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Age-related changes in myosin-V myenteric neurons, CGRP and VIP immunoreactivity in the ileum of rats supplemented with ascorbic acid

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Summary. We examined the effects of ascorbic acid supplementation on myosin-V, calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP) immunoractivities in the myenteric neurons in aging rats. Male rats were divided into groups: young 90-day-old rats (E90), 345-day-old control rats (E345), 428-day-old control rats (E428), 90- to 345-day-old rats treated with ascorbic acid (1 g/L) (EA345), and 90- to 428-day-old rats treated with ascorbic acid (1g/L) (EA428). The quantitative results showed that aging reduced the number of myosin-V-immunoreactive neurons compared with young animals (E90). Ascorbic acid supplementation in the EA345 and EA428 groups increased the average area of myosin-V neurons by 24.6% and 24.1% compared with the E345 and E428 groups, respectively. When all groups were compared, we observed significant differences for the CGRP- and VIP-immunoractive varicosities of nerve fibers from myenteric neurons. Ascorbic acid supplementation had a neurotrophic effect on all neurons studied, suggesting a neuroprotective role.

Key words: Aging, Ascorbic acid, CGRP, Myenteric neurons, VIP, Myosin V

Introduction

The enteric nervous system (ENS) is located in the layers that form the wall of the gastrointestinal tract and functions independently of the central nervous system (CNS) (Brehmer, 2006). Enteric neurons are heterogeneous with regard to their physiological roles and neurotransmitters (Saffrey, 2004). Chemical, histochemical, immunohistochemical, and physiological studies have confirmed the morphological diversity of the ENS. Nerve cells can express various neurotransmitters in the intestine. ENS nerve cells may undergo structural or functional changes as a result of adaptive responses to different stimuli in an attempt to maintain homeostasis of intestinal function (Giaroni et al., 1999; Lomax et al., 2005). Some pathological conditions also involve changes in enteric neurons and other autonomic neurons, including bowel disease (e.g., ulcerative colitis or Crohn's disease), extraintestinal disease (e.g., Parkinson's disease), and enteric neuropathy (Giaroni et al., 1999; Micieli et al., 2003; Vinik et al., 2003). The aging process can also induce such changes (Schneider et al., 2007).

Aging is associated with a variety of gastrointestinal disorders (Talley et al., 1992; Majumdar et al., 1997). For example, advancing age is associated with an increased incidence of problems related to motility, including delayed gastric emptying and increased intestinal transit time associated with fecal stasis (Jost, 1997; O'Mahony et al., 2002; Hays and Roberts, 2006; Norton, 2006). These aging-related gastrointestinal problems may significantly impact the well-being of aging individuals. The causes of gastrointestinal disorders in the elderly are not clear, but the symptoms suggest an underlying etiology involving a loss or distortion of functional neural mechanisms of the intestine that normally contribute to gastrointestinal function and health.

Several gastrointestinal diseases are associated with aging (Talley et al., 1992; Majumdar et al., 1997), resulting in impaired function of the mucosa or motility of the gastrointestinal tract, which can lead to dysphagia, gastroesophageal reflux disorders, difficulty in relaxing gastric fundus during food intake, atrophic gastritis,

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delays in the digestive period, intestinal diverticula, diarrhea, and fecal incontinence, depending on the affected area (Wade and Cowen, 2004; Phillips and Powley, 2007).

The main factor associated with neuronal death is the generation of free radicals. Nervous tissue has high oxygen consumption (Halliwell, 1992; Kuyvenhoven and Meinders, 1999). Reactive oxygen species, generated mainly in mitochondria during the cellular respiration process, have unpaired electrons, rendering them highly reactive to other biological substances (Kuyvenhoven and Meinders, 1999).

Drugs that reduce oxidative stress, such as ascorbic acid, may play a significant role in the treatment of intestinal complications caused by aging. Ascorbic acid can prevent the reduction in the number of myenteric neurons resulting from the action of free radicals (Zanoni and Freitas, 2005). Antioxidants maintain internal balance in the body, prevent the formation of free radicals, neutralize species that have already formed, and undergo a reduction during the aging process, further promoting the occurrence of oxidative stress (Kuyvenhoven and Meinders, 1999). The modest level of antioxidants associated with increased iron concentration (Benkovic and Connor, 1993) and polyunsaturated fatty acids (Rice-Evans and Burdon, 1993) render the neural tissue more susceptible to reactive oxygen species.

Ascorbic acid is a water-soluble vitamin that has shown antioxidant effects in neutralizing free radicals, as have vitamins A and E (Caldeira, 1989; Deplabos and González, 2000). O'Donnell and Lynch (1998) reported a significant decrease in ascorbic acid concentration in the cerebral cortex in aged rats. Thus, ascorbic acid may also be used to prevent the occurrence of intestinal complications caused by aging because it prevents the decrease in the number of myenteric neurons resulting from the action of free radicals.

The objective of the present study was to investigate the general population of myenteric neurons (myosin-V-immunoreactive) and the varicosities of the subpopulations of nerve fibers in myenteric neurons and muscle layer (i.e., calcitonin gene-related peptide-immunoreactive [CGRP-IR] and vasoactive intestinal polypeptide-immunoreactive [VIP-IR]) in aged rats receiving ascorbic acid supplementation.

