Materials and Methods

Sample calculation

Sample Size: The calculation of the sample size (n) was performed according to Vieira and Hossne [19] and Pereira [20], and based on the study by Freir and Costello [21], who showed a prevalence level of 99% in mice with Alzheimer's disease after the application of the Beta-Amiloid 25-35 peptide (Sigma-Aldrich), significance level (1.96), and the accuracy required for estimation (0.1), according to the CEUA-UNICENTRO committee's approval number 018/2014.

Sample

The samples consisted of 50 *Rattus norvegicus* animals, Wistar strain, weighing between 200 and 250 grams. The animals were divided into four groups:

Control 21 group (C21): Composed of 10 animals with induction of senile plaques in the CA1 region and not treated; euthanized on day 22;

Control 42 group (C42): Composed of 10 animals with induction of senile plaques in the CA1 region and not treated; euthanized on day 43;

Treated 21 group (T21): Composed of 10 animals with induction of senile plaques in the CA1 region and treated with *Ginkgo biloba* (EGb 761) at the concentration of 100 mg/kg, diluted in 1 ml of drinking water, and administered by gavage only on day 21; euthanized on day 22;

Treated 42 group (T42): Composed of 10 animals with induction of senile plaques in the CA1 region and treated with *Ginkgo biloba* (EGb 761) at the concentration of 100 mg/kg, diluted in 1 ml of drinking water, and administered by gavage from day 21 to 42; euthanized on day 43;

Normal group (GN42): Composed of 10 animals without induction of senile plaques in the CA1 region and not treated; euthanized on day 43.

Experimental surgery

The animals underwent stereotaxic surgery when cannulas were implanted into the hippocampal region and directed to the CA1 unilateral area following the coordinates of AP=-3.12 mm, ML=± 3.6 mm, and DV=3.0 mm and considering the bregma as a reference with lambdoid and bregmatic sutures in the same horizontal plane. Tramadol hydrochloride was used for analgesia at the dose of 10 mg/kg, every 12 hours, and intramuscularly for 7 (seven) days [22].

Surgical procedure for Aβ 25-35 (Aβ-25-35) inoculation

The protein Aβ-25-35 1 MG was acquired from the Sigma-Aldrich company, diluted to 0.1 µl of DMSO, and 0.9 µl of distilled water was added. It was then stored for 72 hours in a refrigerator at 4 degrees Celsius for incubation.

The animals were anesthetized intra-abdominally with a solution in the proportion of 80 mg/kg of Hydrochloride Ketamine (Ketamine, 10 ml bottle) to 15 mg/kg of Hydrochloride Xylazine (Dopaser, 10 ml bottle) and taken to a stereotaxic apparatus (David Kopf, EUA), where their heads were fixed by the temporal bone and the upper incisors. Cannulas were made with 30 × 09 needles (5 mm long), implanted in the hippocampal region and directed to the CA1 area of the hippocampus, according to the stereotaxic coordinates in the atlas by Paxinos and Watson [23]. The stereotaxic coordinates used were the following: AP=-3.12 mm; ML=±1.8 mm and DV=2.8 mm, using bregma as a reference and the lamboid and bregmatic sutures on the same horizontal plane. After the implants were put in place, the cannulas were fixed in the calvaria using a self-curing acrylic prosthesis. Stainless steel wire was inserted in order to prevent occlusion of the cannula. A screw was placed in the anterior section of the skullcap to fix the wire. The animals were allowed to rest for five days and were then anesthetized once again and taken for stereotactic processing. Using a Hamilton syringe, they received 2 µl of the peptide Aβ 25-35 (Sigma-Aldrich) in the CA1 region of the hippocampus, as described by Freir and Costello [21]. Injected with 0.1 µl per minute (with a total time of 20 minutes), thereby avoid backflow and allows the toxin being absorbed into the brain parenchyma (Figure 1).

