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Evaluation of the effect of *Ginkgo biloba* extract (EGb 761) on the myenteric plexus of the small intestine of Wistar rats

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Background. The aging process causes a reduction in the myenteric neuronal population, related to oxidative stress, resulting in malfunctioning of the digestive tract. The purpose of this study was to evaluate the action of *Ginkgo biloba* extract (EGb 761), an important antioxidant drug, on the myenteric plexus of the jejunum and ileum of rats after treatment for 120 days. ***Methods.*** Fragments of the jejunum and ileum were collected from three groups of rats: a 90-day-old group (group Y), a 210-day-old group (group A), and a 210-day-old group treated daily with the extract EGb 761 (50 mg/kg body weight) (group TA). The analysis was carried out by using the myosin-V immunohistochemical technique. Neuronal densities were estimated, and a study of the neuronal profile area of 500 neurons from each group was carried out. ***Results.*** In the jejunum, there was a significant neuronal population reduction of 17% only in group A compared with group Y. In the ileum, there was a significant neuronal reduction of 36% in group A compared with group Y, and a significant reduction in group TA of 20%. The difference in the reduction between groups A and TA in the ileum was also significant. In the jejunum, only group A showed a significant increase in neuronal profile area, but in the ileum, there was a significant increase in both groups A and TA. ***Conclusions.*** A daily dose of 50 mg/kg body weight of *Ginkgo biloba* extract has a significant neuroprotector effect on the myenteric plexus of the ileum during the aging process in rats.

Key words: neuroprotection, myenteric plexus, *Ginkgo biloba*, aging

Introduction

Aging is a life process during which a series of alterations occur that represent the regression of practically the whole organic system, and it involves cell death. The accumulation of these cell losses is characteristic of aging tissues; although such losses can occur throughout the life of an individual, they are accentuated in individuals of advanced age.

Neuronal losses can occur from various types of injury, such as from the use of drugs or the toxic action of reactive oxygen species (ROS). With increased public concern about the maintenance of health and vitality during aging, the study of the effects of ROS on aging and in the development of diseases has become an area of great interest. ROS are formed as a consequence of the incomplete conversion of oxygen to water during respiration in aerobic organisms.[1] An example is the hydroxyl radical, which when present in excess in the brain causes apoptosis of nerve cells.[2] Because of the instability of ROS, they can react with any cellular component, altering its structure. This reaction can occur with lipids in the membranes of the cells, or with the nucleotides of DNA molecules, changing their genetic sequences.[3]

ROS help the organism during inflammatory processes, destroying the inflamed cells and also parasites,[4] but an excess of these radicals provokes what is known as oxidative stress, which can alter the physiology of a tissue, attacking the cells and causing their death during the aging process.

The action of antioxidants, which have polyphenolic constituents (flavonoids), can combat oxidative stress. These are present in vegetables and fruits and in drinks such as wine.[5] Vitamins A, E, and C, carotenoids,[5] and vegetable extracts such as from *Ginkgo biloba* show excellent antioxidant action against free radicals,[6–8] and have become promising candidates for antiaging "drugs."

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Ginkgo biloba yields an ancient vegetable extract that originated in China and is an excellent antioxidant because of its flavonoid components,[9] which have the important antiapoptotic action of reducing the production of ROS.[6–8] Various studies have also demonstrated the important role of *G. biloba* extract in the prevention and treatment of some types of cancer.[10] It protects neurons by blocking the action of ROS, preventing neurological problems in advanced age and in diseases such as Alzheimer's.[11] This extract also has a role in increasing neuronal plasticity.[12,13] Furthermore, it possesses vasodilator properties,[14] increasing the flow of blood to the brain, oxygenating the neurons.[15]

The enteric nervous system (ENS), owing to its location and its easy access, has been the object of various studies on neuronal plasticity in neuronal degenerative diseases[4,16] and aging.[17–21] The ganglia of the ENS have an important role in the control of intestinal motility produced by peristaltic movements, which function to propel the contents of the small intestine, and they coordinate the contractions and relaxation of the circular and longitudinal muscles, as well as control the action of the intestinal glandules.[22]

During aging, the number of these nerve cells decreases by up to 60%, depending on age and the segment studied.[17] These losses prejudice the motility and normal functioning of the digestive tube.

