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The impact of low and high doses of acrylamide on the intramural neurons of the porcine ileum

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ABSTRACT

The present study was designed to assess the influence of acrylamide supplementation, in tolerable daily intake (TDI) dose and a dose ten times higher than TDI, on the neurochemical phenotype of the ENS neurons and synthesis of proinflammatory cytokines in the wall of the porcine ileum. The study was performed on 15 juvenile female Danish Landrace pigs, divided into three groups: C group- animals receiving empty gelatine capsules, LD group- animals receiving capsules with the TDI dose (0.5 μg/kg b.w./day) of acrylamide and HD group- animals receiving acrylamide in a dose ten times higher than the TDI (5 μg/kg b.w./day) in a morning meal for 28 days. It was established that supplementation of acrylamide led to an increase in substance P (SP)-, calcitonin gene-related peptide (CGRP)-, galanin (GAL)- and vesicular acetylcholine transporter (VAChT)-like immunoreactive (LI) neurons as well as a decrease in neuronal nitric oxide synthase (nNOS) -like immunoreactivity in all types of ileum intramural plexuses. Moreover, using ELISA method, an increase in the level of proinflammatory cytokines (IL-1β, IL-6 and TNF- α) was noted in the ileum wall. The results suggest that SP, CGRP, GAL, nNOS and VACHT participate in the regulation of inflammatory conditions induced by acrylamide supplementation.

1. Introduction

Acrylamide, (acrylic acid amide, ACM) is a synthetic chemical compound used in industry for the production of plastics, paints, varnishes, paper and cosmetics (El-Mehi and El-Sherif, 2015). In 2002, the presence of acrylamide in food products was demonstrated for the first time. ACM is formed in food products subjected to high temperature (e.g. chips, fries, coffee) in a process called the Maillard reaction (Acaroz et al., 2018). This discovery led to a series of studies on the toxicity of acrylamide. Numerous reports confirmed that exposure to ACM leads to reproductive disorders, tumours and peripheral neuropathy (Dybing et al., 2005; Matoso et al., 2019; Shipp et al., 2006). It has been demonstrated that ACM is absorbed into the human body through the digestive tract, the respiratory system, and the skin. It then undergoes biotransformation and elimination, mainly in the liver. Metabolic conversion of acrylamide to a more reactive epoxide derivative (glycidamide), is mediated by the cytochrome P450 enzyme (Zödl et al., 2007). The gastrointestinal (GI) tract is the first line of defence of the living organism against food toxins. In recent years, an increase in the pace of life and a consequent change in eating habits have been observed. There has been a significant increase in the consumption of products containing large amounts of ACM, especially among young people and children, which contributed to the selection of young animals for this experiment. It cast doubt on whether the contents of ACM allowed for consumption are safe for public health. This is particularly important because proper assessment of human exposure to ACM delivered via food is difficult to estimate. The World Health Organization (WHO) assessed that dietary exposure for acrylamide in humans ranges between 0.3 and 0.8 μg/kg of body weight per day (WHO, 2002). The choice of pigs in the present experiment was also not accidental. Due to the high similarity in anatomical, histological and physiological properties to humans, the pig is an excellent animal model in biomedical research, especially in GI pathology (Brown and Timmermans, 2004).

The enteric nervous system (ENS) located in the wall of the GI tract participates in many physiological functions as well as defence reactions during GI disorders (Furness et al., 2014). Intramural neurons connected by a dense network of neuronal processes form plexuses whose occurrence have been described from the oesophagus to the anus. The spatial arrangement of ENS plexuses depends both on the part of GI tract and the species of the animal under investigation. In the small and large intestines of large mammals, including pigs, the myenteric plexus (MP) (located between the longitudinal and circular

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Abbreviations

ACh	acetylcholine
ACM	acrylamide
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CRP	C-reactive protein
ENS	enteric nervous system
GAL	galanin
GALT	gut-associated lymphoid tissue
GI	gastrointestinal
GSH	cellular glutathione reduced form
HE	haematoxylin and eosin
IL-1 β	interleukin 1 β
IL-1α	interleukin 1α
IL-6	interleukin 6
IL-8	interleukin 8
ISP	inner submucous plexus
LDH	oxidized form of cholesterol
LI	like immunoreactive
LPS	lipopolysaccharide
MP	myenteric plexus
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NPY	neuropeptide Y
OSP	outer submucous plexus
SP	substance P
TDI	tolerable daily intake
TNF- α	tumour necrosis factor- α
VAChT	vesicular acetylcholine transporter
VIP	vasoactive intestinal peptide
WHO	World Health Organization

muscle layers) and two submucous plexuses: outer submucous plexus (OSP) (located near the external circular muscle layer) and inner submucous plexus (ISP) (located between the muscularis mucosa and lamina propria) were distinguished (Gonkowski, 2013; Nezami and Srinivasan, 2010). It is well known that the ENS neurons may play several biological roles in the GI tract thanks to the content of many neuroactive substances forming the chemical code of neurons. The ENS is referred to as the intestinal brain due to plenty of neurons in its structure, numerous neurotransmitters synthesized, as well as the performance of numerous regulatory functions independent of the central nervous system (CNS) (Furness et al., 2014). In addition, close cooperation of ENS neurons with the intestinal immune system has been demonstrated. The presence of the receptors for neurotransmitters in immune cells has been confirmed (Cervi et al., 2014). It is known that the lymphoid tissue in the gastrointestinal mucosa contains 70% of whole-body lymphocytes and forms a gut-associated lymphoid tissue (GALT) system. The GALT consists of individual or grouped follicles forming Peyer's patches (Jung et al., 2010). It represents the first line of defence of the body against harmful factors. In the porcine ileum, GALT is organized in the lymphoid continuous lymphoid follicle (lymphatic plate) (Wąsowicz et al., 2018). It should be emphasized that ACM stimulates an increase in the synthesis of pro-inflammatory cytokines in both animal and human organisms (Naruszewicz et al., 2009; Santhanasabapathy et al., 2015). However, available data describes only the level of proinflammatory cytokines in the blood serum, and there is no data on their synthesis in the gastrointestinal wall.