Materials and methos

Study groups

Intestinal segments were obtained from 85 male albino Wistar rats (Rattus norvegicus) that were 90 days old and obtained from the Central Biotery of Universidade Estadual de Maringá. All animal procedures were approved by the Committee of Ethics in Animal Experimentation, Universidade Estadual de Maringá (protocol no. 024/2004; approved May 27, 2004). All techniques were carried out in accordance with the ethical principles of the Brazilian Academy of Animal Experimentation.

The animals were weighed and divided into five groups: E90 (young- 90-day-old rats), E345 (345-day-old control rats), E428 (428-day-old control rats), EA345 (345-day-old aged rats treated with ascorbic acid since 90 days old) and EA428 (428-days-old aged rats treated with ascorbic acid since 90 days old). Ascorbic acid supplementation was conducted for 255 days (EA345 group) and 338 days (EA428 group) by adding it to the animals' water daily (1 g/l) in black painted bottles. The solution was offered *ad libitum*. During this period, the rats were kept in individual cages with lights on from 6:00 h to 18:00 h and constant temperature (24±2°C). Treatment was always administred in the afternoon (15:00-18:00 h).

Material resection and processing

After the experimental period, the animals were sacrificed under thiopental anesthesia (40 mg/kg body weight, i.p.), after which nasal-caudal lengths were obtained. Two hours preceding sacrifice, the animals were injected with vincristine sulfate (0.5 mg/kg) to stabilize the microtubules of the cytoskeleton.

Nerve fiber immunoreactive for CGRP and VIP

The ileum was removed, washed with 0.1 M phosphate-buffered saline (PBS), pH 7.4, tied on one end of the segment, injected with Zamboni fixative, and closed at the other end. The segments were maintained for 18 h in the same solution. The ends were cut, opened on the mesenteric border, dehydrated in an increasing series of alcohol (80, 95 and 100%), clarified in xylol, and rehydrated in a decreasing series of alcohol (100, 95, and 80%). The segments remained in each solution for 30 min and were stored in PBS with the addition of 0.08% sodium azide at 4°C. Subsequently, the segments were dissected under a stereomicroscope by removing the mucosa and submucosa, resulting in two prepared segments for each animal's total muscular layers. Separate preparation techniques were used for CGRP and VIP immunohistochemistry.

The preparations were initially washed three times in 0.1 M PBS Triton X-100 (0.3%) solution for 5 min and incubated in 10% goat serum blocking solution for 2 h. The preparations were then incubated in a solution containing anti-CGRP primary antibody (1:200; Peninsula Labs, USA) or VIP (1:200, Peninsula Labs) produced in rabbits, at room temperature for 24 h. After this period, the preparations were washed three times in 0.1 M PBS for 5 min and incubated with anti-rabbit conjugated fluorescein secondary antibody (1:200; Peninsula Labs) for 2 h at room temperature and protected from light. The preparations were then washed three times in 0.1 M PBS for 5 min and mounted in

glycerol gel (9:1). A negative control was performed with the omission of the primary antibody.

Analysis of CGRP-IR and VIP-IR neuron varicosities

The images of CGRP-IR and VI-IR nerve fibers were captured by an AxioCam (Zeiss, Jena, Germany) high resolution camera coupled to an Axioskop Plus (Zeiss) light microscope, digitized on a computer using AxioVision version 4.1 software, and recorded on a flash drive. Ten microscopic fields were captured for each preparation, obtained by sampling, to yield a total of 300 images. The image analysis program Image-Pro Plus version 4.5.0.29 (Media Cybernetics, Silver Spring, MD, USA) was used to measure the area (μm^2) of 400 varicosities in each animal for a total of 2400 varicosities per group. Varicosities are symmetric expansions along the nerve fiber which accumulate neurotransmitters. The contour of the circumference of each varicosity was performed manually. Measurements of the varicosities were performed using 800x digital zoom, keeping the original calibration of the captured image.

Immunolocalization of neuronal myosin V: study of general myenteric neuron population