The search for mechanisms that can prevent the reduction of neuronal density during aging, through medicines that reduce collateral effects and improve the quality of life of the individual, is of great scientific importance. The objective of this study was to evaluate the possible neuroprotector effect of the purified extract of the *G. biloba* plant (EGb 761) on the myenteric plexus of the jejunum and ileum of rats over a period of 120 days.

Materials and methods

Antibodies

Anti-tail polyclonal antibody, generated against a chicken myosin-V recombinant protein, has been previously characterized.[23] A cDNA fragment (corresponding to amino acids 899–1830) of a myosin-V tail clone from a chicken brain was subcloned into a pGEX vector to generate a glutathione transferase (GST)/myosin medial tail (corresponding to amino acids 1117–1435) fusion protein in XL1-Blue bacteria. This GST/myosin-V medial tail was purified over glutathione resin. The fusion protein was used as an antigen for antibody production in rabbits. The immune serum was purified using a PLM/myosin-V tail protein affinity column to enrich IgG antibodies directed against the myosin-V medial tail. Goat anti-rabbit IgG secondary antibody conjugated to peroxidase was used (Pierce, Rockford, IL, USA).

Animal procedures

The animals used in this study were treated in accordance with the guidelines of the Committee on Care and use of Laboratory Animals of the National Research Council.

Ninety-day-old male Wistar rats (*Rattus norvegicus*) weighing about 300 g were used. The rats were kept in individual metabolic cages, in a room with a maintained photoperiod (6:00 a.m.–6:00 p.m.) at room temperature (RT) (24 ± 2°C), with water and food (Nuvital lab chow; Nuvilab, Colombo, Paraná, Brazil) ad libitum. Three groups of the five animals each were used: a young group, 90 days old (group Y); an adult group, 210 days old (group A); and an adult group orally given the purified extract of *G. biloba* (EGb 761) (Tebonin, Altana Pharma, Jaguariúna, São Paulo, Brazil) daily for 120 days at a dose of 50 mg/kg body weight (group TA). Group Y rats were killed at the age of 90 days, and after a further 120 days, the animals of groups A and TA were also killed.

Immunohistochemistry of the myenteric plexus

To collect the samples, the rats were anesthetized intraperitoneally with thionenbutal (Abbott Laboratories, North Chicago, IL, USA) (40 mg/kg body weight). The animals were perfused with 1 ml/g body weight of saline solution followed by perfusion with 1 ml/g body weight of fixing solution (10 mM sodium periodate, 75 mM lysine, and 1% paraformaldehyde in 37 mM phosphate buffer, pH 7.4).[24] Immediately after perfusion, a piece of the jejunum and of the ileum were removed from the rats, flushed with fixing solution, immersed in the same fixative, and, after 15 min, opened and left in the solution for 1 h. Samples were then dehydrated through a graded alcohol series (50%, 70%, 80%, 90%, 95%, and 100%), being kept in each solution for 10 min, cleared in xylol (10 min), and rehydrated back through the ethanol steps to 70% ethanol, in which the tissue was stored. At this step, the jejunum and ileum fragments were dissected, removing the muscle wall containing the myenteric plexus of the submucosal layer. Dissection was followed by resumption of hydration through 60% and 50% ethanol to phosphate-buffered saline (PBS 0.1 M, pH 7.4). The tissues were washed twice in PBS and blocked for 3 h with PBS containing 2% bovine serum albumin (BSA), 2% goat serum, and 0.5% Triton-X-100 at room temperature. Immunostaining was carried out by incubating the tissue fragments in 0.89 µg/ml of affinity-purified antibody specific to the myosin-V

medial tail domain diluted in PBS containing 1% BSA, 2% goat serum, and 0.1% Triton-X-100, with shaking for 12 h at 4°C and for another 18 h at room temperature. Following incubation, the fragments were washed in PBS containing 0.1% Triton-X-100 and then in PBS with 0.05% Tween-20. They were then incubated with 10 µg/ml secondary antibodies conjugated with peroxidase in PBS for 24 h at room temperature under agitation. Next, they were washed four times for 15 min in PBS containing 0.05% Tween-20. Immunoreaction with peroxidase-conjugated antibodies was developed by incubation with 0.75 mg/ml diaminobenzidine in PBS and 0.03% H_2O_2 for 10 min at room temperature under agitation. Samples were mounted in a gel mounting medium containing 50% glycerol and 0.07 g/ml gelatin in PBS and 2 µl/ml phenol. In control samples, the primary antibody was substituted by goat serum.