On the other hand, the nervous system is characterized by high plasticity in response to various pathological stimuli, and the ability to adapt to the changing environmental conditions. This adaptation involves alterations in the chemical phenotype of neurons by increased expression of some neurotransmitters and reduced expression of others, or activation of the expression of previously inactive genes (Ekblad and Bauer, 2004; Furness et al., 2014; Palus et al., 2018). In recent years, there has been an increasing amount of literature describing changes in the chemical coding of neurons innervating the gastrointestinal tract during inflammation, metabolic disorders, neuronal damage and intoxication with different natural and synthetic substances (Bulc et al., 2019; Gonkowski, 2013; Szymanska et al., 2018; Makowska and Gonkowski, 2018). Previous toxicological research reported that acrylamide intoxication leads to an increase in the density of fibres immunoreactive to calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and substance P (SP) in the rat ileum (Belai and Burnstock, 1996). Alterations in the neurochemical characteristics of the intestinal neuronal structures (both neurons and neuronal processes) may be the first subclinical symptoms of various disorders within the GI tract. Thus, the present study was designed to assess the influence of acrylamide supplementation, in a tolerable daily intake (TDI) dose and a dose ten times higher than TDI, on the neurochemical phenotype of the ENS neurons and synthesis of proinflammatory cytokines in the wall of the porcine ileum.

2. Materials and methods

2.1. Animals and experimental procedures

The study was performed on 15 juvenile female pigs of the Danish Landrace, approximately 8 weeks old, weighing ca. 20 kg. All animals were kept under standard laboratory conditions, fed a commercial grain mixture and had free access to water. After seven days of acclimatization, the pigs were randomly assigned to one of three experimental groups: control (C group, n = 5), in which empty gelatine capsules were administered, a low dose group (LD group, n = 5)- animals receiving capsules with the tolerable daily intake (TDI) dose (0.5 μg/kg b.w./day) of acrylamide (> 99%; Sigma-Aldrich, Poznań, Poland), and

Table 1
Antibodies used in immunofluorescence method.

Antigen	Host species	Cat No.	Dilution	Supplier
Primary antibodies				
PGP 9.5	Mouse	7863–2004	1:1000	Bio-Rad, Hercules, CA, USA
SP	Rat	8450–0505	1:150	AbD Serotec, Raleigh, NC, USA
CGRP	Rabbit	MAB317	1:4000	Millipore
GAL	Rabbit	RIN7153	1:3000	Peninsula, San Carlos, CA, USA,
nNOS	Rabbit	AB5380	1:2000	Sigma-Aldrich, Saint Louis, MO, USA
VACHT	Rabbit	H–V007	1:2000	Phoenix Pharmaceuticals
Secondary antibodies				
Alexa Fluor 488 donkey anti- mouse IgG		A21202	1:1000	Thermo Fisher Scientific, Waltham, MA, USA
Alexa Fluor 546 goat anti- rabbit IgG		A11010	1:1000	Thermo Fisher Scientific, Waltham, MA, USA
Alexa Fluor 546 goat anti- rat IgG		A11081	1:1000	Thermo Fisher Scientific, Waltham, MA, USA

a high dose group (HD group, n = 5) - animals receiving capsules with acrylamide in a dose ten times higher than the TDI (5 μg/kg b.w./day). All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the Local Ethical Committee for Experiments on Animals in Olsztyn (Approval No.: 11/2017). To the appropriate dose of acrylamide, all pigs were weighed once a week. Capsules were administrated with the morning meal for 28 days. After this time, animals of all groups were treated with azaperone (Stresnil, Jansen Pharmaceutica N.V., Belgium, 4 mg/kg of body weight, i.m.) and euthanized after 15 min using a lethal dose of sodium pentobarbital (Morbital, Biowet Puławy, Puławy, Poland; 0.6 ml/kg of body weight, i.v.). Directly after euthanasia, ileum fragments (about 8 cm long, situated 2 cm before the ileocecal valve) from each animal were collected. Fragments for the immunofluorescence technique were fixed by immersion in a 4% buffered solution of paraformaldehyde (pH = 7.4) for 1 h, washed three times in 0.1 M phosphate buffer (pH 7.4, every 24 h) and transferred to an 18% buffered solution of sucrose (pH = 7.4) for two weeks. Fragments for histopathological evaluation were put into a 10% formalin solution.

2.2. Double-labelling immunofluorescence

Fourteen-micrometre-thick cryostat sections of the tissue samples were processed for routine double-labelling immunofluorescence technique (as described previously by Palus et al., 2018), using primary antisera raised in different species and species-specific secondary antibodies (Table 1). Sections were dried at room temperature for 45 min and washed three times in 0.1 M phosphate-buffered saline (PBS, pH = 7.4, 10 min). Subsequently, they were incubated with buffered blocking mixture (containing 10% horse serum and 0.1% bovine serum albumin in 0.1 M PBS, 1% Triton X-100, 0.05% Thimerosal and 0.01% sodium azide) for an hour in a humid chamber at room temperature, rinsed in PBS (3 × 10 min) and incubated overnight with primary antisera raised against the protein gene-product 9.5 (PGP 9.5 used here as a pan-neuronal marker) and substance P (SP), calcitonin gene-related