Another 25 rats were perfused with 1 mL/body weight of saline solution followed by perfusion with 1 mL/g per body weight of fixation solution containing sodium periodate (10 mM), lysine (75 mM), paraformaldehyde (1%) in phosphate buffer (PB) (37 mmol/L, pH 7.4). Immediately after perfusion, each treated fragment of jejunum was removed, rinsed with saline solution, flushed with fixative solution and tied in its extremities (balloons). Thirty minutes after immersion in fixative solution the jejunum was opened and left in this solution for an additional 30 min. Subsequently, the segments were opened along the mesenteric border and dehydrated in alcohols (50%, 70%, 80%, 90%, 95% and 100%), kept in each solution 10 min, cleared in xylol (10 min), rehydrated back through the ethanol 100%, 95%, 90%, 80%,70%, and stored in 70% ethanol. Afterwards, the segments were dissected under stereomicroscopy with trans-illumination, through the removal of the mucosa and submucosa layer, obtaining muscular layer whole mounts. These mixtures were washed four times in phosphate buffered saline (PBS) (0.1 M, pH 7.4) and blocked for 2 h with PBS containing bovine serum albumin (2%), goat serum (2%) and Triton X-100 (0.5%) at room temperature. Later, the segments were incubated in eppendorfs in a solution containing 0.86 μg/mL of the myosin-V primary antibody (extracted from rabbits) (1:750) diluted in PBS, BSA (1%), Triton X-100 (0.1%) and goat serum (2%) at room temperature under agitation (48:00). After incubation, the tissues were rinsed twice in PBS (0.1 M), Triton X-100 (0.1%) and twice in a solution of PBS (0.1 M) + Tween-20 (0.05%). Then, the tissues were incubated in peroxidase conjugated secondary antibodies (1 μg/mL) (1:1000, Pierce Lab) at room temperature under shaking (24 h). Finally, they were rinsed four times for 15 min in PBS (0.1 mol/L)+ Tween-20 (0.05%). The staining with peroxidase conjugated antibody was developed by incubation with 0.75 mg/mL diaminebenzidine and 0.03% of H_2O_2 in water (1 mL) and PBS 0.1 mol/L (1 mL) for 15 min at room temperature under shaking. Samples were mounted in glycerol gel, containing glycerol (50%), gelatin in water (0.07 g/mL) and phenol (2 μL/mL). The negative control was performed with the omission of the primary antibody.

Quantitative analysis of myosin-V-immunoreactive myenteric neurons

The counts of myosin-V were made in images captured by an AxioCam (Zeiss, Jena, Germany) high resolution camera coupled to an Axioskop Plus (Zeiss) light microscope, digitized on a computer using AxioVision version 4.1 software, and recorded on a flash drive. Image-Pro Plus version 4.5.0.29 image analysis software (Media Cybernetics, Silver Spring, MD, USA) was used for the neuronal quantification of the images recorded on the flash drive. Ten ganglia neurons of the myenteric plexus were quantified per animal.

Morphometric analysis of myosin-V-immunoreactive myenteric neurons

The measurement of the areas of myosin-V-immunoreactive myenteric neuronal cell bodies was performed using the same images captured using a 20x objective, similar to the quantitative analysis. The area (μm^2) of 100 neuronal cell bodies was measured using Image-Pro Plus for each animal for each technique, yielding 500 areas per group.

Statistical analysis

Data were analyzed using Statistica and GraphPad Prism software. Morphometric data were set in delineation blocks followed by Tukey's *post hoc* test. For the other data, we used one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. The significance level was set at 5%. The results are expressed as mean ± standard error.

Results

Table 1 shows the average initial body weight, final body weight, and nasal-caudal length of all groups. The average initial weight of all groups was 328.5±13.6 g. A weight increase of 41.24% and 59.2% was found in aged animals without supplementation (E345 and E428 groups, respectively) compared with the average initial

weight ($p<0.05$). An aging period of 83 days was associated with a 12.12% weight increase between the E345 and E428 groups ($p<0.05$). Ascorbic acid supplementation in the EA345 and EA428 groups did not alter body weight compared with controls ($p>0.05$).

The nasal-caudal length increased in the E345 and E428 groups compared with the E90 group ($p<0.05$). The supplemented groups (EA345 and EA428) presented a nasal-caudal length similar to the group of young animals (E90; $p>0.05$). The average ascorbic acid intake during the experiment in the EA345 and EA428 groups was 39.98±1.456 and 41.7±1.362 mg/l/day.

The quantitative results showed that aging reduced the number of myosin-V-immunoreactive neurons compared with young animals (E90; $p<0.05$) (Fig. 1). Ascorbic acid supplementation did not act in the recovery of neuronal loss ($p>0.05$). Photomicrographs showing myosin-V-, VIP-, and CGRP-immunoreactive myenteric neurons are shown in Fig. 2, 3 and 4, respectively.

Aging did not alter the size of myosin-V-immunoreactive neurons ($p>0.05$). Ascorbic acid supplementation in the EA345 and EA428 groups increased the average area of neurons by 24.6% and 24.1% compared with the E345 and E428 groups, respectively ($p<0.05$) (Table 2). Increases in areas in the EA345 and EA428 groups were also observed, reflected by a shift to the right in the relative frequency curve (Fig. 2F).

The area of varicosities (μm^2) of CGRP-IR and VIP-IR nerve fibers is shown in Table 2. When all groups were compared, we observed significant differences ($p<0.05$) for the two neurotransmitters. In the E345 and E428 groups, we found a reduction in the area of CGRP-IR nerve fiber varicosities compared with the young group (E90). The initial size of varicosities of VIP-IR neurons was reduced in the E345 group, and subsequently the areas recovered in the E428 group with values higher than those of young animals.

Treatment with ascorbic acid (EA345 and EA428 groups) increased the area of varicosities of CGRP-IR and VIP-IR neurons compared with the E345 and E428 groups, also reflected by a shift to the right in the relative frequency curve (Figs. 3F, 4F).

