

Analysis of Nuclear Volume and Morphology of Neuronal Brain Tissue of Rats Trained

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Abstract

The aim of the present study was to analyze neuronal volume, nervous tissue cell density, and cell proliferation in the hippocampus, striatum, and sensorimotor cortex of the brains of rats trained before cerebral ischemia. Twenty-four Wistar rats were used in the experiment and were divided into two groups: animals that performed physical exercise on a treadmill, and animals that were not submitted to exercise and formed the sedentary group. Each group was then divided into two subgroups, one that underwent transient middle cerebral artery occlusion (MCAO) for 60 minutes, and another that was submitted to false surgery. The rat brains were removed and the tissue was processed by the Nissl method to calculate neuronal volume. Hematoxylin and eosin staining was also conducted for the labeling of general nerve tissue cells, and cell proliferation analysis was performed using the silver impregnation method of proteins associated with nucleolar organizer regions (AgNORs). The obtained results showed that the animals exercised before cerebral ischemia presented a significant difference in number of AgNORs in the hippocampus when compared to the sedentary group. Differences between groups were not found in the cortex and striatum. A significant difference was observed in the neuronal volume of the cerebral cortex and striatum. It can be concluded that exercise performed prior to cerebral ischemia promoted neuroprotection by increasing neuronal volume and cellular proliferation in areas of the brain that regulate movement.

Keywords: Brain Ischemia; Exercise; Silver Staining; Neuroprotection

Introduction

Cerebrovascular diseases constitute brain dysfunctions that are linked to the blood supply of the brain. The study of such diseases is of fundamental importance due not only to the high incidence in the population but also to the effects they cause, including death, physical incapacity, and dementia, which lead to social and economic losses [1,2].

A stroke is a cerebrovascular disease that can occur when there is obstruction or bleeding in the arteries that irrigate the brain. Such obstruction, which may be caused by a clot, leads to decreased oxygen supply in brain tissue, causing its failure. In turn, hemorrhaging, which refers to extravasation of blood in nerve tissue, causes hematomas or clots, damaging nerve cells [3-5]. Ischemic stroke or brain ischemia is the largest cause of death worldwide [4].

Brain ischemia, which results in neuronal death, consists of the obstruction of blood supply to the brain, which is part of the encephalon. Thus, studying the nucleoli of nerve tissue cells is crucial to understand the process of cell death [6] and may clarify some of its triggering mechanisms. There are histochemical techniques that allow the study of neuronal nuclei and nucleoli, such as the Nissl method

[7] and silver staining (AgNORs) [8]. The Nissl technique enables the labeling of Nissl corpuscles present in neurons, allowing the quantification and visualization of these cells [7,9]. The AgNORs method, in turn, allows the identification of nucleolar activity regions in the neurons [10].

The regions of the brain that are most vulnerable to cerebral ischemia include the striatum, hippocampus, and motor cortex. These areas are essential in the planning and execution of movement [11] and are extensively studied in ischemic lesions of the brain [9,12-14].

Research has been conducted aiming at preventing and treating the impacts of cerebral ischemia on neuronal tissue. Physical exercise has been proven to promote the prevention of these disorders, potentially inhibiting stroke precursors [2].

Studies have also confirmed that physical exercise can foster brain protection in cases of ischemia since it leads to positive regulation of the brain-derived neurotrophic factor (BDNF) and proteins involved in synaptic function [3]. Recent advances in elucidating the role of neurotrophins in physical activity-dependent plasticity have provided insight into how behavior may affect specific aspects of neuronal biology [15].

Experimental evidence that physical activity performed before or after ischemia promotes neuroprotection has been found, including reduction of the cerebral infarction area, neuronal survival, neurotrophic factor release, increased angiogenesis, and release of stress-protective proteins [12,16]. Other mechanisms, such as collateral recruitment, arteriogenesis, vasculogenesis, and angiogenesis, are crucial in maintaining cerebral blood flow and, consequently, aid in protecting the brain against stroke [17,18].