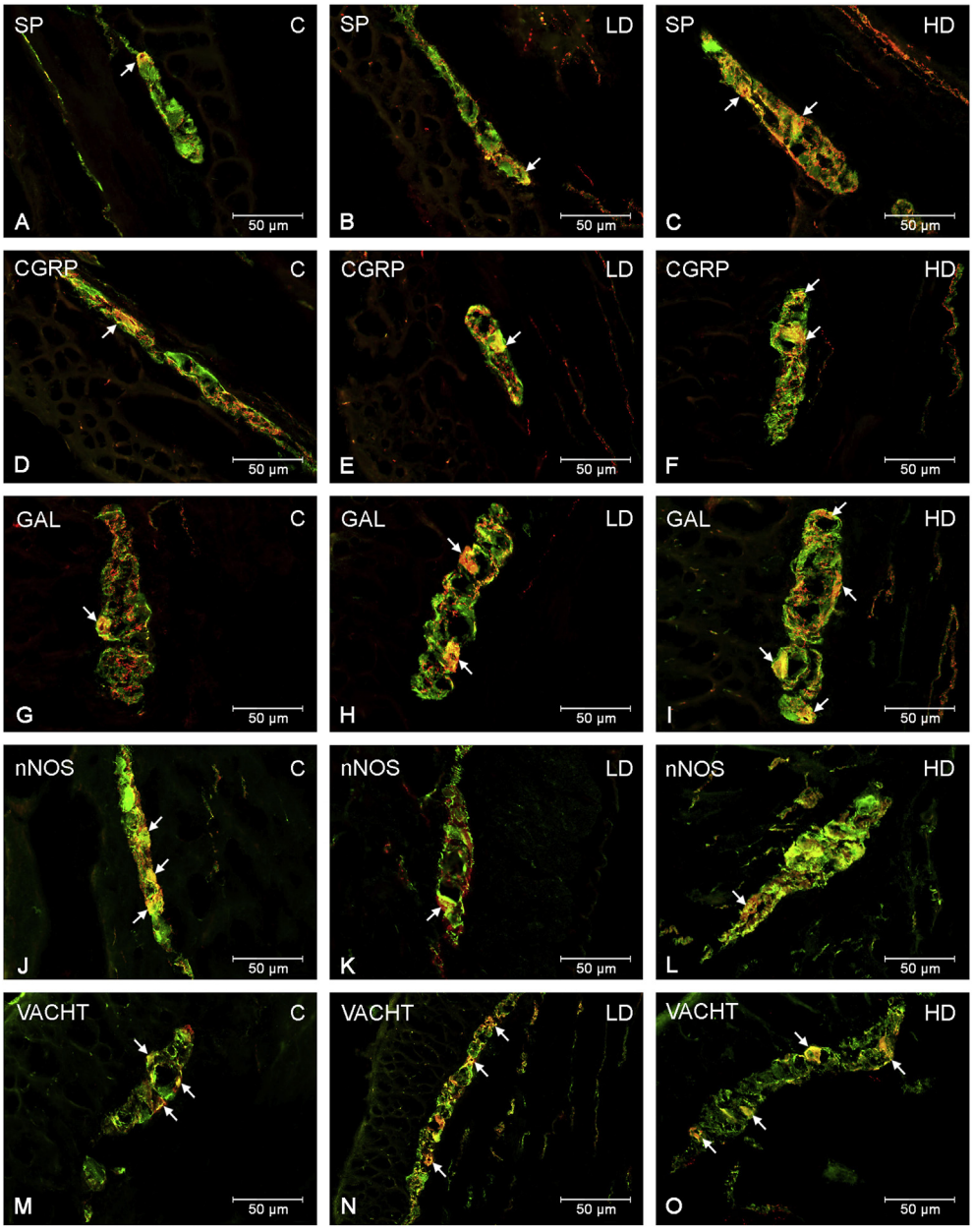

Fig. 1. Myenteric neurons in the porcine ileum immunoreactive to SP, CGRP, GAL, nNOS and VACHT.
Distribution pattern of neurons immunoreactive to protein gene-product 9.5 (PGP9.5)—used as a panneuronal marker and/or SP, CGRP, GAL, nNOS and VACHT in myenteric plexuses in the porcine ileum under physiological condition (A, D, G, J, M), after low (B, E, H, K, N) and high (C, F, I, L, O) doses of acrylamide administration. Photographs A–C showing myenteric neurons immunoreactive to SP, D–F— myenteric neurons immunoreactive to CGRP, G–I—myenteric neurons immunoreactive to GAL, J-L myenteric neurons immunoreactive to nNOS and M-O- myenteric neurons immunoreactive to VACHT. All photographs have been created by digital superimposition of two color channels (green for PGP 9.5 and red for SP, CGRP, GAL, nNOS and VACHT, respectively). Neurons immunoreactive to particular substance studied are indicated with arrows.

peptide (CGRP), galanin (GAL), neuronal nitric oxide synthase (nNOS), vesicular acetylcholine transporter (VAChT) (Table 1). Following subsequent rinsing in PBS (3 × 10 min), the sections were incubated with a mixture of secondary antibodies (Table 1) for 1 h at room temperature. Finally, the washed sections were cover-slipped in carbonate-buffered glycerol (pH8.6). Additionally, pre-absorption for the neuropeptide antisera with appropriate antigens, as well as the omission and the replacement tests were performed to eliminate non-specific labelling. There was no fluorescence observed in any of these control staining.

In order to establish the percentage of neurons displaying immunoreactivity to particular neuroactive substance studied, at least 500 PGP 9.5 LI cell bodies with a clearly visible nucleus in all types of ileum plexuses were evaluated (the number of PGP 9.5 was considered as 100%). To avoid double-counting, the same combination of antigens assigned to quantitative investigations were separated by at least 200 μm. The obtained data were pooled and expressed as a mean ± standard error of mean (SEM). The results from each group were analysed statistically with Statistica 12 (Stat Soft Inc., Tulsa, OK, USA). Significant differences were assessed with a one-way analysis of variance (ANOVA) with Dunnett's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

2.3. Determination of interleukin levels by the ELISA method

Peyer's patches were isolated from the collected segments of the ileum using the mechanical macroscopic method previously described by Obremski et al. (2013). After fixing in liquid nitrogen, they were stored at −80 °C until analysis. Individual samples (1 g) were homogenized in 2.5 mL of extraction buffer with the following composition: PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4), 0.5% sodium citrate, 0.05% (POCH, Gliwice, Poland), Tween 20 (Sigma Aldrich, Saint Louis, MO, USA), and protease inhibitors (Ref. 11 697 498 001, Roche, Basel, Switzerland) using a homogenizer (Omni-TipsTM Disposable, Omni International, Kennesaw, USA). Immediately after homogenization, samples were centrifuged for 60 min at 8600 g (Eppendorf 5804 R) and the obtained supernatant was subjected to routine ELISA tests according to the manufacturer's instructions. The concentration of cytokines was determined by an ELISA