Table 1. Initial weight (IW), final weight (FW), and nasal-caudal length (NCL). E90, young rats; E345, aged 345-days-old rats; EA345, aged 345-days-old rats supplemented with ascorbic acid; E428, aged 428-days-old rats; EA428, aged 428-days-old rats supplemented with ascorbic acid (n=6 per group).

	IW (g)	FW (g)	NCL (cm)
E90	-	367.7±6.8*	42.83±0.3#
E345	314.3±10.6	464.0±11.3	45.50±0.6
EA345	336.1±14.1	490.7±13.5	45.58±1.0
E428	335.5±17.4	523.0±15.6**	45.75±0.5
EA428	328.1±12.3	497.9±21.5	45.33±0.7

*: p<0.05 when compared to group E90; **: p<0.05 when compared to group E345; #: p<0.05 when compared to group E345 and E428

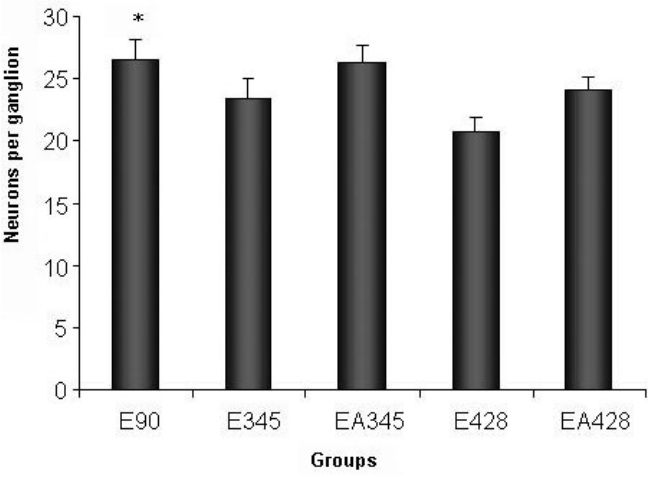

Fig. 1. Number of myenteric neurons immunoreactive for myosin-V ganglia using a 20x objective in the intermediate region of the ileum in young rats (E90), aged rats with no supplementation (E345 and E428), and aged rats supplemented with ascorbic acid (EA345 and EA428). n=5 animals per group. *: p<0.05, compared with E428 group.

Table 2. Cell body area (μm^2) of myosin-V-immunoreactive myenteric neurons and area of varicosities (μm^2) of CGRP- and VIP-immunoreactive nerve fibers. E90, young rats; E345, aged 345-days-old rats; EA345, aged 345-days-old rats supplemented with ascorbic acid; E428, aged 428-days-old rats; EA428, aged 428-days-old rats supplemented with ascorbic acid (n=6 per group).

	E90	E345	EA345	E428	EA428
Myosin V	202.4±3.5	192.1±4.3	255.0±2.63*	201.6±3.5	265.5±2.97*
CGRP	3.8±0.02	3.4±0.02**	3.7±0.02#	3.2±0.02**	4.2±0.02##
VIP	3.8±0.02	3.7±0.02**	5.2±0.03#	4.0±0.02**	4.6±0.02##

*: p<0.05 when compared to group E90, E345 and E428; **: p<0.05 when compared to group E90; #: p<0.05 when compared to group E345; ##: p<0.05 when compared to group E428

Discussion

In the present study, a significant increase was observed in weight between the aged (E345 and E428) and young animals, which was consistent with nasal-caudal length and reflected normal growth. According to