Thus, it has become fundamental to study neuronal volume, cell density and cell proliferation in the brains of rats submitted to moderate treadmill training prior to cerebral ischemia, since few studies that analyze these parameters in important areas of motor movement planning have been carried out.

Materials and Methods

A total of twenty-four 30-day-old Wistar rats, with a mean weight of 138.7 ± 2.18 g were utilized in the present study. The animals were maintained in cages, with free access to water and feed, a 12-hour photoperiod, room temperature between 21 and 22°C, and relative air humidity of 60 - 70%. The rats were weighed at the beginning of the experiment (30 days), immediately before (76 days) and after (81 days) the surgical procedure, and at the end of the trial (126 days).

Twelve animals were submitted to ischemia by transient middle cerebral artery occlusion (MCAO) and distributed in the following subgroups: EBI (n = 6): exercised on the treadmill (Insight Instrumentos® - Ribeirão Preto, SP, Brazil) before ischemia, and SI (n = 6): did not undergo training.

The other twelve rats (control) were subjected to surgical access, but not to MCAO, and were distributed in the following subgroups: EBS (n = 6): exercised before surgery, and SC (n = 6): were not submitted to exercise on the treadmill.

The animals that exercised prior to the surgical procedure underwent a five-day treadmill adaptation period at a speed of 10 - 12 m/min, for 12 min/day. Thereafter, the rats from the groups that trained before surgery (EBI and EBS) were submitted to exercise on the leveled treadmill for six weeks, five days a week, for 30 min/day, at a speed of 15 m/min. The training velocity was gradually increased, with the animals running at 10 m/min on the first day, 12 m/min on the second day, and from the third day on they ran at a speed of 15 m/min [19].

In the middle cerebral artery occlusion procedure, the animals received pre-emptive analgesia with fentanyl (0.3 mg/Kg/IM), were sedated with Diazepam (2.5 mg/Kg/IP) and anesthetized with isoflurane in an anesthetic breathing system with 100% oxygen. Antimicrobial prophylaxis was performed intramuscularly with enrofloxacin (10 mg/Kg).

After anesthesia, atropine diluted in 2% saline (0.1 mL per animal) was administered intraperitoneally to prevent cardiac arrhythmias and bronchial hypersecretion secondary to mechanical stimulation of the vagus nerve during the operation.

The surgical procedure used to perform the occlusion was described by Longa, *et al* [20]. After trichotomy and antisepsis of the ventral cervical region, a median sagittal incision of approximately 3.0 cm in length was conducted, and the tissues were divulsed until exposure of the left common carotid artery (CCA) bifurcation. The pterygopalatine branch of the internal carotid artery (ICA) was clamped at its origin, and the external carotid artery (ECA) was ligated with vicryl 0, at the distal portion of the CCA bifurcation. After the CCA and ICA were clamped, the ECA was sectioned for passage of the occlusion wire (monofilament for silicone occlusion, 5-0 in diameter and 0.12 mm long, Doccol®). The wire was introduced through the left ECA inside the ICA until the origin of the MCA, approximately 20 - 21 mm away from the CCA bifurcation. In order for occlusion wire passage, the ICA clip was removed. The distances were determined by previously made markings on the wires at 18, 20 and 22 mm in length. Introduction of 20 mm of the occlusion wire, associated with discrete resistance to wire passage, indicated the location of the MCA origin. The surgical incision was then closed, maintaining the occlusion wire and the clip in the CCA.

The wire and the clip remained in place for 60 minutes. Subsequently, the animal was again anesthetized, the wire was removed, and the portion of the ECA proximal to the CCA bifurcation (through which the occlusion wire was introduced) was ligated. Tissue approximation and dermorrhaphy were carried out using 3-0 nylon thread.

After surgery, the animals were maintained under observation during anesthetic recovery for two hours, with body temperature between 36 - 38°C and water and feed at will. In order for volumetric replenishment, 10 mL of intraperitoneal saline was injected daily during the first 72 hours [21].