Study characterization

The animals were undisturbed for five days after surgery and subsequently received the Beta-Amiloid 25-35 peptide (Sigma-Aldrich) through a Hamilton syringe in the hippocampal CA1 region as described by Freir and Costello [21]. Animals rested for 21 days after the intracerebral injection. One animal from each group was sacrificed, and their brains were analyzed to confirm the presence of senile plaques (Figure 2).

Cytokines determination

One milliliter of blood was collected from each animal. Blood was maintained in a water bath for 15 minutes, and centrifuged at 300*g* for 5 minutes at 18°C. After centrifugation, the supernatant (serum) was stained for the detection of IL-2, IL-4, IL-6, TNF-α, and interferon-gamma (IFN-γ) using the BD™ Cytometric Bead Array Mouse Th1/Th2 Cytokine kit (Becton Dickinson, USA) according to the manufacturer's instructions; these samples were Analyzed in a BD™ Accuri C6 Flow Cytometer (Becton Dickinson, USA).

The theoretical detection limit for each cytokine using the BD™ Cytometric Bead Array Mouse Th1/Th2 Cytokine Kit is defined as the concentration that corresponds to two standard deviations above the fluorescence average of

30 negative control replicates (0 pg/ml). These limits were: IL-2=0.1 pg/ml; IL-4=0.03 pg/ml, IL-6=1.4 pg/ml, IFN-γ=0.5 pg/ml, IL-10=16.8 pg/ml, and TNF-α=0.9 pg/ml. The readings were manually performed through the acquisition of 10,000 events in each sample. Flow cytometry data were analyzed in the FCap 3.0 Array software (Becton Dickinson, USA) and results were plotted in graphs showing averages and standard deviations.

Water maze

The maze is located in a 3 × 3 m room and consists of a circular fiberglass pool, approximately 1.80 m in diameter and 43 cm height containing water at 25°C at the level of 30 cm deep. This pool is divided into four imaginary quadrants named NW (northwest), SW (southwest), NE (northeast), and SE (southeast). A circular acrylic platform (15 cm in diameter) is placed at 2 cm below the water level. The pool has no track or internal marks, but there are stable visual tracks outside the labyrinth, labeled in blue, yellow, and red that serves as a reference for the animal to locate the platform. The rat's ability to locate the platform depends on the development of a cognitive spatial map of the environment in the animal's brain. A television camera with angular lens was fixed on the ceiling, directly above the pool, to record the tests. All tests were performed at the same time of day with the same room temperature associated with the water temperature [24].