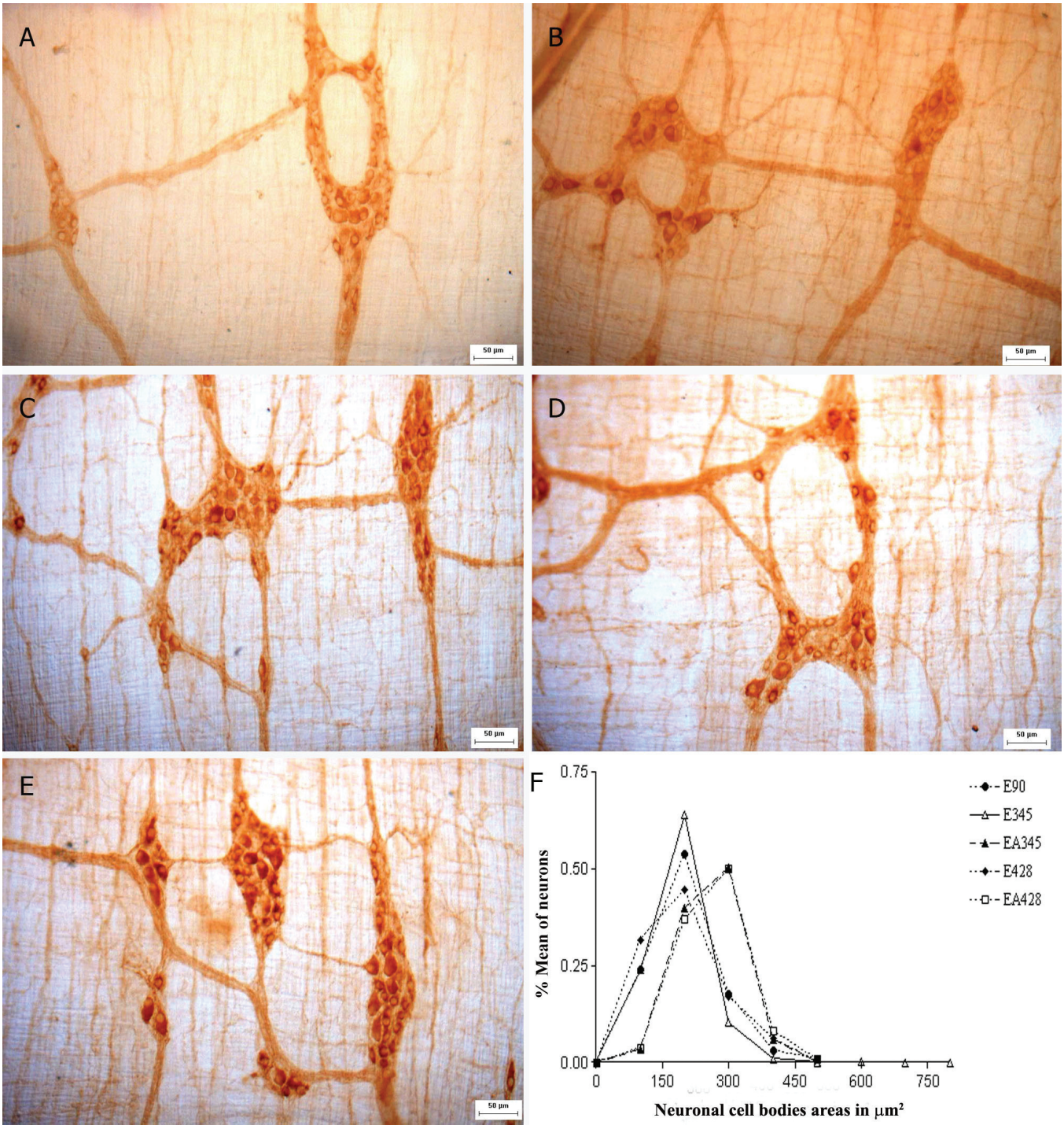

Fig. 2. Photomicrograph of Myosin-V-immunoreactive neurons of the intermediate region of the ileum of rats in the following groups: E90 (young) **(A)**, E345 (aged 345-days-old rats) **(B)**, EA345 (aged 345-days-old rats supplemented with ascorbic acid) **(C)**, E428 (aged 428-days-old rats) **(D)**, and EA428 (aged 428-days-old rats supplemented with ascorbic acid) **(E)**. **F.** Relative frequency distribution of cellular profile of myosin-V-immunoreactive myenteric neurons in the ileum. Bars: 50 μm.

Phillips and Powley (2001), body weight varies with age, but the animals gain weight until they are approximately 21 months old and subsequently quickly lose weight with age. Ascorbic acid supplementation had no effect on weight gain. The age of rats used in this experiment were similar to those used by Marese et al.