Next, the scale for the MCAO rat model proposed by Menzies, *et al.* [22], with scores ranging from 0 to 4, was utilized, in which the higher the score, the worse the neurological deficit. On the first day after surgery, the animals that presented score 4 were included in the experiment and used in the SI (sedentary and MCAO) and EBI (exercise before MCAO) subgroups.

After 126 days, all animals were anesthetized with 1% ketamine (30 mg/Kg) and xylazine (4.0 mg/Kg) intraperitoneal injection. Soon after, they were perfused transcardially with heparinized saline solution (0.9% NaCl containing 5000 IU of heparin/L) for 5 minutes, followed by infusion with 4% paraformaldehyde solution for another 5 minutes. The brains were then removed and sliced into 1.0 mm sections in the coronal plane. The paraffin slices of the sections (3.20 mm, 0.20 mm, and -2.80 mm, in relation to the bregma), were selected for labeling using the silver staining/AgNORs method [6,10]. These sections were again sliced (Leica® rotary microtome), obtaining 6 µm-thick cross-sections, which underwent deparaffinization in xylene I for 5 minutes, xylol II for 5 minutes and xylol III for 10 minutes. Subsequently, the sections were rehydrated in absolute alcohol for 10 minutes, 70% alcohol for 5 minutes, 50% alcohol for 5 minutes, and deionized water for 10 minutes. Thereafter, the sections were stained with a 50% silver nitrate solution (Sigma-Aldrich) and a solution of 1% formic acid containing 2% colorless gelatin, dissolved for 35 minutes. After incubation in the silver nitrate solution, the sections were washed in deionized water for 15 minutes, followed by dehydration in 70% alcohol for 3 minutes, and absolute alcohol for another 3 minutes. Next, they were dipped in xylene I for 2 minutes and xylol II for another 2 minutes. The slides were then assembled and analyzed.

In order to quantify the neurons and the AgNORs of the silver-stained neuronal cells [6], four images of each histological slice were captured using the Motic Images Plus 2.0 software and a digital camera (Moticam 580) attached to a microscope (new Optical Systems 1801), amplified at 40X and 100X. Next, the selected images were analyzed using the Image J software (Image-Pro Plus, version 4.5, Windows 98), thus obtaining the number of neurons and AgNORs of each histological section.

For general cell counting, some paraffin sections were re-sectioned into 5 µm-thick slices and stained with hematoxylin and eosin for cell quantification in regions of the cerebral cortex, striatum, and hippocampus.

In order to conduct the Nissl staining method (Cresyl Violet), some slices underwent re-sectioning (Leica® rotating microtome, model RM2255), resulting in 10 µm-thick cross-sections, which were then submitted to Nissl staining, followed by slide assembly and analysis. In the staining procedure, the slides were immersed in a cresyl violet solution to reveal the cytoplasm of the neurons and the Nissl corpuscles. In neuronal lesions, corpuscles can disappear, a phenomenon known as chromatolysis. Thus, staining serves as an indicator of neuronal viability. The slides containing the sections were submitted to Nissl staining according to the following protocol: immersion for 5 minutes in xylol 1, xylol 2, 100% alcohol, 100% alcohol, 95% alcohol, and 70% alcohol, then for 30 minutes in the 0.5% cresyl violet solution. The slides were then dehydrated for 5 minutes in 70% alcohol, 95% alcohol, 100% alcohol, 100% alcohol, and finished with diaphanization in xylol 1 and xylol 2, followed by assembly and analysis [7,9].

For the quantitative analysis of the cells, two images of each histological section were taken - sensorimotor cortex, striatum, and hippocampus, totaling six images from each animal. The Axio Vision program (version 4.8) and a digital camera (AxionCam ERC5S) attached to a microscope (Zeiss Axio Lab A1), amplified at 200 X, with an image analysis area of 50 x 10⁶ µm², were used to capture the images.