Behavioral and locomotor measurement

The Open Field Test provides simultaneous measurements of

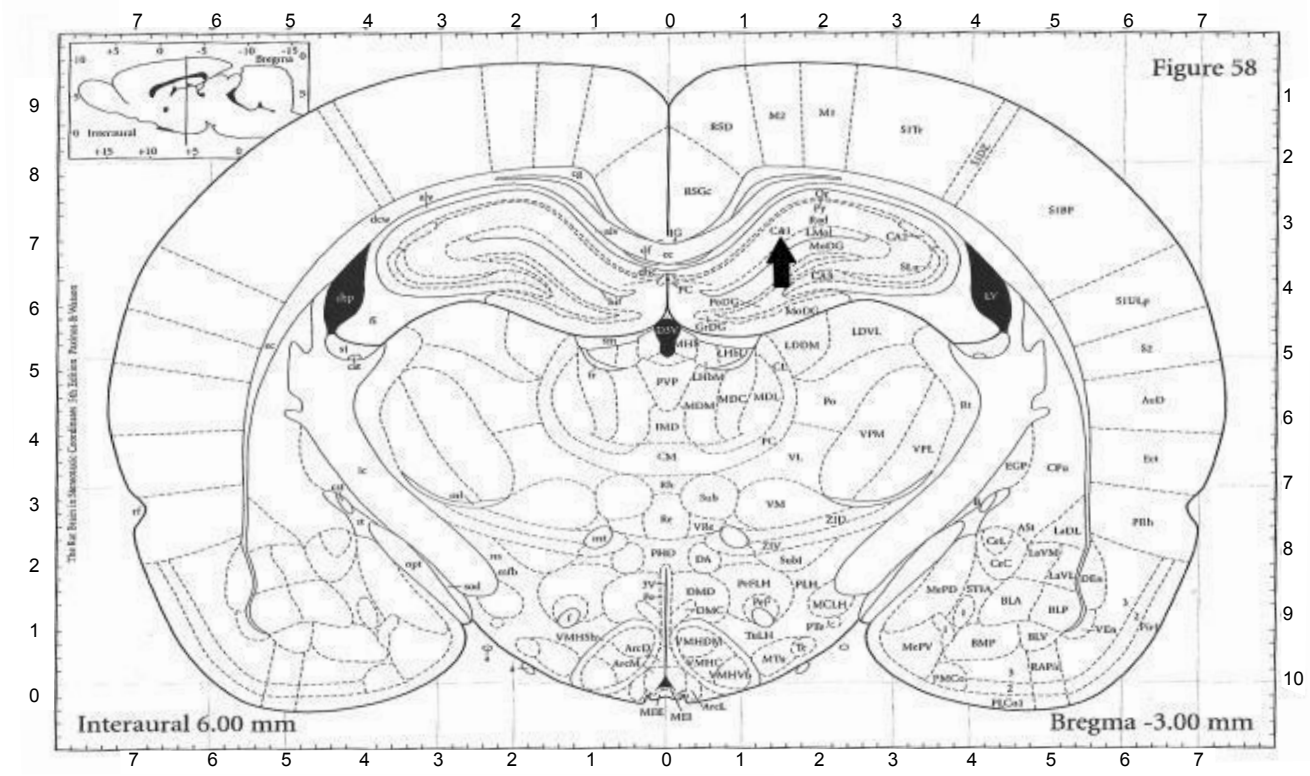

Figure 1: Brain parenchyma.

locomotion, exploration, and anxiety [25]. To conduct the test, the animals were individually placed in the center of an arena and exposed to the Open Field for a period of 5 minutes, during which time their behavior was filmed. The etiological analysis of their behavior assessed the following: the frequency and duration of walking, standing, and grooming; and the number of fecal boli. The number of rectangles covered by four paws corresponded to the locomotor measurement. Standing was classified as animals solely on hind paws. Grooming was classified as movements of front paws along the head or body. Fecal boli were counted after the animal was removed from the arena. The arena's floor was cleaned with alcohol and extensively dried through air circulation after each rat.

Euthanasia

The animals were anesthetized with 80 mg/kg of ketamine and 15 mg/kg Xylazine; they received 1 ml of a lethal dose of thiopental (100 mg/kg) intraperitoneally after the verification of their anesthetized state.

Histology

Brains were collected from euthanized animals; they were cut near the cannulas' path and placed in 15% formalin. The cuts were embedded in paraffin, sliced 2 microns thick, and stained with haematoxylin and eosin (H.E.) for verification of the presence of senile plaques. Although animals that did not have these characteristics would not be further evaluated, all animals evaluated at this stage showed the presence of senile plaques.

Statistical analysis

The Graph Pad 6.0 and Origin 8.0 software were used for graphic construction and data analysis, respectively. The Shapiro-Wilk test was used to verify sample normality, and the Kruskal-Wallis test, with the Dunn's post-test at $p<0.05$, was used in the analysis of samples showing normality.

Results

Numerous alterations were observed between the control and treated groups in the histological analysis. The control group showed intense gliosis, reactive neurons associated with intense picnosis, malacia diffuse areas, multiple fibrotic astrocytes, hyperplastic and hypertrophic ependymal cells with perivascular astrocytes (Figure 2a). Intense vacuolation of gray substance with multifocal hypertrophic motor axons associated with intense spongiosis and malacia, and presence of hypertrophic astrocytes with diffuse spongiosis and diffuse senile plaques were observed in the treated group (Figure 2b).