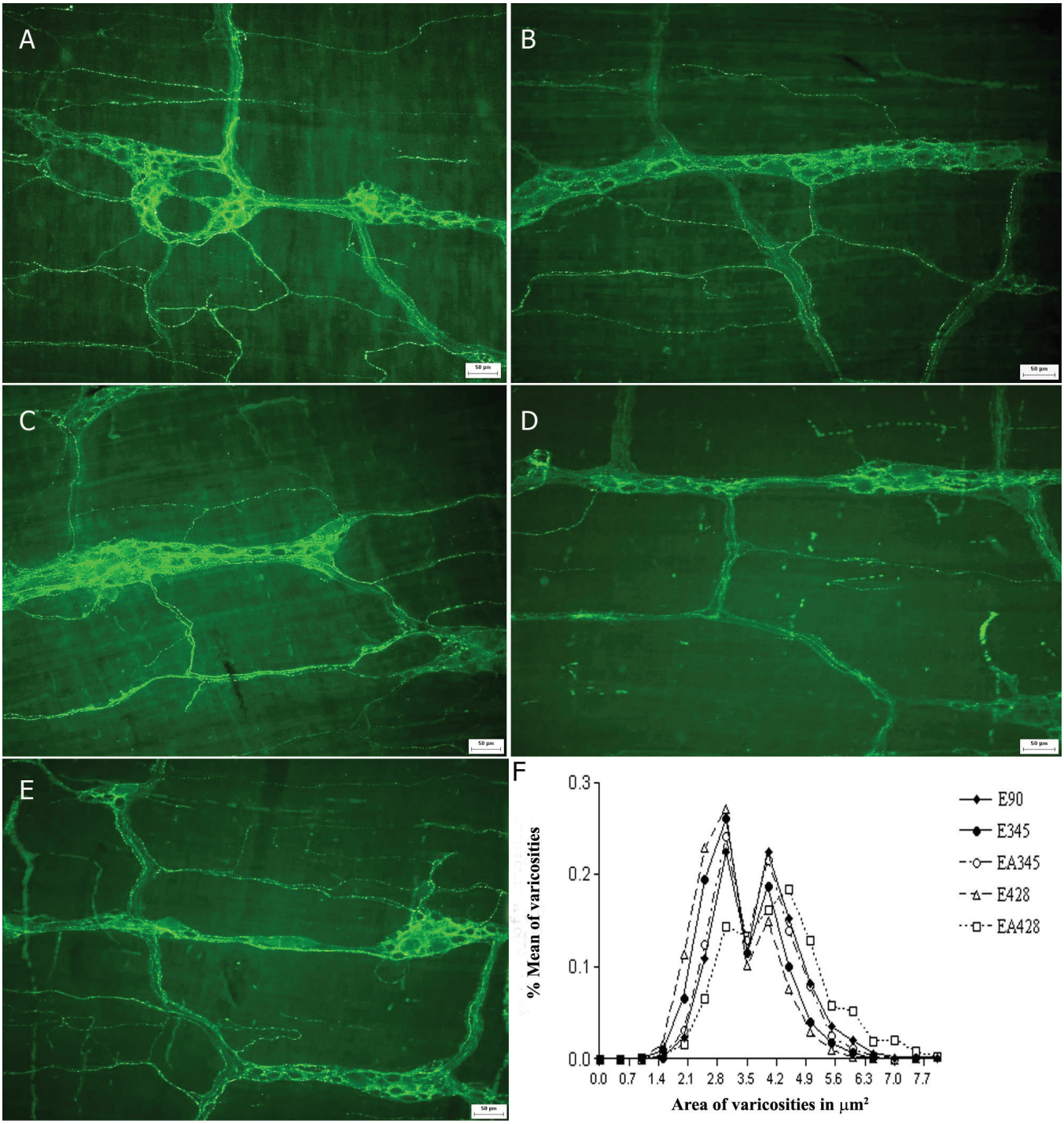

Fig. 3. Photomicrograph of varicosity of CGRP immunoreactivity in the myenteric plexus of the ileum in young and aged rats: E90 (young) **(A)**, E345 (aged 345-days-old rats) **(B)**, EA345 (aged 345-days-old rats supplemented with ascorbic acid) **(C)**, E428 (aged 428-days-old rats) **(D)**, and EA428 (aged 428-days-old rats supplemented with ascorbic acid) **(E)**. **F.** Relative frequency distribution of varicosity of CGRP-immunoreactive myenteric neurons in the ileum. Bars: 50 μm.

(2007).

The technique used for determining the general population of myenteric neurons was myosin-V. Myosin-V is a motor protein found in neuron cell bodies and extensions (Drengk et al., 2000) and is specifically present in neuronal cytoplasm which confers specificity