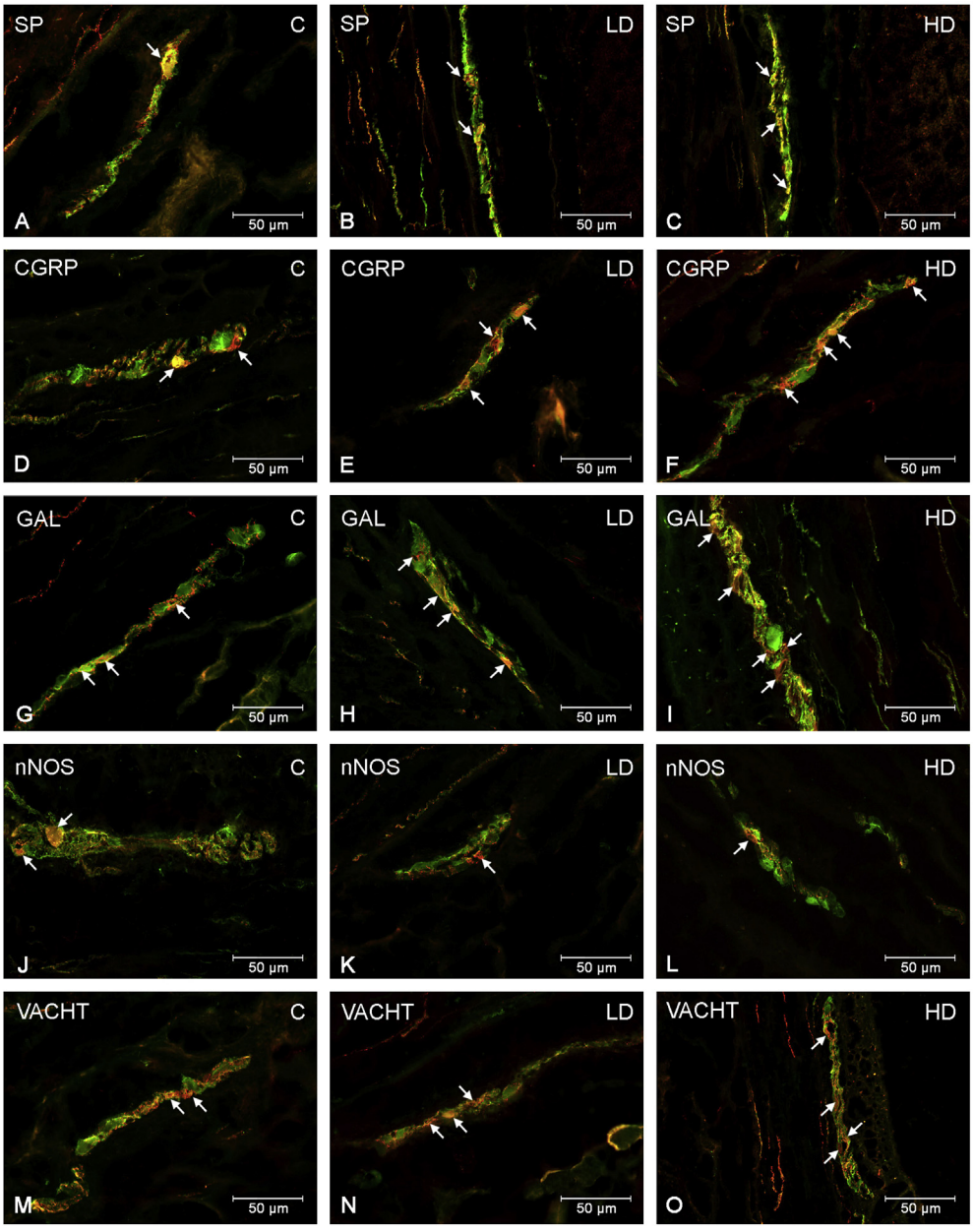

Fig. 2. Inner submucous neurons in the porcine ileum immunoreactive to SP, CGRP, GAL, nNOS and VACHT.
Distribution pattern of neurons immunoreactive to protein gene-product 9.5 (PGP9.5)—used as a panneuronal marker and/or SP, CGRP, GAL, nNOS and VACHT in inner submucous plexuses in the porcine ileum under physiological condition (A, D, G, J, M), after low (B, E, H, K, N) and high doses (C, F, I, L, O) of acrylamide administration. Photographs A–C showing inner submucous neurons immunoreactive to SP, D–F— inner submucous neurons immunoreactive to CGRP, G–I— inner submucous neurons immunoreactive to GAL, J-L- inner submucous neurons immunoreactive to nNOS and M-O- inner submucous neurons immunoreactive to VACHT. All photographs have been created by digital superimposition of two color channels (green for PGP 9.5 and red for SP, CGRP, GAL, nNOS and VACHT, respectively). Neurons immunoreactive to particular substance studied are indicated with arrows.

kit using a multifunctional plate reader (TECAN Infinite M200, Männedorf, Switzerland).

ELISA polystyrene 96 well microplates (NUNC, Denmark) were coated with a solution of capture antibodies in carbonate buffer (100 µl per well, overnight, at 4 °C). Next, the plate was washed three times in PBS with 0.05% tween 20 (tPBS) and blocked in 1% of BSA (Sigma Aldrich, USA) in PBS (90 min at 37 °C). Afterwards, the extract samples and standards were added and incubated by 90 min. A solution to detect antibodies in PBS with 1% BSA (Sigma Aldrich, USA) was added and incubated for 90 min. Following this, the solution of streptavidin conjugated with HRP enzyme was added. The next step was incubation with substrate (OPD or TMB, Sigma Aldrich, USA) for 30 min. The enzymatic reaction was stopped by the addition of 2.5 M HCl (POCH, Poland). The plate was then measured in a plate reader at 492 nm or 450 nm (depending on substrate OPD or TMB). The measurement results were calculated in Excel (Microsoft, USA) and Statistica 12 software (Stat Soft Inc., Tulsa, OK, USA) and re-calculated for 1 g of the fresh tissue and per 1 mg of protein. Significant differences were assessed with a one-way analysis of variance (ANOVA) with Dunnett's test ($^*p < 0.05$).

2.4. Histopathological evaluation

Fragments of ileum were subjected to histopathological assessment using routine histological methods (haematoxylin and eosin staining [HE]), as described previously by Przybylska-Gornowicz et al. (2018), in the Histological Laboratory at the Department of Histology and Embryology, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn.

3. Results

3.1. Neurochemical characteristic of the ENS neurons

Under physiological conditions, neurons immunoreactive to SP, CGRP, GAL, nNOS and VACHT were detected in all types of enteric plexuses (MP, ISP and OSP) (Table 1, Figs. 1–3). The most numerous groups of SP-positive neurons were observed in submucous plexuses

