Administering ascorbic acid to rats undergoing ageing processes: effects on myosin-V immunoreactive myenteric neurons

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ABSTRACT

During the ageing process the enteric nervous system undergoes morphofunctional changes, such as enteric neurodegeneration. Neuronal death can be attributed to increase radicals free, and ascorbic acid (AA), known antioxidant, could minimize damage cause by oxidative stress. The objective of this study is to analyse the behaviour of morphoquantative myenteric neurons in the duodenum of adult Wistar rats with aged 90 (C90), 345 (E345) and 428 (E428) days, as well as animals of the same age who received ascorbic acid supplementation for 120 days (EA345 and EA428). Whole-mount preparations of muscle layer from the duodenum of the animals were immunostained by the method myosin V. 80 microscopic fields were quantified (14.8 mm²/animal) and measured 100 neuronal cell bodies per animal. During the aging process, there was a reduction in neuronal density in all animals groups, indicating that the effects of age were not attenuated with AA supplementation. The increase in the neuronal area of the cell bodies in 428-day-old animals proved the influence of age on this parameter. There was no observed a neuroprotective effect of AA (1 mL/g body weight) on the neuronal population myenteric myosin V immunoreactive.

Key words: aging, antioxidant, enteric nervous system, immunohistochemistry.

INTRODUCTION

The enteric nervous system (ENS) is formed by a complex neuronal network that controls motility, gland secretion and blood flow in the wall of the gastrointestinal tract (GIT). This network features two ganglionated plexuses – the submucosal plexus located between the muscle layer of the mucosa and the submucosa, and the myenteric plexus located between the inner and longitudinal outer muscle layers of the digestive tube – which are able to mediate reflexes that are relatively independent from the central nervous system (Costa et al. 1980, Gershon 1981, Wade 2002, Wade and Cowen 2004).

Changes to the ENS lead to compromised functioning of the digestive system, with negative effects on other body organs, as this system is responsible for providing nutrients, water and electrolytes. Studies show that ageing leads to
changes in the morphology and density of ENS neurons, influencing GIT functioning (Hansen 2003, Phillips and Powley 2007).

Neurodegeneration in the myenteric plexus is a characteristic of ageing observed in rodents (Saffrey and Burnstock 1994, Phillips and Powley 2001, Phillips et al. 2003; Thrasivoulou 2006), with an approximate reduction of 50% in the neuron population for rats between 3 and 30 months of age (Gabella 1996, Phillips and Powley 2007). However, the life stage in which this loss begins is still questioned. An analysis of the duodenal myenteric population of rats between 21 days and 14 months of age showed that progressive and significant neuronal loss occurs starting at 210 days (Marese et al. 2007). This reduction occurs differently in each region of the GIT, and is constant within a given region (Saffrey and Burnstock 1994, Wade 2002, Marese et al. 2007). Thus, neuronal loss is equal to 40% and 50% in the small intestine, and 50% and 60% in the colon, when comparing adult (Gabella 1971) and old rats (Santer and Baker 1988), respectively.

In addition to quantitative changes, alterations in the profile of myenteric neurons are observed; larger neurons represent the largest contingent of the ganglionic population in aged animals (Gabella 1971, Thrasivoulou et al. 2000, Marese et al. 2007, Phillips and Powley 2007). The cellular death of myenteric neurons in the small intestine of rats, attributed to age, is linked to an increase in free radicals caused by oxidative stress (Joseph et al. 2000, Wiley 2002). In vitro studies have shown an increase in free radical levels in myenteric neurons, unleashing the process of apoptosis, which supports the concept of neuronal vulnerability in ageing (Soubeyre et al. 2001, Golden and Hinerfield 2002).

Antioxidant substances such as ascorbic acid (AA) have a neutralising effect on free radicals, which suggests their protective effect against oxidative damage (Greene and Latimer 1983, Feldman et al. 1997). The addition of AA into apoptosis-induced rat cerebellum granule cell cultures showed that this vitamin is capable of preventing apoptosis, even when apoptosis-inducing biochemical events had already begun (De Ruvo et al. 2000). Other authors further consider that AA acts as a natural antioxidant in neuronal extracellular fluid, in the ascorbate ionized form, representing an important neuroprotection site (Rice 2000, Joseph et al. 2000).

