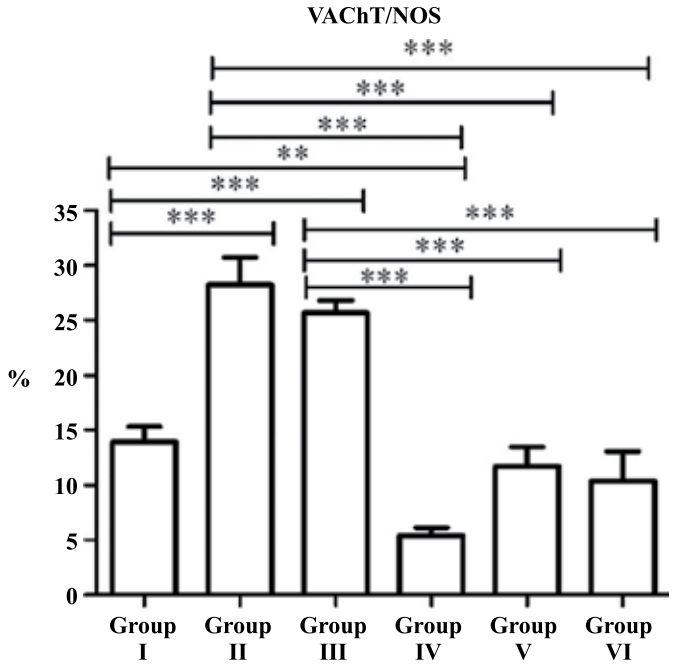

Fig. 4. Percentages of nerve cells expressing VAChT and NOS in the EG region. Differences were considered significant when $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Table 4. Arbitrary evaluation of amount of nerve fibers supplying the region of the EG.

	Group I	Group II	Group III	Group IV	Group V	Group VI
VAChT	++	++	++	+++	+	+
NOS	+	+	+	+	+	+

+ single nerve fibers, ++ moderate amount of nerve fibers, +++ large amount of nerve fibers

est in group I. The levels of ChAT mRNA expression in groups II, III, IV, V, and VI were reduced by a factor of 2.5, 6, 9, 28, and 10, respectively, when compared with the levels of ChAT mRNA expression in group I (Fig. 5).

The highest expression of mRNA encoding NOS was present in group I. The levels of NOS mRNA expression in groups II, III, IV, V, and VI were reduced by a factor of 1.7, 3, 9.5, 45, and 30, respectively, when compared with the levels of NOS mRNA expression in group I (Fig. 6).

The differences in the expressions of both, ChAT and NOS, mRNAs were statistically significant. However, there were no statistically significant differences between the cranial, middle, and caudal portions of the EG in the expressions of genes encoding investigated substances. Statistical significances of the obtained results are shown in the graphs.

Discussion

Development of the ruminants forestomachs has already been thoroughly studied in many aspects using various methodologies. Studies were mainly focused on morphometric characterization of the developing organs (Franco et al. 2004a, Redondo et al. 2005, Redondo et al. 2011, Franco et al. 2012, Garcia et al. 2013), as well as on histochemical and immunohistochemical characterization of its innervation (Harrison and Wathuta 1987, Kitamura et al. 1993, Groenewald 1994, Yamamoto et al. 1994, Teixeira et al. 1998, Pfannkuche et al. 2002, 2003a, Franco at al. 2004b, Franco et al. 2012, Garcia et al. 2013). Authors of previous investigations have only managed to evaluate the total number of nerve cells per slide (Lalatta-Costerbosa et al. 2011) and the number of neurons in ganglia (Pfannkuche et al. 2003a, 2003b). However, their works were mainly conducted in suckling calves or focused on innervation of a rumen wall, not especially of EG itself. Therefore, the present paper is the first comprehensive study describing the development of the gastric groove innervation.

The highest number of cholinergic nerve cells was present in the second trimester fetuses. From this developmental stage onward, their amount was gradually decreasing and reached the lowest value in 4-year-old cows. The same developmental pattern was observed for nitrergic nerve structures with the highest percentage of NOS-IR neurons in the third trimester fetuses. It is unquestionable that, as the main inhibitory compo-