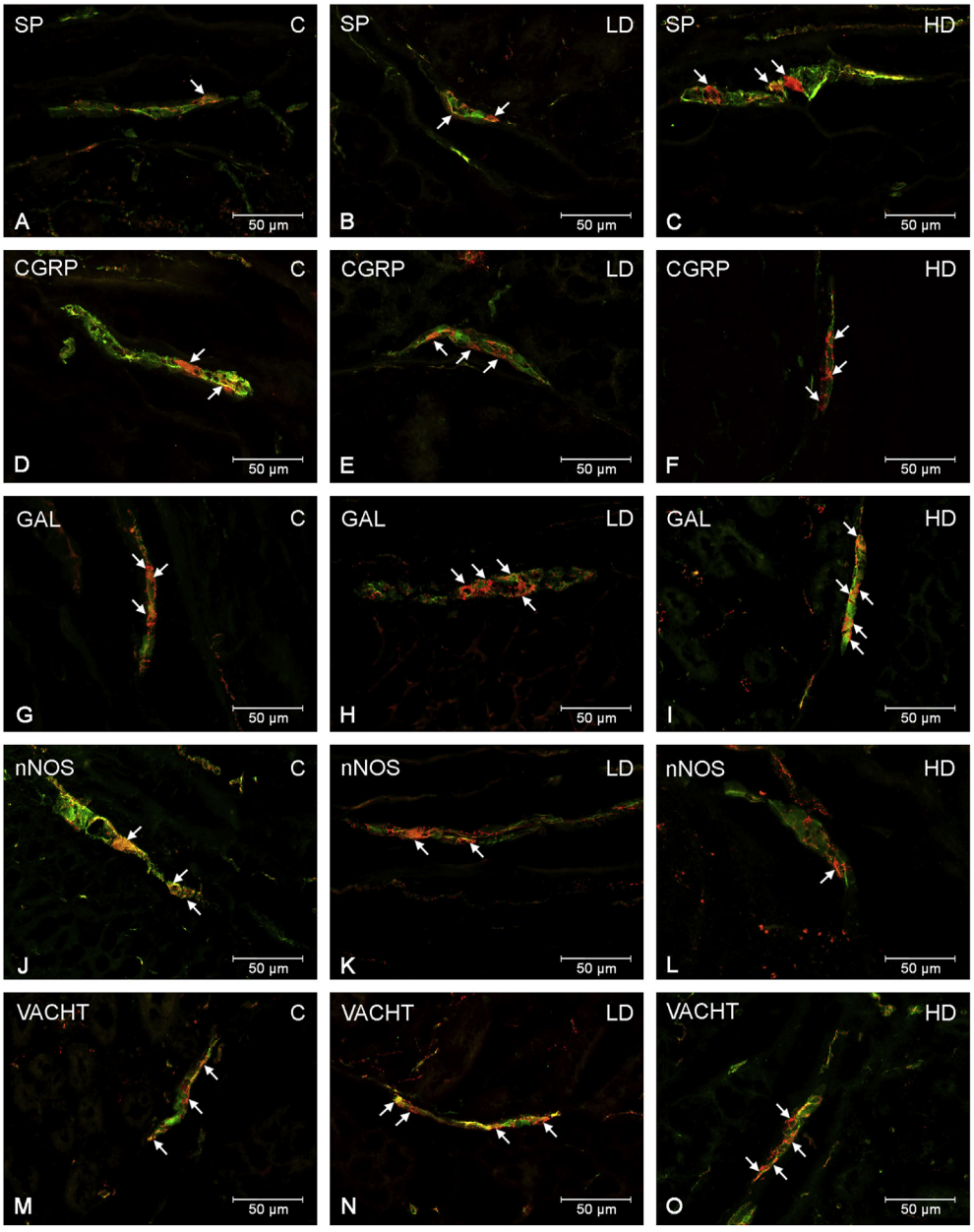

Fig. 3. Outer submucous neurons in the porcine ileum immunoreactive to SP, CGRP, GAL, nNOS and VACHT.
Distribution pattern of neurons immunoreactive to protein gene-product 9.5 (PGP9.5)—used as a panneuronal marker and/or SP, CGRP, GAL, nNOS and VACHT in outer submucous plexuses in the porcine ileum under physiological condition (A, D, G, J, M), after low (B, E, H, K, N) and high doses (C, F, I, L, O) of acrylamide administration. Photographs A–C showing outer submucous neurons immunoreactive to SP, D–F— outer submucous neurons immunoreactive to CGRP, G–I— outer submucous neurons immunoreactive to GAL, J-L- outer submucous neurons immunoreactive to nNOS and M-O- outer submucous neurons immunoreactive to VACHT. All photographs have been created by digital superimposition of two color channels (green for PGP 9.5 and red for SP, CGRP, GAL, nNOS and VACHT, respectively). Neurons immunoreactive to particular substance studied are indicated with arrows.

(15.47 ± 1.64% in the OSP and 14.79 ± 1.23% in the ISP) (Figs. 2A and 3A). However, in the MP only 0.53 ± 0.14% of all cells immunoreactive to PGP 9.5 simultaneously expressed SP (Fig. 1 A). Similarly, CGRP-positive neurons were the most abundant in the ISP (23.44 ± 1.23%) (Fig. 2D). A slightly smaller number of CRRP-LI cell bodies were noted in the OSP (20.14 ± 1.32%) (Fig. 3D)and the least were in the MP (16.07 ± 1.04%)(Fig. 1D). Moreover, a significant number of ISP and OSP neurons were GAL- positive (49.45 ± 0.63% and 38.84 ± 1.16%) (Figs. 2G and 3G), while only 3.01 ± 0.44% of MP neurons displayed GAL immunoreactivity (Fig. 1G). In turn, the largest population of nNOS-positive neurons were observed in the MP (40.62 ± 1.33%) (Fig. 1 J). However, a significantly smaller number of nNOS-LI neurons were present in submucous plexuses (12.52 ± 0.76% in the ISP and 11.80 ± 1.17% in the OSP) (Figs. 2J and 3J). Finally, VACHT-LI neurons were present in the largest number in the ISP (50.22 ± 1.23%) (Fig. 2M), slightly less in the MP (40.92 ± 2.45%) (Fig. 1M) and the lowest were in the OSP (30.77 ± 0.71%) (Fig. 3M).