Considering this evidence, our objective is to evaluate the myosin-V immunoreactive myenteric population in the duodenum of rats undergoing the ageing process with daily AA supplementation.

MATERIALS AND METHODS

ANIMAL PROCEDURE

All the procedures of this study regarding the use of animals were in accordance with the ethical principles adopted by the Brazilian School of Animal Experimentation (Colégio Brasileiro de Experimentação animal - COBEA) and approved by the Animal Experimentation Ethics Committee of the Universidade Estadual de Maringá (UEM), Brazil.

Duodenum segments from 25 male Wistar rats (Rattus norvegicus) from the Central Biotery of the Universidade Estadual de Maringá were used. The rats were kept in polypropylene boxes under controlled temperature (24 ± 2°C) and illumination (12 h/12h light/dark cycles) with ad libitum access to water and food. The animals were distributed in the following experimental groups: group C90 (adult), E345 and E428 (aged), without the supplement of AA; group EA345 and EA428 received the supplements of AA diluted in water (1 g/L daily prepared) after 90 days of age.

After 90, 345 and 428 days of experimentation, the animals were weighed and intraperitoneally anesthetised with thiopental (40 mg/Kg body weigh, i.p.; Abbott Laboratories, Chicago, IL, USA), and euthanised. After laparotomy, the small intestine was collected and its length and circumference were measured to obtain the area.
MYOSIN-V NEURONAL STAINING

The antibody of choice, anti-Myosin-V, described for the immunostaining of neurons from the enteric nervous system (Drengk et al. 2000), is specific and it has the advantage of staining only neurons (sensorial, motor or interneuron) and their processes. The animals were perfused with saline solution (1 mL/g body weight) followed by fixative solution containing 10 mM sodium periodate, 75 mM lysine, 1% paraformaldehyde in 37 mM phosphate buffer, and pH 7.4. Immediately after perfusion, the duodenum was removed and the fixative solution was gently injected into the lumen, distending the muscular layer.

After applying ligatures to maintain the distension, the samples were post-fixed in the same solution as above for 1 h, dehydrated in ethanol (50, 70, 80, 90, 95 and 100%), cleared in xylol, rehydrated in ethanol (100, 95, 90, and 80%) and stored in 70% ethanol. Afterwards, the segments were dissected under stereomicroscopy with transillumination through the removal of the mucosa and submucosa layer, thus, obtaining whole-mount muscular layer preparations containing the myenteric plexus. The tissues were washed four times in PBS (0.1 M, pH 7.4) and blocked for 1 h in PBS with 2% BSA, 2% goat serum and 0.5% Triton X-100 at room temperature. Immunostaining proceeded with the incubation of tissues in 0.89 µg/mL of affinity-purified antibody specific to the myosin-V medial tail domain (Espreaifico et al. 1992, Buttow et al. 2003) diluted in the PBS with 2% BSA, 2% goat serum and 0.1% Triton X-100 at room temperature and under agitation (48 h). After incubation, the fragments were washed twice in PBS with 0.1% Triton X-100 and twice in PBS with 0.05% Tween 20.

Soon after, the tissues were incubated with 10 µg/mL secondary antibody conjugated with peroxidase for 24 h at room temperature under agitation and washed four times for 15 min in PBS with 0.05% Tween 20. The immunoreaction was developed with 0.75 mg/mL diaminebenzididine in PBS and 0.03% H₂O₂ for 10 min. Samples were placed in a gel mounting medium containing 50% glycerol, 0.07 g/mL gelatin in PBS and 2 µL/mL phenol. The negative control was performed with the omission of the primary antibody.

QUANTITATIVE ANALYSIS AND NEURONAL MORPHOMETRY

The quantification of myosin-V immunostained myenteric neurons was performed in 80 random microscopic fields for each animal (Miranda-Neto et al. 2001), with the aid of a light microscope (Olympus BX41) and a 40x objective. We analysed the intermediate and antimesenteric regions of the intestinal circumference (Natali et al. 2000). Half-neurons were counted in alternate fields. The area of each microscopic field was 0.185 mm², with a total area of 14.8 mm². The results were shown in mm².