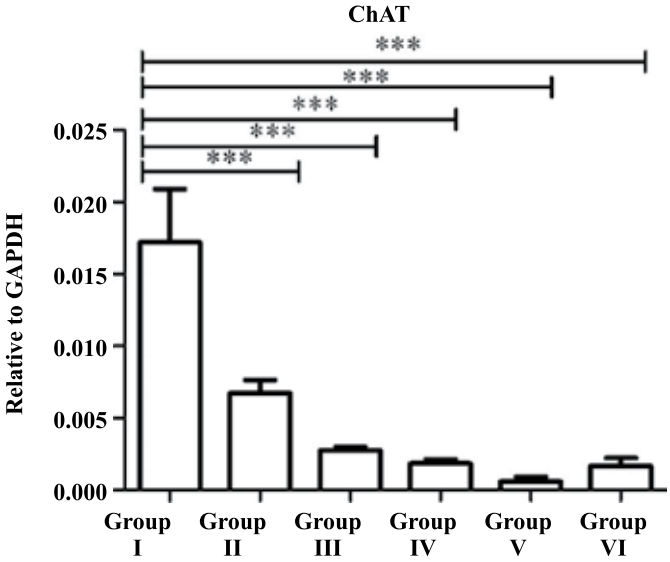

Fig. 5. Expression of mRNA encoding ChAT in EG tissue samples from animals of different age groups. Level of ChAT mRNA were measured by Real-Time PCR. The data obtained from each sample were normalized to GAPDH. Relative quantities (RQ) of mRNA were analysed using the comparative Ct method. Each cDNA sample was amplified in triplicate and all data are expressed as the mean ± SEM. Differences were considered significant when p≤0.001 (***).

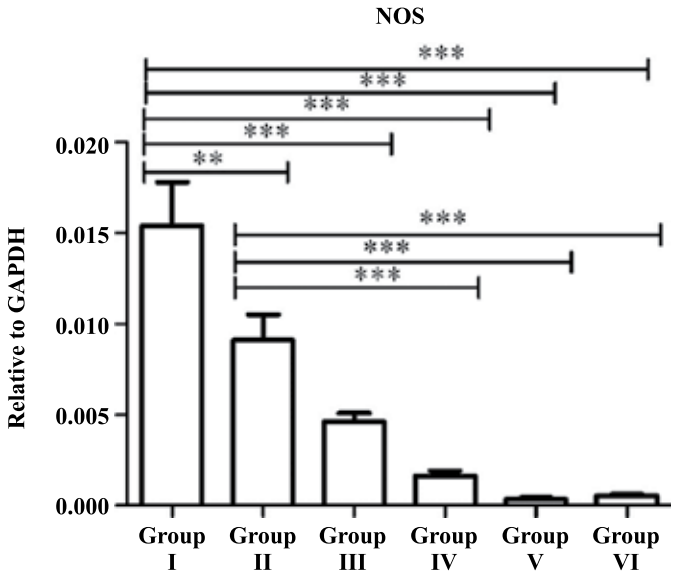

Fig. 6. Expression of mRNA encoding NOS in EG tissue samples from animals of different age groups. Level of NOS mRNA were measured by Real-Time PCR. The data obtained from each sample were normalized to GAPDH. Relative quantities (RQ) of mRNA were analysed using the comparative Ct method. Each cDNA sample was amplified in triplicate and all data are expressed as the mean ± SEM. Differences were considered significant when p≤0.01 (**), p≤0.001 (***).

nents of EG innervation, nitrergic nerve cells are vital for a proper relaxation of the gastric groove fundus when receiving liquid food; their percentages, therefore, decrease with age since the postpartum period. In studies on the effect of colostrum or fodder with the addition of exogenous IgG on the morphology of the small intestine and the expression of neuronal nitrergic structures it was found that IgG as well as colostrum had significant impact on morphological features and nitrergic innervation of the duodenum in piglets (Woliński et al. 2012). This suggests that dietary factors can be important for maintaining the activity of the nitrergic neurons in the gastrointestinal tract.

Nerve cells simultanously positive for VAChT and NOS were particularly numerous in the second and third trimester fetuses, whereas they were underrepresented in other developmental stages compared to single-positive populations of neurons. However, as shown in Fig. 4, no unique pattern can be applied to the alterations in VAChT/NOS-IR nerve cells percentages. Based on the chemical profile of ENS neurons thoroughly described in previous studies (Furness 2000,

Arciszewski and Zacharko-Siembida 2008), we presume that most of these cells are descending interneurons involved in local motility reflexes.

Nevertheless, our results indicate an important decrease in the total number of cholinergic and nitrergic nerve cells considered as primary excitatory motor neurons and primary inhibitory motor neurons (Bult et al. 1990) in the ENS, respectively. It corresponds with progressive development of forestomachs and declining importance of the gastric groove that occur with age.