Acrylamide supplementation, in both the TDI and doses ten times higher, led to changes in the neurochemical phenotype of the ENS neurons in the porcine ileum (Table 2, Figs. 1–3). The character of the observed changes clearly depended on the type of enteric plexus, dose of acrylamide and neuroactive substance under investigation. The most remarkable increase in the number of SP-LI neurons was noted in the MP (almost two-fold in the LD group and nearly four-fold in the HD group) (Fig 1 B, C). Significant changes were also noted in the OSP (an increase of about 9 percentage points (pp) in LD and 16 pp in HD groups, respectively) (Fig. 3 B, C). However, in the ISP only in the HD group was the change significant (an increase of about 7 pp) (Fig. 2C). In turn, the most important changes in the number of CGRP-LI neurons were observed in the ISP, where in both LD (about 7 pp) (Fig. 2E) and HD (about 12 pp) (Fig. 2F) groups the increase was highly statistically significant. A slightly smaller increase was noted in MP (about 5 pp in LD group and about 7 pp in HD group, respectively) (Fig. 1E and F). In the OSP, only supplementation with a high dose of acrylamide caused changes in the population of CGRP-positive neurons (an increase of about 7 pp) (Fig. 3F). Comparable changes were observed in the population of GAL-LI neurons. The most important alterations were detected in the ISP (an increase about 9 pp in LD group and 16 pp in HD group) (Fig. 2H and I) and slightly less in MP (an increase of about 4 pp in LD group and 14 in the HD group) (Fig. 1 H, I). Whereas a significant increase in the OSP was only noted in the HD group (about 17 pp) (Fig. 3 I). In turn, a decrease in the number of neurons displaying immunoreactivity to nNOS was observed in all types of enteric plexuses. The changes were most strongly expressed in the OSP, where significant changes were noted in both the LD and HD group (a decrease of about 4 pp and 9 pp) (Fig. 3K and L). In MP and ISP, notable changes were only noted in the HD group (a decrease of about 9 pp and 5 pp, respectively) (Fig. 1L and 2L). Finally, changes in VACHT-immunoreactivity were also important during acrylamide supplementation. Changes were the greatest in the OSP (an increase of about 10 pp in the LD group and 20 pp in the HD group) (Fig. 3N and O) and in the ISP (an increase of about 6 pp in the LD group and 12 pp in the HD group) (Fig. 2N and O). The increase was statistically important only in MP in the HD group (about 9 pp) (Fig. 1O).

3.2. Changes in cytokine levels

Supplementation of acrylamide elicited fluctuation in the level of proinflammatory cytokines synthetized in the ileal Payer patches (Fig. 4). In the C group, the level of interleukin 1 β (IL- 1β) amounted to 63.38 ± 3.74 pg/mg. A low dose of ACM did not cause statistically significant changes in the levels of the IL-1β with regard to group C (78.07 ± 3.70 pg/mg), although in the HD group the increase was statistically significant (to 90.20 ± 11.38 pg/mg) (Fig. 4 A). Similarly, only supplementation of a high dose of ACM led to an increase in interleukin-6 (IL-6) (from 107.17 ± 9.70 in the C group to

Table 2
Neurons immunoreactive to particular neuroactive substance studied in the porcine ileum under physiological conditions (C group) and after low dose (LD group) or high dose (HD group) of acrylamide administration.

	ISP			OSP			MP		
	C group	LD group	HD group	C group	LD group	HD group	C group	LD group	HD group
SP	14.79 ± 1.23	20.26 ± 1.31	21.52 ± 1.05 *	15.47 ± 1.64	24.77 ± 1.26 *	31.25 ± 1.11 ***	0.53 ± 0.14	0.95 ± 0.13 **	1.93 ± 0.18 ***
CGRP	23.44 ± 1.23	30.50 ± 0.63 ***	35.28 ± 1.29 ***	20.14 ± 1.32	22.99 ± 1.06	27.42 ± 0.35 ***	16.07 ± 1.04	20.65 ± 1.16 *	23.41 ± 0.79 ***
GAL	49.45 ± 0.63	58.87 ± 1.72 *	65.70 ± 3.04 ***	38.84 ± 1.16	41.11 ± 0.45	56.52 ± 1.89 ***	3.01 ± 0.44	7.44 ± 0.42 **	17.34 ± 1.01 ***
nNOS	12.52 ± 0.76	10.96 ± 0.95	8.01 ± 1.32 *	11.80 ± 1.17	7.69 ± 0.61 **	3.20 ± 0.45 ***	40.62 ± 1.33	33.14 ± 2.00	31.46 ± 1.34 **
VACHT	50.22 ± 1.23	56.05 ± 1.03 **	62.22 ± 1.04 ***	30.77 ± 0.71	40.38 ± 1.47 ***	51.16 ± 1.02 ***	40.92 ± 2.45	43.87 ± 1.16	49.77 ± 0.91 **

ISP- inner submucous plexus, OSP- outer submucous plexus, MP- myenteric plexus.
*p < 0.05, **p < 0.01, ***p < 0.001 indicate differences in expression of particular substance studied in comparisons to the control animals. Relative frequency of particular neuronal subclasses is presented as % (mean ± SEM) of all neurons counted within the ganglia stained for PGP 9.5 (used as pan-neuronal marker). At least 500 PGP 9.5-positive cell bodies were evaluated in particular types of enteric plexuses in each animal.

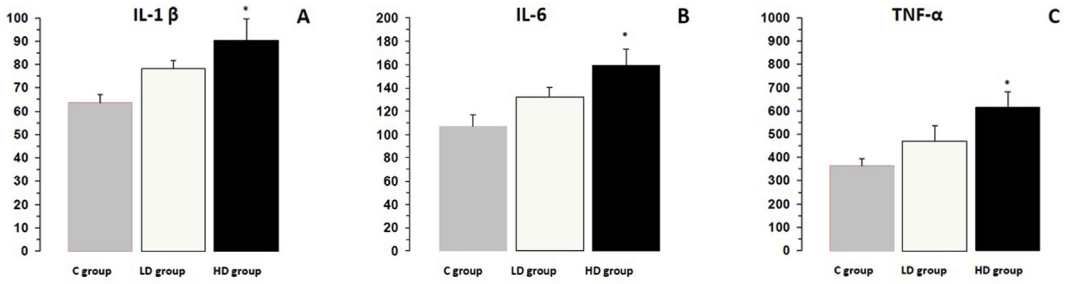

Fig. 4. The concentration of cytokines in the ileal Payer's patches in control animals (gray bars) and after low (white bars) and high (black bars) doses of ACM supplementation. A- concetration of IL-1β in control (C group) and experimental groups (LD group and HD group); B- concetration of IL-6 in control (C group) and experimental groups (LD group and HD group); C- concetration of TNF-α in control (C group) and experimental groups (LD group and HD group).

159.31 ± 13.76 pg/mg in the HD group) (Fig. 4 B) and tumour necrosis factor- α (TNF- α) (from 365.03 ± 28.09 in the C group to 617.20 ± 65.93 pg/mg in the HD group) (Fig. 4 C) levels in a statistically significant manner, although in the LD group there was also an upward trend (IL-6: an increase to 130.97 ± 9.20 pg/mg, TNF-α: an increase to 467.90 ± 67.90 pg/mg) (Fig. 4 B, C).