We randomly measured the area of the cellular bodies (cellular profile) of 500 immunoreactive myosin-V cells of the duodenum per group. Images were obtained using a high-resolution camera (Q Color 3, Olympus America) coupled to a light microscope (Olympus BX41). Morphometric analyses were performed using Image-Pro Plus R 4.5 image analysis software (Media Cybernetics, Silver Spring, MD, USA). The neurons were classified according to their size in class intervals of 100 µm².

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism® v.3.0 (GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) was followed by Tukey’s post hoc test to compare mean values. Levels of $p < 0.05$ were considered statistically significant. Results are presented as mean ± standard deviation.

RESULTS

Mean values of body weight, length, width and area of the small intestine between the different groups
are shown in Table I. The results indicate that age led to an increase in body weight, regardless of whether AA supplementation was received. This difference was statistically significant when comparing adult animals (90 days) with animals at a more advanced age (428 days) and supplemented animals (EA345, EA428).

Conversely, there was a significant reduction in intestinal length in 428-day-old rats that did not receive AA supplementation compared to adult animals (90 days); there was no statistically significant variation in intestinal length among the other groups (Table I).

With regard to small intestine width, we observed a significant increase for animals in groups E345, E428 and EA345 when compared to group C90.

When analysing the total small intestine area, it was observed that there was a significant increase in that parameter throughout the ageing process for animals in groups E345, EA345 and E428. Only group EA428 did not show a significant increase in intestine area, which was expected given that the width and length did not show any significant alteration in those animals.

### Quantification of the Neuronal Myenteric Population

The duodenal membrane mounts from all groups showed typical ganglionic arrangement, composed of cellular bodies of different sizes. Nevertheless, it was evident that the ganglia of 90-day-old animals showed closer and more numerous cellular bodies, whereas there was greater spacing and lower amounts in the ganglia of 345 and 428-day-old rats (Figure 1).

The mean value for the number of immunoreactive myosin-V neurons are expressed in Table II and Figure 2. The obtained data demonstrate that during the ageing process of the animals, there was a reduction in the mean neuron population for advanced age groups (E345 and E428) in a 14.83 mm² area, or even when projecting for the total intestine area. In the animals supplemented with AA (EA345 and EA428), the reduction in neuronal density was significant when compared to group C90, not differing from their respective controls. This makes possible to affirm that the effects of age were not attenuated with AA supplementation.

### Morphometry of the Neuronal Cell Body

Table III shows the mean values obtained by measuring 500 cells per group, indicating a significant increase in the cellular profile for animals from groups E345 and E428 when compared to group C90. Among the animals supplemented with AA, group EA345 maintained the same neuronal profile as group C90, and a significant increase in the neuronal profile was observed in EA428 animals in relation to adult animals (C90). The distribution of neurons in 100 µm² class intervals revealed that the predominant cellular population in the myenteric ganglia for animals of the different groups is presented in Figure 3.

### Discussion

The obtained results showed an increase in body weight ($p < 0.05$) when comparing 90-day-old animals with 428-day-old animals, supplemented...
EFFECT OF ASCORBIC ACID ON MYENTERIC NEURONS OF RATS

or not with AA. Similar results were obtained by Natali et al. (2000) and Marese et al. (2007) in male Wistar rats from different age groups; these researchers observed that the body weight of adult animals increases gradually with age. The present study compared animals at 3, 11 and 14 months of age, evidencing greater weight gain in animals between 11 and 14 months of age.

A gradual increase in body weight with ageing was reported in mice from 2 to 24 months of age, with the greatest weight gain recorded between 10 and 20 months (Miller et al. 2002).

Those authors suggest that weight gain with age is controlled genetically, and that the decrease in this parameter is linked to animal longevity.

In the present study age also interfered in small intestine length. Animals at 428 days showed significantly shorter small intestine length than 90-day-old animals. Similar results were observed in same-age animals (Marese et al. 2007). Increased intestinal length in old rats compared to young rats is found in the literature (Johnson et al. 1998, Phillips et al. 2003), which differs from the results shown in the present work. However, the lineages

Fig. 1 - Myenteric ganglia in the duodenum of rats at different ages. (A) 90 days, (B) E345 days, (C) E428 days and supplemented with ascorbic acid (D) EA345, (E) EA428 days. Myosin-V immunohistochemistry. Image captured using a 40x lens. Calibration bar = 10µm.