Quantitative analysis of the myenteric plexus

The quantitative analysis was performed in the intermediate region (60°–120°; 240°–300°), considering the mesenteric insertion as 0°.[25] Myenteric neurons were counted with a BX 40 Olympus microscope under a 40× lens. Forty microscope fields were randomly counted for each preparation. The area of each microscope field was 0.229 mm^2. The results are shown in cm^2.

Neuronal cell profile analysis

Images were taken with a high-resolution camera and transferred to a microcomputer. The cell profiles (μm^2) of 100 cell bodies for each animal were measured with image analysis software (Image-Pro-Plus 4.5; Media Cybernetics, Silver Spring, MD, USA), for a total of 500 neurons for each studied group. Neurons were classified into 10 μm^2 classes, and the percentage of each group was calculated for each interval.

Statistical analysis

Data obtained from the groups Y, A, and TA were compared by analysis of variance (ANOVA). If the ANOVA results indicated a difference among values, the data were further subjected to a Tukey multiple comparison test. In all cases, P values less than 0.05 were considered to be significant.

Results

Myenteric neuron density

Table 1 shows the neuronal density of the jejunum and ileum for the three groups studied. In the jejunum, a greater neuronal density was observed in group Y than in groups A or TA. Group A showed a significant neuronal reduction of 17% compared with group Y ($P < 0.05$), whereas group TA showed an increase of 10.6% compared with group A ($P < 0.05$).

In the ileum, group A showed a significant reduction in neuronal density of 36% compared with group Y ($P < 0.001$) (Table 1, Fig. 1). Group TA showed a significant recovery of 20.6% compared with group A ($P < 0.05$).

Neuron size

In the analysis of the neuronal profile area of 500 myenteric neurons from each group, it was observed that in the jejunum, group A showed a significantly larger mean area than group Y ($P < 0.01$) (Table 1). However, the increase in the area of group TA compared with group Y was not significant ($P > 0.05$). The area of the neurons in group Y varied from 117.5 to 801.0 μm^2, in group A from 81.0 to 812.5 μm^2, and in group TA from 23.7 to 754.2 μm^2. Neurons 200 μm^2 in area were most frequent in group Y, 240 μm^2 in area in group A, and 200 μm^2 in group TA (Fig. 2a). More neurons with an area of less than 150 μm^2 were observed in group Y, leading to a reduction in the mean area of neurons. In group TA, neurons with an area between 150 and 250 μm^2 were more frequent than in groups Y and A.

In the ileum, group A had a significantly larger (41.3%) mean neuronal profile area than group Y ($P < 0.001$). Treatment with *G. biloba* extract (group TA)

Table 1. Density and myenteric neuronal cell profile area in the jejunum and ileum of rats

	Jejunum		Ileum	
Group	Neuronal density (cm^2)	Profile area (μm^2)	Neuronal density (cm^2)	Profile area (μm^2)
Y	14 923 ± 1308	254.7 ± 117.4	21 507 ± 1142	204.8 ± 110.5
A	12 434 ± 812*	278.6 ± 119.8*	13 777 ± 2637*	349.0 ± 126.5*
TA	13 908 ± 1143	264.4 ± 104.9	17 358 ± 1728*·**	312.1 ± 133.1*·**

Values are means ± SD ($n = 5$/group)
* Significant difference compared with group Y ($P < 0.05$)
** Significant difference compared with group A ($P < 0.05$)