3.3. Histopathological evaluation

Low and high doses of acrylamide supplementation caused microscopic changes in the mucosa and submucosa of the ileum (Fig. 5). Superficial erosions (Fig. 5B), focal changes in the surface epithelium structure, infiltration of the mucosa and submucosa by leucocytes, especially eosinophils and lymphocytes (Fig. 5C and E,F), as well as hyperaemia in the mucosa and submucosa were observed. Changes were more strongly expressed in the HD group.

4. Discussion

The present experiment, for the first time, demonstrated the impact of low (TDI) and high doses of acrylamide supplementation on neurochemical characterization of the intramural neurons in the porcine ileum. Even a TDI dose of ACM led to alterations in the expression of the investigated neuroactive substances. The character and force of changes were dependent on both the plexus and the neuroactive substance studied. Additionally, changes in the expression of neuroactive substances were accompanied by an increase in the level of proinflammatory cytokines synthesized by ileum Peyer patches. These observations correlate well with reports describing the common interaction between the ENS and the immune system of the intestine (Margolis and Gershon, 2016). This co-operation included bidirectional communication involving numerous neurotransmitters/neuromodulators as well as cytokines (Verheijden et al., 2015). Recent reports underline the important role of this interaction in modulation of intestinal inflammation (Margolis et al., 2011). In turn, the results of the present study suggest that changes caused by ACM supplementation may be associated with inflammation. This is well in line with previous reports on ACM toxicity. Namely, it has been shown that long-term consumption of potato chips containing high doses of ACM leads to an increase in the secretion of inflammatory mediators (IL-6, C-reactive protein (CRP)) and oxidized form of cholesterol (LDH) in the human body. Additionally, *in vitro* studies have demonstrated the increase of reactive oxygen species in isolated human monocytes and reduced concentration of cellular glutathione reduced form (GSH) (Naruszewicz et al., 2009). It has also been demonstrated that ACM stimulates an increase in the synthesis of proinflammatory cytokines, such as TNF-α, IL-1β and an induced form of nitric oxide synthase (iNOS) in the CNS structures of albino mice (Santhanasabapathy et al., 2015).

Alterations in the neurochemical phenotype of the ENS neurons observed in the present study may be an adaptation of neurons to inflammatory conditions accompanying ACM intoxication. In recent years, participation of individual neurotransmitters/neuromodulators in intestinal regulation has been confirmed in many animal models (Costes et al., 2013; Margolis et al., 2011; Steinhoff et al., 2014). It was also shown that neurotransmitters secreted by neurons may positively or negatively affect the severity of inflammatory responses of the bowel leading to changes in GI tract secretion and motility (Mawe, 2015). It should be emphasized that the contribution of the neuroactive substances in this experiment to the regulation of inflammatory conditions was also described.

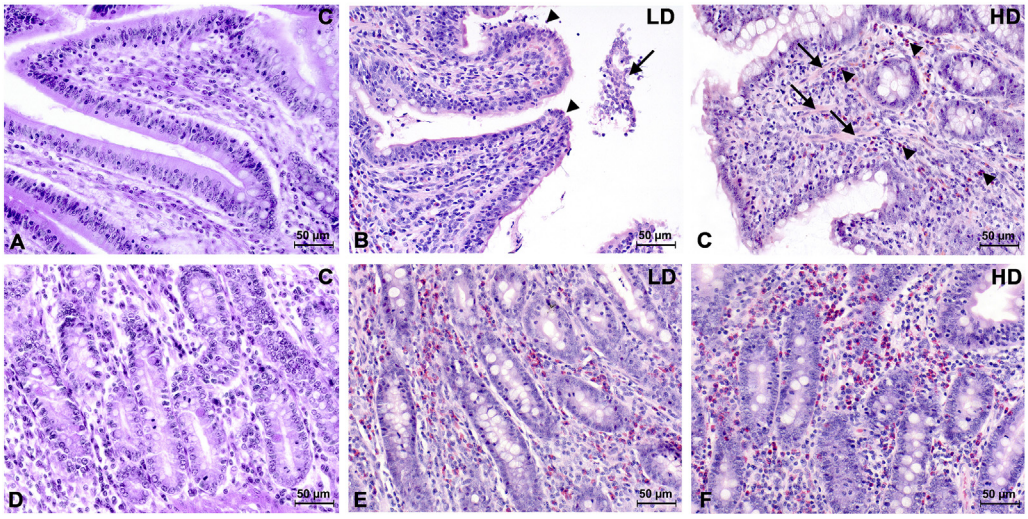

Fig. 5. Histopathological evaluation of the ileum sections from control (A, D), LD (B, E) and HD (C, F) groups: A- mucosa in physiological state; B- superficial erosions (arrowheads) and exfoliated cells (arrow); C- increased number of smooth muscle cells within the intestinal villi (arrows) and infiltration of eosinophils (arrowheads); D- submucosa in physiological state; E – infiltration of eosinophils and leukocytes; F- infiltration of eosinophils and leukocytes.

Namely, GAL is a neuropeptide that is known for its regulatory properties in the course of various inflammatory conditions. An upregulated level of GAL-like immunoreactivity was noted following pathological states within the GI tract, including natural and experimentally induced inflammation (Gonkowski et al., 2010; Palus and Całka, 2015; Matkowskyj et al., 2009). It was also shown that GAL influences the secretion of cytokines involved in inflammatory response, like TNF-á, interleukin 1á (IL-1á) and interleukin 8 (IL-8) (Dallos et al., 2006). Our findings support previous reports appearing to confirm the anti-inflammatory properties of GAL in the intestine.

It should be underlined that one of the components of inflammation is pain. Pain reactions lead to an increase in the expression of sensory neuropeptides. Tachykinins are unquestionably engaged in the regulation of intestinal inflammation. SP participates in the regulation of inflammation via binding to NK1 receptors (Shimizu et al., 2008). The presence of NK1 receptors on macrophages and T lymphocytes was confirmed (Castagliuolo et al., 1997). SP, which is synthetized and released in the ENS structures, affects various immune cells and causes changes in the levels of cytokines including interleukin-1 (IL-1), IL-6, IL-8 and TNF-α mainly by activation of the transcription factor NK-κB in target cells (Azzolina et al., 2003). Upregulated expression of NK1 receptors was observed in inflammatory bowel disease, which suggests its participation in immune response in the GI tract (Shimizu et al., 2008). Furthermore, previous reports demonstrated that an elevated level of SP expression accompanies GI tract disorders, such as childhood chronic gastritis and duodenitis (Islek et al., 2016), gastric ulcers (Erin et al., 2012) and *Helicobacter pylori* infection (Mönnikes et al., 2005).