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Sprague-Dawley and Fischer 344) respectively used by those authors differ from that used in this study. Animal lineage and longevity are of fundamental importance in this type of evaluation, as the mortality rate is significantly lower in Wistar rats when compared to Sprague-Dawley rats of the same age (Johnson et al. 1998). It should also be considered that those authors worked with extreme age periods (3 and 24 months), differing from the procedure adopted in this study, which evaluated the ages of 3, 11 and 14 months.

AA supplementation enabled the maintenance of intestinal length, both in 345 and 428-day-old animals, in relation to 90-day-old animals. Considering that the maintenance of intestinal length for animals supplemented with AA was accompanied by body weight gain for the same group, it is possible to infer that the intestinal length verified in these animals led to increased body weight, probably by providing greater absorption area. Although no data were found in the literature specifically for this parameter, and considering the role of AA as an antioxidant agent that inhibits apoptosis and improves the physiological conditions of ageing (Golden and Hinerfield 2002, Junqueira et al. 2004), a positive influence of this antioxidant on intestinal length is suggested in our study.

### Table II

Number of myosin-V immunoreactive myenteric neurons quantified in 14.8 mm² of duodenum of rats at different ages: C90 days, E 345 days, E428 days, and with ascorbic acid supplementation: EA 345 and EA 428 days (n = 5). Data were expressed as mean ± standard deviation (n = 5 animals/group).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of myenteric neurons</th>
<th>Projection of the number of neurons for the small intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>C90</td>
<td>4,199 ± 283.7a</td>
<td>2,794,495 ± 226,300 a</td>
</tr>
<tr>
<td>E345</td>
<td>2,438 ± 224.4b</td>
<td>2,172,544 ± 267,700 ab</td>
</tr>
<tr>
<td>E428</td>
<td>1,507 ± 247.8c</td>
<td>1,468,238 ± 319,100 ac</td>
</tr>
<tr>
<td>EA345</td>
<td>2,051 ± 603cb</td>
<td>2,161,576 ± 624,400 b</td>
</tr>
<tr>
<td>EA428</td>
<td>1,607 ± 356.2c</td>
<td>1,245,370 ± 246,200 c</td>
</tr>
</tbody>
</table>

Means followed by the same lowercase letters in the same column do not differ from one another according to Tukey’s test (p < 0.05).

### Table III

Cellular profile area (cell body) of the myosin-V immunoreactive myenteric neurons in the duodenum of rats at different ages: C90 days, E 345 days, E428 days, and supplemented with ascorbic acid: EA 345 and EA 428 days (n = 500 cells/group). Data were expressed as mean ± standard deviation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cellular profile area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C90</td>
<td>301.1 ± 94.66 c</td>
</tr>
<tr>
<td>E345</td>
<td>336.5 ± 119.3 b</td>
</tr>
<tr>
<td>E428</td>
<td>353.9 ± 117.5 ab</td>
</tr>
<tr>
<td>EA345</td>
<td>305.7 ± 106.2 c</td>
</tr>
<tr>
<td>EA428</td>
<td>357.0 ± 121.7 a</td>
</tr>
</tbody>
</table>

Means followed by the same lowercase letters in the same column do not differ from one another according to Tukey’s test (p<0.05).
EFFECT OF ASCORBIC ACID ON MYENTERIC NEURONS OF RATS

QUANTIFICATION OF THE MYENTERIC NEURONAL POPULATION

The myosin-V immunohistochemical technique used in our study is highly restricted to the neuronal cytoplasm, cellular bodies and nerve fibers, without marking glial cells, and is indicated by several authors as a specific marker for neurons (Drengk et al. 2000, Buttow et al. 2003, Zanoni et al. 2003, Schoffen et al. 2005, Marese et al. 2007).

Myosin-V protein, present in the cytoplasm of neurons, is linked to cell transport involving exocytosis, endocytosis, neurotransmitter release and axoplasmic transport, capable of creating movement through the actin filaments (Hasson and Mooseker 1997, Langford and Molyneaux 1998).

Neuronal population was evaluated by quantification in the intermediate and antimesenteric regions (Natali et al. 2000). However, given that there were no significant differences between them, their data were grouped together because, in the duodenum, the distributions of muscle fibres in circular and longitudinal strata as well as the neuronal distribution are homogenous throughout the intestinal circumference (Santer 1994, Araújo et al. 2003, Natali et al. 2003).