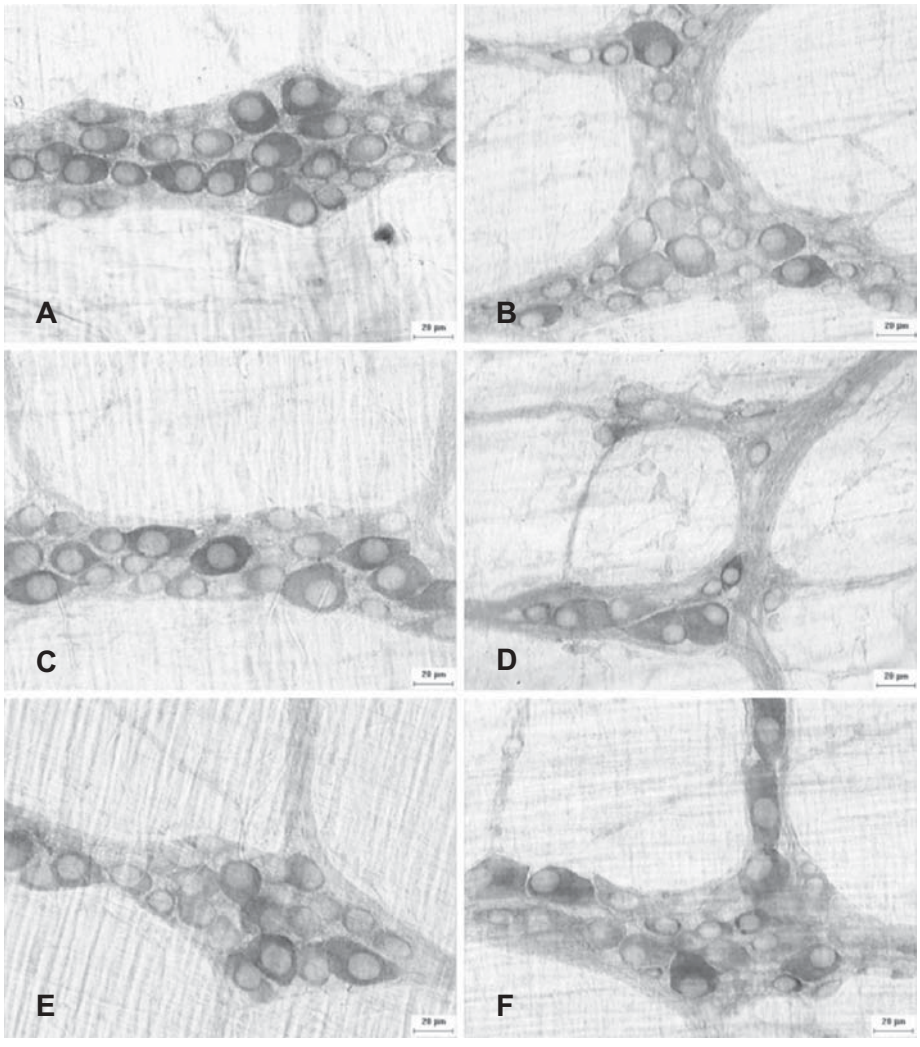

Fig. 1A–F. Immunoreactive myosin-V neurons in the jejunum (**A, C, E**) and ileum (**B, D, F**). There is a significant reduction in neuronal density and an increase in the neuronal profile area in group A, in the jejunum (**C**) and ileum (**D**), and in group TA, in the ileum (**F**). The *bars* correspond to 20 µm

significantly reduced this increase to 10.6% ($P < 0.001$). The neuronal profile area in the ileum in group Y varied from 18.06 to 607.3 µm^2, in group A from 105.06 to 833.4 µm^2, and in group TA from 21.43 to 804.1 µm^2. The frequency distribution of neuronal size showed that neuron size was increased in groups A and TA relative to group Y (Fig. 2b), as reflected by the shift of the curve to the right on the graph. In group Y, neurons of 150 µm^2 were most frequent, in group A, neurons of 300 µm^2, and in group TA, neurons of 200 µm^2.

In the two segments of the intestine analyzed, similar results were observed in the analysis of the neuronal profiles: group A profiles were larger than group Y profiles, with a concomitant reduction in neuronal density. However, group TA showed a reduction in the neuronal profile concomitant with the maintenance of neuronal density in comparison to group A.

Discussion

Myenteric neuron density

One of the consequences of aging is the loss of various types of cells, including neurons. The discovery of medicines that can ease neuronal loss, with the objective of reducing the damage caused by aging, is valuable for improving the quality of life of elderly people. In this study, an experiment was carried out over a period of 120 days, starting with young rats (90 days old) and continuing until they reached adulthood (210 days old). In this period, it is already possible to observe a significant loss of neurons in the small intestine of these animals.[25,26]

The results of this study showed a reduction in the number of neurons in the myenteric plexus of the small