The highest expressions of mRNAs encoding ChAT and NOS were present in the first trimester fetuses, whereas in the subsequent developmental stages the expressions of genes encoding those enzymes were gradually decreasing up to the second year of life. In 4-year-old cows we have observed a slight increase in expressions of the investigated genes. High expressions of mRNAs encoding VAChT and NOS in the first trimester fetuses are probably due to the high concentration of nerve elements in a relatively small organ. Along with the growth of an animal accompanied by an increase in EG size, the concentration of nerve structures is decreasing, resulting in a substantial decline in expressions of investigated genes. This study provides for the first time data on ChAT gene expression at the region of EG of cattle during its development. Until now ChAT expression within the gastrointestinal tract has been analysed solely in a transgenic zebrafish (Danio rerio) (Nikaido et al. 2018). However, the structure of a digestive tract in the zebrafish differs significantly from that of mammals, thus the data obtained by the aforementioned authors cannot be extrapolated to cattle.

The outcomes presented in this paper imply that the age-related reduce of EG motility results from the gradual loss of cholinergic and nitrergic neurons, as well as from the decline in the concentration of nerve elements. Our observations prove that both neuronal populations are crucial for a proper functioning of a perinatal sucking reflex in calves. Therefore, their contribution to a general neuronal activity in the ENS diminishes with age as the high motility of the gastric groove is not necessarily required in older cattle.

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References

Arciszewski MB, Zacharko-Siembida A **(2008)** Układ nerwowy jelitowy. (Enteric nervous system). 1 st ed., Novae Res, Gdynia

De Bruin MG, Wyman WEA (1901) The age of the foetus. In: Bovine obstetrics. WR Jenkins, New York, pp 23-25.

Budras KD, Habel RE **(2003)** Bovine anatomy : an illustrated text. 1-st ed. Schlütersche GmbH & Co. KG, Verlag und Druckerei, Hannover, Germany.

Bult H, Boeckxstaens GE, Pelckmans PA, Jordaens FH, von Maercke YM, Herman AG **(1990)** Nitric oxide as an inhibitory non-adrenergic non-cholinergic neurotransmitter. Nature 345: 346-347.

Dreher C, Wellmann R, Stratz P, Schmid M, Preuss S, Hamann H, Bennewitz J **(2019)** Genomic analysis of perinatal sucking reflex in German Brown Swiss calves. J Dairy Sci 102: 6296-6305.

Franco AJ, Masot AJ, Aguado MC, Gomez L, Redondo E **(2004a)** Morphometric and immunohistochemical study of the rumen of red deer during prenatal development. J Anat 204: 501-513.

Franco AJ, Redondo E, Masot AJ **(2004b)** Morphometric and immunohistochemical study of the reticulum of red deer during prenatal development. J Anat 205: 277-289.

Franco A, Masot J, García A, Redondo, E **(2012)** Ontogenesis of the Reticulum with Special Reference to Neuroendocrine and Glial Cells: A Comparative Analysis of the Merino Sheep and Iberian Red Deer. Anat Histol Embryol 41: 362-373.

Furness JB **(2000)** Types of neurons in the enteric nervous system. J Auton Nerv Syst 81: 87-96.

Furness JB **(2006)** The enteric nervous system. blackwell publishing Ltd, 9600 Garsington Road, Oxford, UK, pp 288.

Garcia A, Masot J, Franco A, Gazquez A, Redondo E **(2013)** Histomorphometric and immunohistochemical study of the goat abomasum during prenatal development. Histol Histopathol 28: 1639-1649.

Gentile A, Sconza S, Lorenz I, Otranto G, Rademacher G, Famigli-Bergamini P, Klee W **(2004)** D-Lactic acidosis in calves as a consequence of experimentally induced ruminal acidosis. J Vet Med A Physiol Pathol Clin Med 51: 64-70.

Goldstein AM, Hofstra RM, Burns AJ **(2013)** Building a brain in the gut: development of the enteric nervous system. Clin Genet 83: 307-316.

Groenewald HB **(1994)** Neuropeptides in the myenteric ganglia and nerve-fibers of the forestomach and abomasum of grey, white and black karakul lambs. Onderstepoort J Vet Res 61: 207-213.

Groneberg D, Voussen B, Friebe A **(2016)** Integrative control of gastrointestinal motility by nitric oxide. Curr Med Chem 23: 2715-2735.

Guilhermet R, Mathieu CM, Toullec R, Beaufils M, Mansion M, Manis Y **(1975)** Transit des aliments liquides au niveau de la gouttiere oesophagienne chez le veau preruminant et ruminant. (Transit of liquid food at the level of the esophageal groove in the preruminant and ruminant calf.) Ann. Zootech 24: 69-79.

Harrington AM, Hutson JM, Southwell BR **(2010)** Cholinergic neurotransmission and muscarinic receptors in the

enteric nervous system. Prog Histochem Cytochem 44: 173-202.