Figure 3 shows the behavior of animals in relation to locomotion in the Open Field and Morris Water Maze tests. Rats treated with EGb 761 showed a greater range of locomotion than the control groups. The T21 and T42 averages after treatment with *Ginkgo biloba* were 1,125 ± 857.1 cm and 1,115 ± 459.6 cm, respectively, compared to C21 and C42 with averages and standard deviations of 899.5 ± 363.2 cm and 907.2 ± 96.12 cm, respectively. The analysis showed statistically significant differences between these results with values of $p=0.0059$ and $p=0.0078$.

Figure 4 shows the results for pro-inflammatory interleukins. The levels of TNF-α were high in the C21 and C42 groups, with the values of 2,663 pg/ml and 2,097 pg/ml, respectively; 1,009 pg/mL and 935.9 pg/ml in T21 and T42 groups, respectively; and 53,879 pg/ml in GN42 group. The IL-2 levels were 2,533 pg/ml and 2,226 pg/ml in the C21 and C42 groups, respectively; 1,168 pg/ml and 869 pg/ml in the T21 and T42 groups, respectively; and 16,89 pg/ml in N42 group. The difference in

IL-2 levels between the C21 and T42 groups was statistically significant. The average values for IL-6 showed significance between the T42 and C21 groups. The same applies to the INF-y values showed statistical difference between the C21 and T42 groups. GN42 group with value of 10,52 pg/ml for IFN-y.

Figure 5 shows the concentration levels of Interleukin 4, an anti-inflammatory mediator. The averages were 3,488 pg/ml and 3,085 pg/ml in the C21 and C42 groups, respectively, 2,168 pg/ml and 2,357 pg/ml in the T21 and T42 groups, respectively, and 16,10 pg/ml in GN42. The average values for IL-4 showed significance between the T42 and C21 groups. The same applies to the values of IL-10 which showed statistical difference between the C21 and T42 groups.

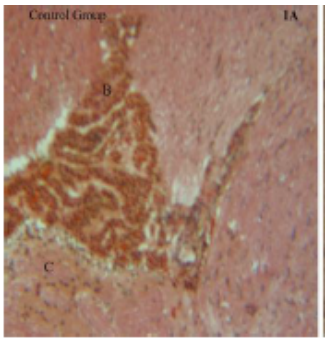

Figure 2a: Photomicrograph of the region where the β-amyloid toxin was injected.

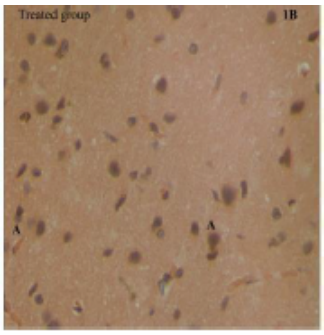

Figure 2b: Presence of senile plaques and some neurofibrillary aspects.

Discussion

The results show some neuronal alterations in the histological samples. The presence of reactive neurons (hypertrophic) is observed in both groups; reactive astrocytes have reduced ability to scavenge reactive oxygen species (ROS). This leads to a significant increase in free radicals and various species of ROS, rendering astrocytes vulnerable to metabolic and mitochondrial alterations and favoring the production of oxidative stress. The production of pro-inflammatory cytokines is favorable under these conditions as observed in the C21 group (without EGb 761 treatment), which shows high concentration levels. In the other groups, the effects of the EGb 761 extract influenced the control of pro-inflammatory cytokines.

Ni et al. [26], Bastianetto et al. [16] suggested that EGb 761 could attenuate apoptosis induced by Aβ25-35. Chen and Herrup [27], demonstrated that cytotoxicity in neural cells is induced in the presence of Aβ and H_2O_2 hydroxyl radicals that are present in AD. In this study, diffuse vacuolization of the gray substance (malacia) and intense gliosis was observed in group treated, i.e., intense neuronal death from lack of oxygen and glucose and the presence of diffuse senile plaques. The results suggest a reduction in tissue damage in hippocampal structures caused by the pro-inflammatory cytokines released during apoptosis. This damaged tissue is subjected to the effect of the β-amiloid 25-35 peptide in the group receiving treatment with the EGb 761 extract.