From the results obtained regarding the quantification, it can be observed that age is a factor that promotes a reduction in the number of myosin-V immunoreactive myenteric neurons. When comparing the numbers of neurons in adult animals (C90) with 345-day-old animals, a reduction of 41.94% was observed; compared to 428-day-old animals, this decrease is 64.11%. Studies reporting the effects of age on the myenteric plexus of the small intestine of rats indicate 40% neuronal loss between the ages of 6 and 24 months (Santer 1994, Araújo et al. 2003, Natali et al. 2003).

Age-related neuronal death may be linked to higher levels of free radicals. Given that the intestine has a large absorption surface area and is probably exposed to high levels of reactive oxygen species (ROS), we can assume that there is a greater possibility of occurrence of oxidative processes in that organ (Dolatshad et al. 2001), which contributes to neuronal loss.

Previous studies using the myosin-V immunohistochemical technique to mark the population of myenteric neurons in the duodenum of rats at different ages have shown that myenteric neuronal loss in Wistar rats begins at around 210 days of age (Marese et al. 2007).

Considering the results obtained in our study, we can agree with both authors, as neuronal loss was indeed ongoing throughout the study. However, as no physiological changes were observed in the animals during the corresponding life period, it is believed that the maintenance of GIT activities remains the same, indicating that the remaining neurons undergo rearrangement to stay in activity. Nevertheless, we highlight the fact that the ganglionic morphology in 345 and 428-day-old animals with greater spacing and lower quantity of cellular bodies were demonstrably distinct from ganglia of group E 90, whose cellular bodies were closer and more numerous.

In the process of neuronal reduction with age, it should be considered that the susceptibility of myenteric neurons to ageing is different, the population of nitrergic neurons is less vulnerable to the ageing process (Johnson et al. 1998, Wade 2002, Phillips et al. 2003, Wade and Cowen 2004, Zanoni et al. 2005).
In addition to the increase in ROS levels, the reduction in neurotrophic derived factors from glial cells due to age can contribute to the vulnerability of enteric neurons with ageing (Dolatshad et al. 2001). Extrinsic innervation also gives trophic support to neurons in the upper GIT during ageing, which indicates that the interaction between these neurons is essential in maintaining a normal neuronal circuit (Santer and Baker 1988, Phillips and Powley 2001).

In the animals that received supplementation with ascorbic acid, the loss in relation to 90-day-old animals was 51.49%, for 345-day-old animals and 62.16% for 428-day-old animals. Our results demonstrated that neuronal loss as a result of age remained, even with ascorbic acid supplementation at a concentration of 1 g/L of water administered daily ad libitum for 255 and 338 days. A neuroprotective effect was found for neuronal density, with an increase in the number of nitrergic neurons in the ileum of rats supplemented with AA at the same concentration used by us (1 g/L) (Zanoni et al. 2005, Pereira et al. 2006). These results cannot be regarded as conflicting, given that the myosin-V population in the same segment did not show that effect. As previously mentioned, neuronal vulnerability is not the same for all neurons; as such, the effect of age varies according to the neuronal chemical phenotype (Phillips and Powley 2007).

The action of converted AA in ionized ascorbate form as a neuroprotective agent is widely discussed in the literature. Studies in a culture of cerebellum granule neurons from 8-day-old Wistar rats revealed that in those cells, AA was able to block the apoptotic process resulting from induced formation of ROS (De Ruvo et al. 2000). Those authors attribute AA with the ability to block the activity of caspase-3, an enzyme responsible for the apoptotic mechanism, reducing neuronal loss.

Other studies have exposed the neuroprotective activity of AA in neurons of the cerebral cortex; however, they consider the hypothesis that ascorbate is oxidised in dehydroascorbic acid (DHA), both in growth medium and in blood plasma, and can enter the cell via the glutamate receptor, competing with glucose. In cytosol, it is again reduced to ascorbate, but that process creates oxidative stress which activates caspase-3, unleashing cell apoptosis (Song et al. 2001.)

Contrariwise, another author who verified a neuroprotective effect for cerebellum granule cells affirms that DHA reduction reactions do not produce ROS, as DHA rapidly reverts to ascorbate, which can be used by the cell or released into the extracellular fluid, features antioxidant properties, and does not generate free radicals (Wilson 2002).