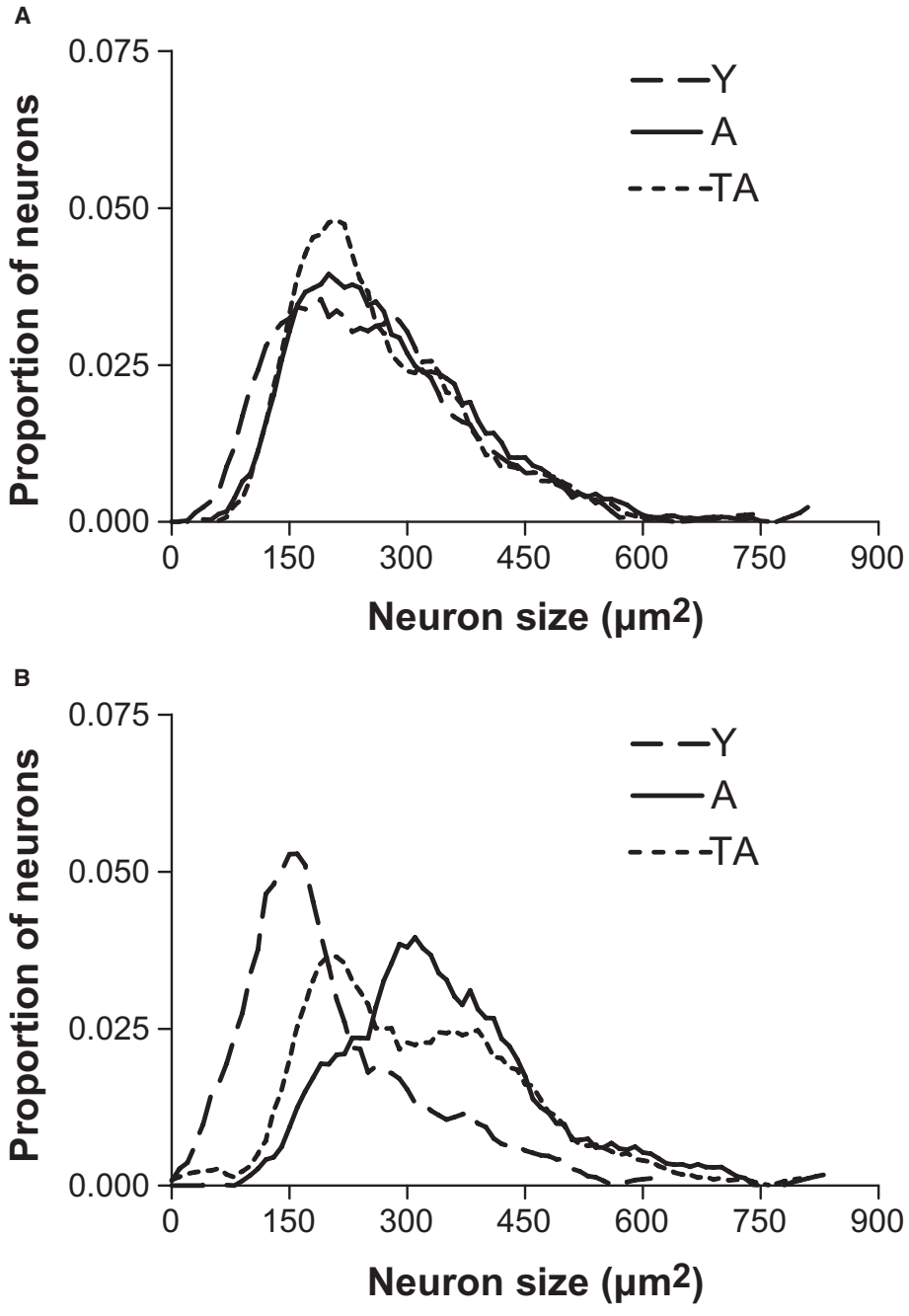

Fig. 2A,B. Neuronal behavior related to age and treatment: size of neurons in the jejunum (**A**) and the ileum (**B**) in groups Y (young group, age 90 days), A (adult group, age 210 days), and TA (adult group, age 210 days and treated daily with *Gingko biloba*)

intestine over the 120-day period. When group A (210-day-old untreated adults) was compared with group Y (90-day-old young rats), in the jejunum a 17% reduction in the neuronal density of the myenteric plexus was found, and in the ileum there was a loss of 36%, a much greater loss than in the jejunum.