Heneka and O'Banion [28] report that, in addition to glial activation, the neuroinflammatory component present in AD is characterized by a local acute response phase mediated by cytokines, activation of the complement system, release of glutamate, and induction of inflammatory enzymes. Increase in oxide nitric synthase (iNOS) and cyclooxygenase-2 (COX-2) result in increase in the generation of nitric oxide (NO) and ROS. This study corroborates the findings of Heneka and O'Banion, who report the effects of *Gingko Biloba* in reducing pro-inflammatory cytokines and subsequent increase in anti-inflammatory cytokines.

Williams et al. [15] showed that EGb 761 could significantly modulate synaptic plasticity in aged rats by the glutaminergic system, both in chronic and acute treatment. In the present study, improvement in spatial memory was observed in the animals treated with EGB761,

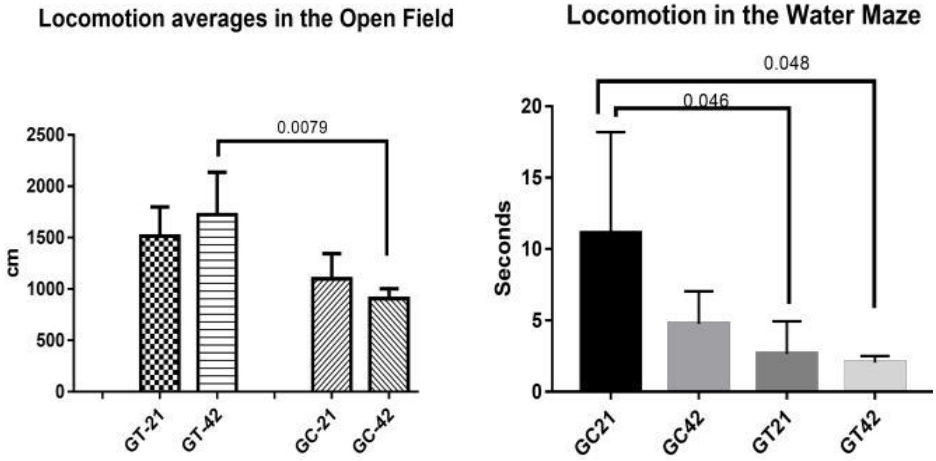

Figure 3: Average values of the range of locomotion in the Open Field and Morris Water Maze tests.

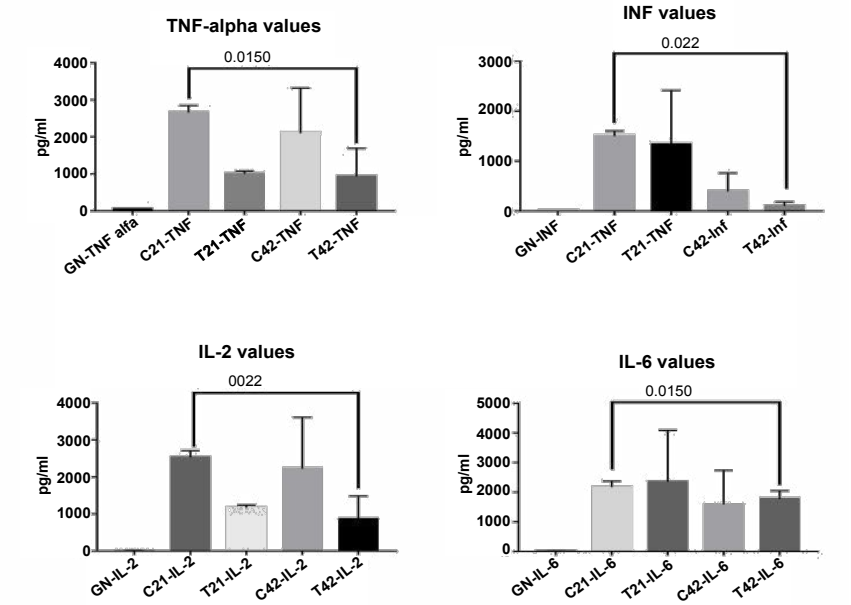

Figure 4: Average values in the Th1 system for proinflammatory interleukins in the 21 and 42 days treated groups.