Herrli-Gygi M, Steiner A, Doherr MG, Blum JW, Kirchhofer M, Zanolari P **(2008)** Digestive processes in ruminal drinkers characterized by means of the acetaminophen absorption test. Vet J 176: 369-377.

Kaba T, Abera B, Kassa T **(2018)** Esophageal groove dysfunction: a cause of ruminal bloat in newborn calves. BMC Vet Res 14: 276.

Kitamura N, Yamada J, Yamamoto Y, Yamashita T **(1993)** Substance P-immunoreactive neurons of the bovine forestomach mucosa: their presumptive role in a sensory mechanism. Arch Histol Cytol 56: 399-410.

Labussiere E, Berends H, Gilbert MS, van den Borne JJ, Gerrits WJ **(2014)** Estimation of milk leakage into the rumen of milk-fed calves through an indirect and repeatable method. Animal 8: 1643-1652.

Lalatta-Costerbosa G, Clavenzani P, Petrosino G, Mazzoni M **(2011)** An immunohistochemical study of the distribution of nitric oxide synthase-immunoreactive neurons and fibers in the reticular groove of suckling lambs. J Anat 218: 439-448.

Majewski M, Sienkiewicz W, Kaleczyc J, Mayer B, Czaja K, Lakomy M **(1995)** The distribution and co-localization of immunoreactivity to nitric oxide synthase, vasoactive intestinal polypeptide and substance P within nerve fibres supplying bovine and porcine female genital organs. Cell Tissue Res 281: 445-464.

Nikaido M, Izumi S, Ohnuki H, Takigawa Y, Yamasu K, Hatta K **(2018)** Early development of the enteric nervous system visualized by using a new transgenic zebrafish line harboring a regulatory region for choline acetyltransferase a (chata) gene. Gene Expr Patterns 28: 12-21.

Olsson C, Holmgren S **(2011)** Autonomic control of gut motility: a comparative view. Auton Neurosci 165: 80-101.

Pfannkuche H, Reiche D, Hoppe S, Schemann M **(2002)** Cholinergic and noncholinergic innervation of the smooth muscle layers in the bovine abomasum. Anat Rec 267: 70-77.

Pfannkuche H, Schellhorn C, Schemann M, Aschenbach JR, Gäbel G **(2003a)** Age-associated plasticity in the intrinsic innervation of the ovine rumen. J Anat 203: 277-282.

Pfannkuche H, Schellhorn C, Schemann M, Gäbel G **(2003b)** Reticular groove and reticulum are innervated by myenteric neurons with different neurochemical codes. Anat Rec A Discov Mol Cell Evol Biol. 274: 917-922.

Redondo E, Franco AJ, Masot A J **(2005)** Morphometric and immunohistochemical study of the omasum of red deer during prenatal development. J Anat 206: 543-555.

Redondo E, Masot J, García A, Franco A **(2011)** Ontogenesis of the omasum: A comparative analysis of the Merino sheep and Iberian red deer. Histol Histopathol 26: 1135-1144.

Sanders KM **(2016)** Enteric Inhibitory Neurotransmission, Starting Down Under. Adv Exp Med Biol 891: 21-29.

Sanders KM, Ward SM **(2019)** Nitric oxide and its role as a non-adrenergic, non-cholinergic inhibitory neurotransmitter in the gastrointestinal tract. Br J Pharmacol 176: 212-227.

Teixeira AF, Wedel T, Krammer HJ, Kühnel W **(1998)** Structural differences of the enteric nervous system in the cattle forestomach revealed by whole mount immunohistochemistry. Ann Anat 180: 393-400.

Wathuta EM, Harrison FA (1987) The ontogeny of vasoactive intestinal polypeptide-like and substance p-like immunoreactivity in the digestive tract of the sheep. Q J Exp Physiol 72(1): 119-128.

Weyns AA **(1988)** The enteric nervous system in the ruminant stomach of the sheep (Ovis aries). Erasmus University Rotterdam.

Woliński J, Słupecka M, Weström B, Prykhodko O, Ochniewicz P, Arciszewski M, Ekblad E, Szwiec K, Ushakova, Skibo G, Kovalenko T, Osadchenko I, Goncharova K, Botermans J, Pierzynowski S **(2012)** Effect of feeding colostrum versus exogenous immunoglobulin G on gastrointestinal structure and enteric nervous system in newborn pigs. J Anim Sci 90: 327-330.

Yamamoto Y, Kitamura N, Yamada J, Yamashita T **(1994)** Immunohistochemical study of the distributions of the peptide-and catecholamine-containing nerves in the omasum of the sheep. Acta Anat (Basel) 149: 104-110.