The cells of the selected images were subsequently quantified with the help of the Image J software (Image-Pro Plus, version 4.5, Windows 98), which obtained the mean number of cells of each histological section, both dead and alive. Cell counting was performed in the two cerebral hemispheres, in specific regions of each section, in relation to the bregma: two areas were considered in the 3.20 mm section: the margin of the ischemic motor cortex region and the area of penumbra; in the 0.20 mm section, the transition between the striatum and the motor cortex was considered, and in the -2.80 mm section, the apex region of the dentate gyrus (hippocampus) was quantified. Quantification of neuronal volume was carried out by comparison with the volume of a sphere, in which the largest and smallest diameters of each neuron were measured, and the mean of these values was used in the formula for calculating sphere volume ($V = \frac{4\pi R^3}{3}$). Thus, after establishing the volumes of the cells, the means of each analyzed area was calculated in the right and left cerebral hemispheres.

Analyses of the number of neurons (Nissl method), general nerve tissue cells, and AgNORs of the hippocampus, striatum, and motor cortex dentate gyrus of the studied groups were performed by one-way ANOVA, followed by the Tukey post-hoc test, considering a significance level of $p < 0.05$. The unpaired t-student test was used to analyze differences between the means of two groups, and the results were expressed as mean ± standard error of the mean (SEM).

Results

The results obtained in this study show that the animals exercised before cerebral ischemia (EBI) presented a significant difference ($p = 0.0241$) regarding the number of AgNORs per field among the cerebral regions of the motor cortex, striatum, and hippocampus. A more substantial number of AgNORs was found in the hippocampus, followed by the striatum, and a smaller amount in the cortex region (Figure 1).

When comparing the hippocampal region of both cerebral hemispheres of the animals exercised prior to ischemia (group EBI) with that of the rats who remained sedentary before the procedure (SI group), a significant difference was not found ($p = 0.3993$) regarding the number of general cells per field. However, when analyzing the number of AgNORs per field in the same region in the two groups, a significant difference ($p = 0.05$) was observed between the obtained values (Figure 2).

In the motor cortex and striatum regions, the difference between the number of general cells per field in the animals exercised before ischemia and the sedentary group was not significant ($p = 0.1257$ in the cortex and $p = 0.9247$ in the striatum). When analyzing the number of AgNORs per field in the same cerebral areas, a significant difference between the EBI and SI groups ($p = 0.1127$ in the cortex) was also not found.

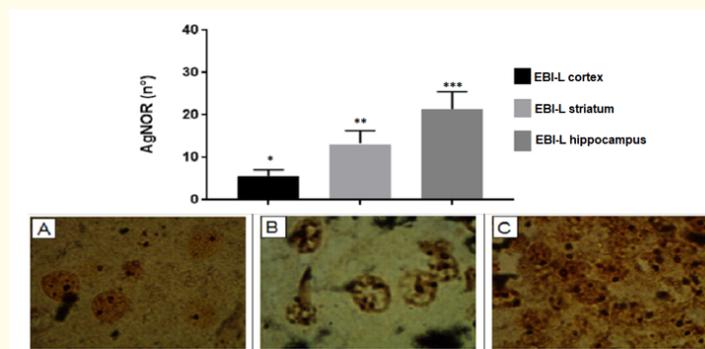


Figure 1: Means of AgNORs per field in the motor cortex, striatum, and hippocampus regions of the left cerebral hemisphere of the animals exercised before cerebral ischemia (EBI-L) and photomicrographies of regions of the left cerebral hemisphere of the animals exercised before cerebral ischemia (EBI-L) - (A) motor cortex region; (B) striatum region, and (C) hippocampal region. Silver staining histological technique (AgNOR). 1000 X.