Studies on aging have demonstrated reductions in the neuronal population during different time periods.[17,18,20,27–30] Marese[26] observed a reduction of around 22% in the neuronal population of the duodenum in 210-day-old Wistar rats compared with 90-day-old rats. In this study, a variation was observed in the neuronal population and in the response to aging in the two segments of the small intestine studied. Phillips et al.[20] also observed a greater reduction in the ileum than in the jejunum. The disposition of the plexus, the size and form of the ganglia and nerve cells, and the quantity of neurons varies according to the species and the

segment studied,[31,32] and the pattern of cellular reduction during aging varies according to the organ studied[19] and the region of the organ studied.[20]

When group TA (210 days, treated with EGb 761) was compared with group Y, it was noted that in the jejunum, there was a nonsignificant loss of 7% in neuronal density, whereas in the ileum, there was a significant loss of 20%. In spite of the fact that neuronal loss related to age had been minimized in group TA in both segments, the reduction, compared with group A was significant only in the ileum. Therefore, the results of this experiment show that treatment with the extract of *G. biloba* was more effective in the ileum than in the jejunum.

During the aging process, oxidative stress, which is considered to be one of the causes of neuronal loss in the digestive tract, occurs.[33] The use of *G. biloba* extract (EGb 761), which has antioxidant[2,9,13,34] and neuroprotector[35] effects, can reduce these neuronal losses. The discovery of the beneficial effects of *G. biloba* extract is a great advance in the use of natural products for the prevention and treatment of diseases, as well as for the general maintenance of health.

The standard extract of *G. biloba* (EGb 761) contains two main active pharmacological groups: glycosidic flavonoids (24%) and terpenoids (6%, subdivided into bilobalides and ginkgolides),[36,37] which have already been studied for their respective actions.

Studies carried out on the myenteric plexus of human colons[27] suggest that problems of intestinal motility in elderly people occur through loss of both intrinsic and extrinsic innervation.

Neuron size

After measuring the area and size frequency distribution of 500 neurons from each group (Fig. 2a), it was confirmed that in the jejunum, the mean area in group A was significantly greater than that in group Y, but that the increase in the mean area in group TA compared with group Y was not statistically significant. These results suggest that an increased neuronal loss promotes an increase in the cellular profile area in the jejunum.

In the ileum as well, group A had a significantly greater mean neuronal area than group Y. Group TA also had a significantly greater mean neuronal area than group Y, but it was also significantly smaller than that of group A. The increase in the neuronal profile area is illustrated by the shift to the right of the curves for groups A and TA (Fig. 2b), with the shift of the curve of group TA being smaller than that of group A. In group A, the mean neuronal profile area was 349 μm^2, similar to that in the ileum of rats of the same age, determined by using the myosin-V immunomarcation technique by Zanoni et al.[25] (340.2 μm^2).

Meciano Filho et al.,[38] who studied aging in the human esophagus, observed that as well as a reduction in the number of neurons, there was also an increase in the neuronal profile area in elderly people. The correlation between the increase in the cytoplasmic area of the neurons and a lower neuronal density was studied by Barbosa,[39] who compared the number and area of neurons in the colon and cecum of rats. He observed a lower number of neurons in the cecum, but with a greater neuronal profile area, and a greater number of neurons with a smaller neuronal profile in the colon. Dahl et al.,[40] who studied the myenteric plexus in the jejunum of rats denervated chemically, observed a compensatory increase in the production of certain neurotransmitters by the surviving neuronal elements, and they believed that the increase in the neuronal profile area might be a compensatory effect for the reduction in the number of nerve cells. Similar observations were made by others.[41,42] However, Santer and Baker[17] and Gomes et al.[43] did not find an increase in neuronal area during aging. Phillips et al.,[20] who studied Fisher 344 rats, observed an increase in the cellular profile area only of myenteric neurons from the large intestine.

The increase in the neuronal profile area observed in group A may be due to the compensatory effect of the reduction in the number of neurons. However, when *G. biloba* extract was administered (group TA), as well as a reduction in neuronal loss, a reduction in the increase of the cellular profile area also occurred. These findings indicate that treatment with 50 mg/kg of body weight of *G. biloba* extract (EGb 761) produces a significant neuroprotector effect on the neurons of the myenteric plexus in the ileum of Wistar rats treated for a period of 120 days. However, a study with higher concentrations of *G. biloba* extract may show a stronger effect of this extract on both of segments of the small intestine tested in this research.

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