**Morphometry of the Neuronal Cell Body (Neuronal Profile)**

Our results reveal there was an increase in neuronal cellular profile with age and that the ganglionic arrangement was maintained, although with visible spaces between the cellular bodies of different sizes in the ganglia of 348 and 428-day-old animals. The maintenance of the ganglionic arrangement and the variety in areas of neuronal cell bodies were also observed in the myenteric ganglia of rats subjected to protein malnutrition using the myosin-V immunohistochemical technique (Schoffen et al. 2005), which evidences the rearrangement ability of these neurons. Several authors describe the great adaptive rearrangement of the plexuses, evidencing enteric neural plasticity (Santer and Baker 1988, Saffrey and Burnstock 1994, Hanani et al. 2003).

In 90-day-old animals, there was a predominance of neurons between 201-300 µm²; in older animals (428 days), the predominant population was of neurons between 301-400 µm². Similar data were observed by authors who detected a significant increase in the population of neurons with larger cellular bodies in the duodenum of aged rats (Marese et al. 2007). This behaviour is not restricted to the small intestine: there are studies...
showing similar results analysing the neuronal profile of the large intestine of rats at 3 and 24 months of age (Phillips et al. 2003). The change in neuronal profile is justified by those authors as an adaptive condition to progressive neuronal loss with age, as greater functional demand is required from these neurons, which could result in a state of cellular hypertrophy. This reflects the plasticity of enteric neurons; thus, the degenerative processes resulting from age can be offset by the plasticity of the ENS, and changes in gastrointestinal motility take place only when the functional reserves of the neurons are spent (Orr and Chen 2002).

In 428-day-old animals, supplemented or not with AA, a significant increase in the neuronal cellular profile was observed when compared to 90-day-old animals. However, when comparing groups E428 and EA428, it was observed that AA supplementation did not interfere with neuronal cellular profile growth, which remained progressive with age. An absence of any effect of AA was also found by Zanoni et al. (2005), with maintenance of the area of immunoreactive myosin-V cell bodies among animals that received supplementation with AA from 90 to 210 days of age.

A different behavior was revealed for 345-day-old rats, for which AA supplementation (EA 345) led to maintenance of the cellular profile at similar levels to the group of 90-day-old animals. If we consider that the conservation of neuronal functions is offset by an increase in the cellular profile, when age related loss takes place (as observed in non-supplemented animals), the return of the mean profile to similar levels as 90 days of age does not characterise a positive effect on that population.

Based on the proposed experimental conditions, it is concluded that age is a determinant factor for body weight gain, intestinal length reduction and lower density, while concomitantly increasing the neuronal profile of immunoreactive myosin-V myenteric neurons. These alterations were not attenuated by supplementation with ascorbic acid at the concentration of 1 g/L.

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RESUMO

Durante o processo de envelhecimento o Sistema Nervoso Entérico sofre alterações morfofuncionais, como a neurodegeneração entérica. A morte neuronal pode ser atribuída ao aumento de radicas livres, e o ácido ascórbico (AA), conhecido antioxidante, poderia minimizar danos causados pelo estresse oxidativo.

O objetivo deste estudo é analisar o comportamento morfoquantitativo dos neurônios mioentéricos no duodeno de ratos Wistar adultos com 90(C90), 345(E345) e 428(E428) dias e animais de mesma idade que receberam suplementação de ácido ascórbico por 120 dias (EA345 e EA428). Preparados totais da túnica muscular do duodeno dos animais foram imunomarcados pelo método Miosina V. Foram quantificados 80 campos microscópicos (14,8 mm²/animal) e mensurados 100 corpos celulares neuronais por animal. Durante o processo de envelhecimento houve uma redução na densidade neuronal nos animais de todos os grupos, indicando que os efeitos da idade não foram amenizados com a suplementação de AA. O Aumento na área dos corpos celulares neuronais nos animais com 428 dias comprovou a influência da idade sobre este parâmetro. Não foi observado um efeito neuroprotetor do AA (1 mL/g peso corporal) sobre a população neuronal mioentérica Miosina V imunoreativa.

Palavras-chave: envelhecimento, antioxidante, sistema nervoso entérico, imunohistoquímica.

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