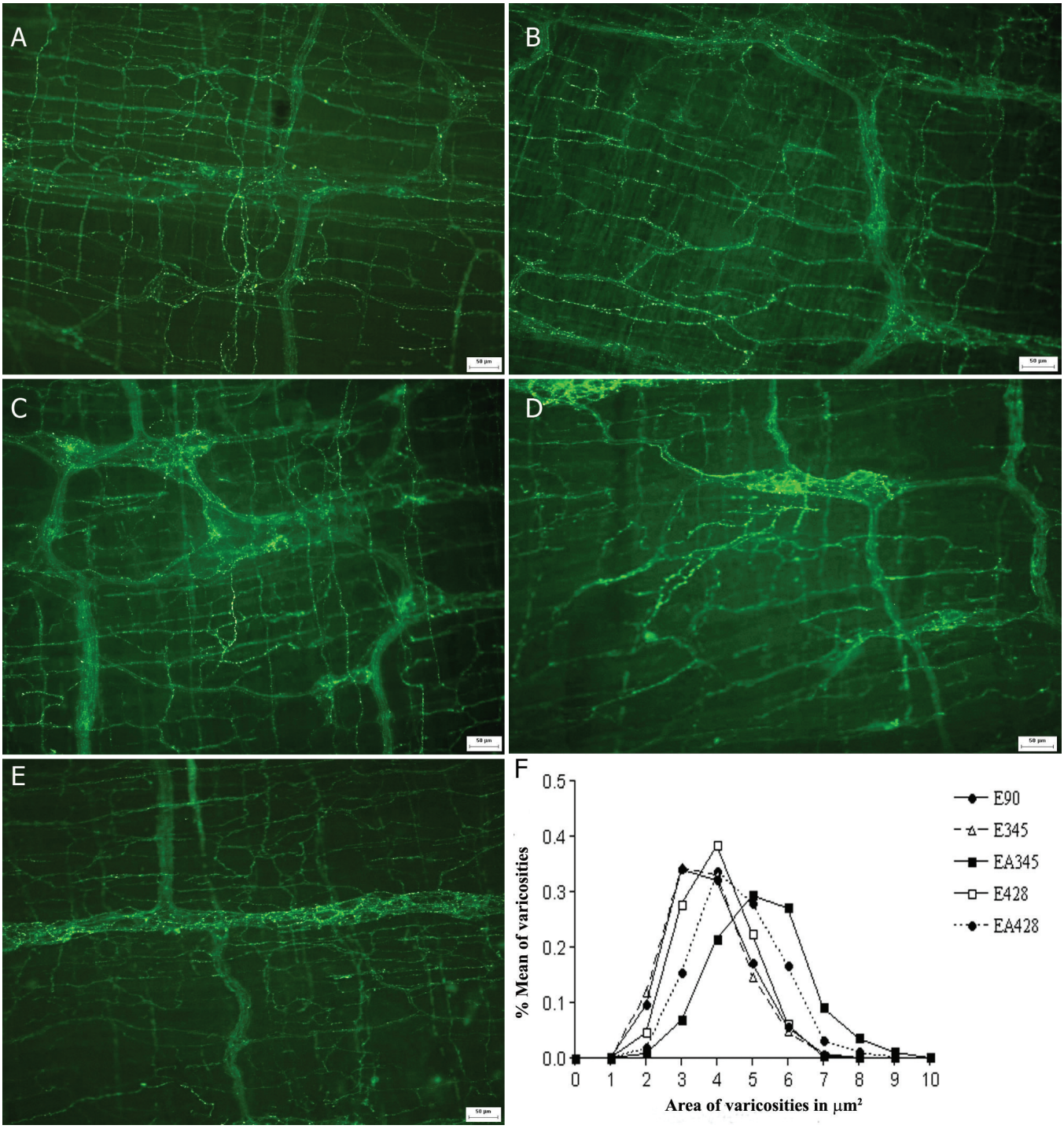

Fig. 4. Photomicrograph of varicosity of VIP immunoreactivity in the myenteric plexus of the ileum in young and aged rats: E90 (young) **(A)**, E345 (aged 345-days-old rats) **(B)**, EA345 (aged 345-days-old rats supplemented with ascorbic acid) **(C)**, E428 (aged 428-days-old rats) **(D)**, EA428 (aged 428-days-old rats supplemented with ascorbic acid) **(E)**. **F.** Relative frequency distribution of varicosity of VIP-immunoreactive myenteric neurons in the ileum. Bars: 50 μm.

to the technique, allowing visualization of cell bodies and their projections (Drengk et al., 2000; Zanoni et al., 2003).

When neuronal density was analyzed, we found a gradual reduction in the number of myenteric neurons during aging, which was significant for animals in the E428 group. Aging promotes changes in the ENS, particularly affecting neurons in the myenteric plexus. Several studies have reported a reduction in the number of neurons as a result of aging in rats, guinea pigs, mice (Santer, 1994; Johnson et al., 1998; Schafer et al., 1999; Wade, 2002; Phillips et al., 2003; Schoffen et al., 2005), and the digestive tract of humans (Meciano Filho et al., 1995; Gomes et al., 1997). Santer and Baker (1988) studied the myenteric neurons of rats at 6 and 24 months and found neuronal loss of 40% in the jejunum in aged rats. The authors emphasized that this loss may be accompanied by concomitant reorganization of the remaining elements, and therefore cannot be considered a degenerative process because the remaining ganglia increase their efficiency. We believe that differences in neuronal density we found when compared to the others is related to species of rat studied. Brownson (1955) conducted a study on brain aging, and defined the Wistar rats a period of 24 months for their senescence. The rats used in this research are considered by us in the aging process. Reduced neuronal density is associated with changes in the mucosa or motility of the gastrointestinal tract and may cause disturbances that, depending on the affected region, include dysphagia, gastro-esophageal reflux disorders, abdominal pain, diarrhea, constipation, and fecal incontinence (Wade and Cowen, 2004).

Neuronal death caused by aging has also been attributed to the large amount of oxidants produced concomitant with decreased antioxidants associated with aging. Aging occurs in most cases because of exposure of cells and their organelles to ionizing radiation, non-enzymatic reactions, and enzymatic reactions that reduce O_2 and water, with the consequent production of free radicals (Caldeira et al., 1989). Myenteric neurons are very susceptible to free radicals that are electronically unstable, highly reactive, and produced under conditions of aging. These radicals can bind to cellular structures, such as membrane lipids and the nucleotides of DNA (Schneider et al., 2007). The high concentration of radicals in situations of ischemia/reperfusion and hyperoxia create mutations in mitochondrial DNA that accelerate the production of additional free radicals, thus creating a cycle of further mutations that lead to detrimental cellular senescence and multiple organ failure. The accumulation of genetic mutations in somatic cells is the specific cause of cell senescence (Fortunato and Pinheiro, 2007). Muller et al. (2007) reported that a chronic state of oxidative stress exists in aerobic cells even under normal physiological conditions, which is attributable to an imbalance between oxidants and prooxidants. Oxidative stress is increased not only by the increased production of reactive oxygen species, but also by a decline in the efficiency of the enzymatic defense system in cells (e.g., glutathione peroxidase, catalase, superoxide dismutase, and so on) (Finkel and Holbrook, 2000).