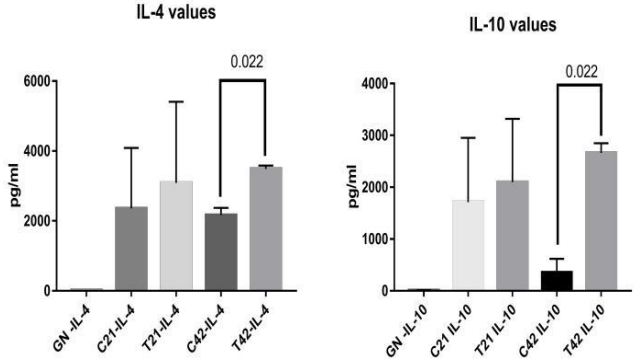

Figure 5: Average values in the Th2 system for the anti-inflammatory IL-4 and IL10 interleukins.

supporting the results of the aforementioned study that EGB761 acts on the glutaminergic system improving synaptic functions.

Pagliarone and Sforcin [29] reported that the IL-6 and IL-10 interleukins are produced by microglia activation and IL-10 acts inhibiting IL-6 and its receptor. The increase in production of inflammatory cytokines may result in an increased inflammatory response by immune and brain cells and may lead to a progression of neurodegenerative diseases.

In the present study, a significant difference was observed between IL-10 and INF-y in the C21 group and T21 and T42 groups while the statistical relationships of IL-10 with IL-6 did not achieve significantly different results. The influence of IL-10 on IL-6 did not produce sufficient immune responses to sustain a balance in the inflammatory process.

Ching-Hsiang et al. [30] found a significant decrease in IL-6 levels with continuous GB treatment for eight weeks, resulting in anti-inflammatory and antioxidant action. Meanwhile, IL-6 produced by several cell types, including endothelial, microglia, neurons, platelets, leukocytes, and fibroblasts cells, is shown as an important regulator of neuroinflammation in neurons and astrocytes.

According to Zhao et al. [31] IL-10 is known for its anti-inflammatory action and ability to inhibit pro-inflammatory cytokines. The reduction of IL-10 may indicate disorders of immunity and acute inflammation. Increased IL-10 levels can modulate abnormal immunity, inhibit inflammation, and promote tissue restoration.

According to Junior et al. [32] many of the protective effects of the CNS associated with the chronic use of EGB are related to the presence of terpenes and flavonoids constituents with antioxidant and anti-inflammatory properties.

The present study demonstrated that animal with the AD-induced neuronal lesion and treated with *Ginkgo biloba* showed a reduction in the levels of pro-inflammatory cytokines and consequent reduction of the inflammatory response.

Davis and Laroche [33] report that the reduction in locomotion can be an adaptation of the animal to a disease state called *Sickness Behavior*: the reduction or loss in IL-10 production leads to behavior with reduced spatial exploration and social activity and reduced food intake. In the present study, although IL-10 levels are high when compared with other cytokines, the animals did not reduce their movements and showed no change in behavior.

Based on a quantitative analysis of the literature, little effect on cognitive function in AD is observed when EGb 761 extract is supplemented for 3 to 6 months at doses between 120 and 240 mg. Petkov et al. (2003) demonstrated that the administration of leaf extracts (GK 501) and roots (PHL-007 010) of *Ginkgo biloba* or their combination improved learning and memory processes, which suggests that the neurotransmission dopaminergic system is involved in the behavioral effects promoted by these components, corroborating the findings in our study.

Cruz et al. [34] reported that the data on this subject are still limited and inconsistent because it involves many variables such as dose, duration of administration, age, individual differences in the level of

cognitive function, testing methodology, and others, which hinders the determination of effects on behavioral, cognitive, and functional measures as well as on the scales used for clinical assessment of the disease stage.

Conclusion

EGb 761 was shown to be effective in reducing serum levels of pro-inflammatory cytokines, promoting a possible reduction in the inflammatory process generated by AD.

Conflict of Interest

The authors have no conflicts of interest to declare.

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