In turn, there is evidence supporting the neuroprotective role of CGRP and its participation in defence mechanisms against harmful factors (Lambrecht et al., 1993). It has been shown that CGRP is involved in the regeneration of gastric mucosa in the course of peptic ulcer disease (Ohno et al., 2008). CGRP release has been also reported to occur in several diseases, such as diabetes or chemically induced inflammation of porcine descending colon as well as ulcerative colitis (Bulc et al., 2018; Li et al., 2013; Makowska and Gonkowski, 2018). It is also known that CGRP participates in the conduction of sensory and pain stimuli (Lambrecht et al., 1993). Additionally, close cooperation between CGRP and the immune system has been reported by many authors. CGRP affects various immune cells and causes inhibition in the levels of cytokines such as TNF- α, IL-1β as well as IL-1β-induced IL-8 release (Li et al., 2004).

On the other hand, a decrease in nNOS-like immunoreactivity was observed in the present study. Previous reports also demonstrated a decrease in nNOS- like immunoreactivity in many GI disorders, such as Crohn's disease, inflammatory processes, diabetes and achalasia (Bulc et al., 2019; Lin et al., 2004; Rivera et al., 2011; Takahashi, 2003). In contrast, inflammatory bowel disease (IBD) caused an increase in the number of nitrergic nerve fibres in the mucosal layer of the canine gastrointestinal tract (Rychlik et al., 2017). Similar changes were observed in course of Crohn's disease and bisphenol A intoxication (Szymanska et al., 2018; Winston et al., 2013). Although both the anti- and pro-inflammatory properties of nitric oxide (NO) were described previously and available data suggest that NO plays a mainly proinflammatory role in the GI tract, the role of NO in neuroprotective and inflammatory response in the GI tract is unclear. We can speculate that the function of NO depends both on the pathological condition and the target tissue.

Finally, VACHT plays a crucial role in the storage and release of acetylcholine (ACh) from synaptic vesicles in neurons and it is known as a marker of cholinergic neurons (de Castro et al., 2009). It was demonstrated that VACHT is a necessary factor in an effective inflammatory response induced by lipopolysaccharide (LPS) administration (Leite et al., 2016). ACh significantly decreased the level of proinflammatory cytokines (TNF-α, IL-6, IL-1β) in human blood macrophages in the presence of LPS (Borovikova et al., 2000). Other authors suggest that cholinergic and nicotinic acetylcholine receptors, choline acetyltransferase and acetylcholinesterase are important factors by which immune cells attenuate inflammatory conditions (Hao et al., 2011). In the present study, it was demonstrated that the ENS neurons increased the synthesis of VACHT in response to ACM intoxication. The readiness by which the neuronal expression of VACHT is upregulated has also been noted in numerous pathological conditions in the intestine (Kolgazi et al., 2013) and it is thought that VACHT plays a crucial role in the control of inflammatory conditions in the gut.

Nevertheless, bearing in mind the neurotoxic activity of ACM described in the peripheral and central nervous systems, we cannot exclude that the observed changes are the result of toxic effects of acrylamide on ENS neurons. Studies on the toxicity of acrylamide have shown that individuals exposed to high doses of ACM have peripheral symptoms such as weakness, tingling and limb endurance, convulsions or ataxia. The destruction of the nervous system, inhibition of axonal transport and neurotransmission are the main consequences of ACM toxicity (Szczerbina et al., 2008; LoPachin, 2004). ACM inhibits creatine kinase, leading to a decrease in ATP in cells and cell death (Sheng et al., 2009). By binding cysteine-rich proteins in the presynaptic membrane, it also reduces the release of neurotransmitters (LoPachin and Barber, 2006). There are also reports confirming that ACM induces the inhibition of slow and fast axonal transports (Sickles et al., 2002). Despite numerous studies on the neurotoxicity of ACM, the mechanism of its toxic effect on neural tissue has still not been sufficiently explained. Nonetheless, we can speculate that supplementation of ACM causes a toxic effect on ENS neurons. Changes in the expression of neuroactive substances in ENS neurons may be the response of neurons to the irritant effect of acrylamide. Numerous previous studies have shown changes in the chemical coding of intramural neurons during neurodegenerative processes in the GI tract (Gonkowski et al., 2010; Liu et al., 2009). In addition, the involvement of neuroactive substances selected for this experiment in the regenerative and neuroprotective processes has also been previously described. Unquestionably, GAL is a peptide with neuroprotective properties whose involvement in trophic and regenerative processes after neuronal crush and axotomy has been described in both central and peripheral nervous systems (Mahoney et al., 2003). Upregulated expression of GAL in ENS structures was also observed in the porcine descending colon following axotomy (Gonkowski et al., 2010). Previous studies also confirmed the neuroprotective effect of SP on the ENS neurons after axotomy and other pathological processes in the GI tract (Bulc et al., 2018; Gonkowski et al., 2010). Similarly, CGRP displays neuroprotective and neurotrophic properties and participates in neuronal repair and regeneration processes (Roza and Reeh, 2001). Additionally, the inhibition of the synthesis and secretion of other neurotransmitters such as nNOS was also described as a CGRP action (Makowska and Gonkowski, 2018). This is consistent with the reduction in the population of nNOS-LI neurons observed in the present study. In turn, previous studies showed that changes in VACHT expression depend both on the part of the GI tract under study and the pathological condition. Nevertheless, ACh shows neuroprotective properties in different parts of the nervous system (Maeda et al., 2004; Nakamizo et al., 2005; Thompson et al., 2006).

5. Conclusion

The current results indicate that supplementation of ACM, even in the TDI dose, led to changes in the neurochemical phenotype of the ENS neurons in the porcine ileum. Changes in the expression of neuroactive substances were accompanied by an increase in the level of proinflammatory cytokines synthesized by ileum Peyer patches. It is reasonable to expect that SP, CGRP, GAL, nNOS and VACHT participate in the regulation of inflammatory conditions induced by ACM supplementation. It is also highly probable that ACM has a direct neurotoxic effect on the ENS neurons in the porcine ileum.

Conflicts of interest

The authors declare no conflicts of interest with respect to the publication of this manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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