The present study used ascorbic acid as an antioxidant. Ascorbic acid is a water-soluble vitamin that has proven effective in neutralizing free radicals, as have vitamins A and E (Caldeira et al., 1989; Deplabos and González, 2000). In aged animals supplemented with ascorbic acid (EA345 and EA428 groups), a tendency was observed toward increased preservation of the number of neurons, reflected in an increase of 14.34% and 13.8%, respectively, compared with animals of a similar age without supplementation. Our research group previously conducted a study of myenteric neurons of the ileum in rats after 120 days of supplementation with ascorbic acid and found myenteric neuron preservation of 18% (Zanoni and Freitas, 2005). Although this was not a significant conservation of the number of neurons, we consider this a positive result because the increased number of neurons per ganglion was accompanied by an increased cell body area of myosin-V-immunoreactive neurons in the EA345 and EA428 groups compared with controls. The increase in area may be related to the functional role of ascorbic acid. It has been reported in the literature that ascorbic acid acts in the synthesis of the neurotransmitter noradrenaline (Levine, 1986) and possibly the synthesis does not occur with the same efficiency without the presence of ascorbic acid. According Luiking et al. (2010) the regulation of nitric oxide production in neuronal cells is mediated by ascorbic acid. These related data (i.e., neuron number and neuronal size) suggest the neuroprotective and neurotrophic effects of ascorbic acid. We did not observe changes when were compared the body cell size from young animals (E90) with the aged (E345 and E428). Marese et al. (2007) observed similar results in the area of the cell body of myenteric neurons of the duodenum of rats in the aging process.

In the present study, we used an immunohistochemical technique to reveal neurons and nerve fibers that express VIP and CGRP. The muscle layers showed a high density of VIP-IR nerve fibers, suggesting that these fibers are motor neurons that play an important role in controlling the activity of gastrointestinal smooth muscles. The nerve fiber varicosities found in the present study were determined to be small dilations observed along the nerve fiber. Limited data in the literature have reported the effect of aging on VIP-IR neurons in the myenteric plexus in rats and the effect of treatment with antioxidants. In the present study, we verified a reduction in the size of varicosities of VIP-IR neurons in the E345 group compared with young animals, but we cannot conclude that this reduction in VIP expression is a result of neuronal death in this subpopulation of neurons because the methodology cannot determine neuronal density. One hypothesis is that a reduction of neurotrophic factors derived from some cells leads to the vulnerability of enteric neurons (Giaroni et al., 1999), such as those

that express VIP. Additionally, because of neural plasticity, these neurons may recover their expression over time. This hypothesis is supported by the fact that E428 animals have greater areas of varicosities than animals in the E345 group. At 83 days, increased activity of synthesis machinery could also occur. By utilizing electrical stimulation, Kasparek et al. (2009) found increased VIP release in the jejunum of Lewis rats during the aging process and an indication of increased synthesis.

Ascorbic acid supplementation increased the size of VIP-IR varicosities in the EA345 and EA428 groups compared with controls. The increased size of varicosities may be related to increased synthesis and release and can be considered positive because, in addition to the role of VIP as a neurotransmitter, recent studies have suggested the importance of VIP in the neuroprotection of central and enteric neurons and as a potent anti-inflammatory peptide. VIP-IR nerve fibers associated with cells in the mucosal immune system participate in the control of mucosal immunity in the gut (Brenneman et al., 2003). Zanoni and Freitas (2005) found similar results in VIP-IR neurons of the submucous plexus in rats with 120 day ascorbic acid supplementation.

When we studied the varicosities of CGRP-IR nerve fibers, we found a reduction in size of 11.9% in the E345 group and 18.9% in the E428 group compared with young animals (E90). CGRP-immunoreactivity in the mammalian small intestine is thought to originate from extrinsic (primary afferent) sources and from intrinsic neurons of the myenteric and submucosal plexuses (Sternini et al., 1987). CGRP is a neuropeptide that plays an important role in many ENS functions in vertebrate species, including the regulation of smooth muscle contractility in the gastrointestinal tract. In circular muscle preparations of the guinea pig ileum, CGRP was found to have both excitatory and inhibitory motor effects (Holzer et al., 1989). This neurotransmitter also exerts a function in the sensory function (Rasmussen et al., 2001), intestinal microcirculation (Kawasaki, 2002) and secretion (Tache, 1992). Recently, Mitsui (2009) reported that peristaltic reflexes evoked by mechanical or chemical stimuli applied to the intestinal mucosa in rat colon may be mediated by CGRP-containing intrinsic primary afferent neurons of the myenteric plexus. Little has been reported in the literature regarding the other phenotypes of enteric innervation, including CGRP, during aging, but possibly a reduction in the size of varicosities induces changes in intestinal homeostasis. Increased production of other neurotransmitters, such as VIP and nitric oxide, may be a compensatory mechanism that reduces CGRP expression.

Ascorbic acid supplementation increased the size of varicosities of CGRP-IR nerve fibers by 7.8% in the EA345 group and 25% in the EA428 group. We hypothesize that ascorbic acid has a neuroprotective effect on these neurons. The varicosities in the EA345 group were similar to those in young animals, and the EA428 group had larger varicosities than the E90 group.

In summary, our results showed that aging reduced the density of myosin-V-immunoreactive neurons and changed the size of varicosities of VIP-IR and CGRP-IR neurons. Supplementation with ascorbic acid had a neurotrophic effect on all neurons studied, suggesting a neuroprotective role.

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