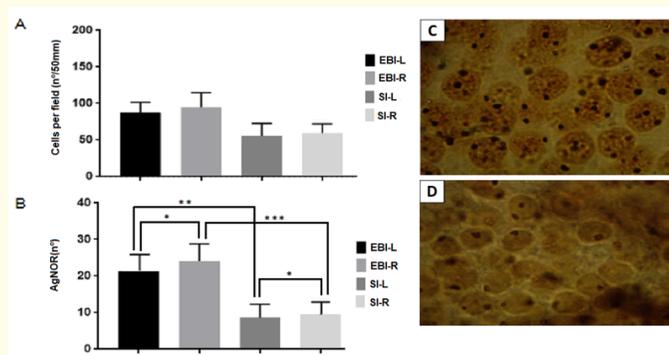


Figure 2: Means of general cells per field and the number of AgNORs in the hippocampus of the sedentary and exercised animals prior to cerebral ischemia. (A) General cells per field in groups EBI (exercised before ischemia) and SI (sedentary/ischemic) in the left (EBI-L; SI-L) and right (EBI-R; SI-R) cerebral hemispheres; (B) Number of AgNORs per field in groups EBI (exercised before ischemia) and SI (sedentary/ischemic) in the left (EBI-L; SI-L) and right (EBI-R; SI-R) cerebral hemispheres. (C) Photomicrographies of the hippocampal region of the left cerebral hemisphere of rats - AgNOR distribution in the hippocampus of rats exercised before cerebral ischemia (EBI-L). (D) AgNOR distribution in the hippocampus of sedentary animals before cerebral ischemia (SI-L). Silver staining histological technique (AgNOR). 1000 X.

The results concerning neuronal volume, obtained by the Nissl staining technique, demonstrated that, in the left cerebral hemisphere, a more significant value was observed in the motor cortex ($5777 \mu\text{m}^3$; $3226 \mu\text{m}^3$; $p < 0.05$) and the striatum ($9148 \mu\text{m}^3$; $6228 \mu\text{m}^3$; $p < 0.05$) of the animals that underwent treadmill exercise prior to cerebral ischemia when compared to the sedentary animals before the ischemic procedure (Figure 3).

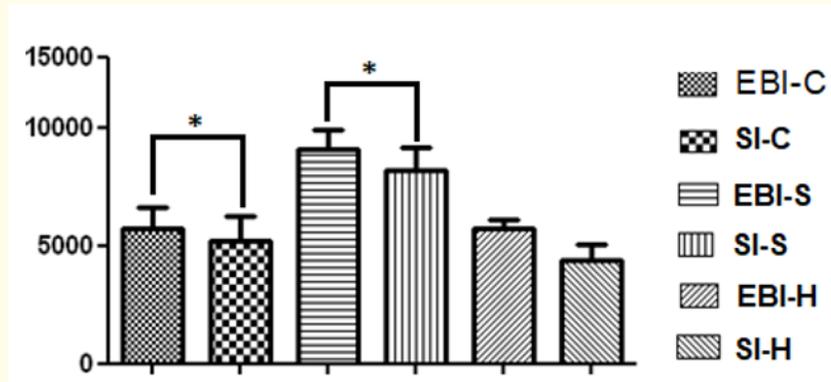


Figure 3: Means of the neuronal volumes, in μm^3 , in the cerebral cortex, striatum, and hippocampus of the left cerebral hemisphere. EBI-C represents the group of slides of the cerebral cortex of rats that exercised before ischemia; SI-C, slides of the cerebral cortex of sedentary rats that were submitted to ischemia; EBI-S, slides of the cerebral striatum of rats that exercised before ischemia; SI-S, slides of the cerebral striatum of sedentary rats that were submitted to ischemia; EBI-H, slides of the cerebral hippocampus of rats exercised before ischemia; SI-H, slides of the cerebral hippocampus of sedentary rats that were submitted to ischemia.

The animals that exercised prior to ischemia presented a higher mean neuronal volume in the motor cortex ($6976 \mu\text{m}^3$; $2810 \mu\text{m}^3$; $p < 0.05$) and the striatum ($11513 \mu\text{m}^3$; $6882 \mu\text{m}^3$; $p < 0.05$) of the right cerebral hemisphere when compared to the sedentary animals regarding the same areas, also in the right hemisphere (Figure 4).

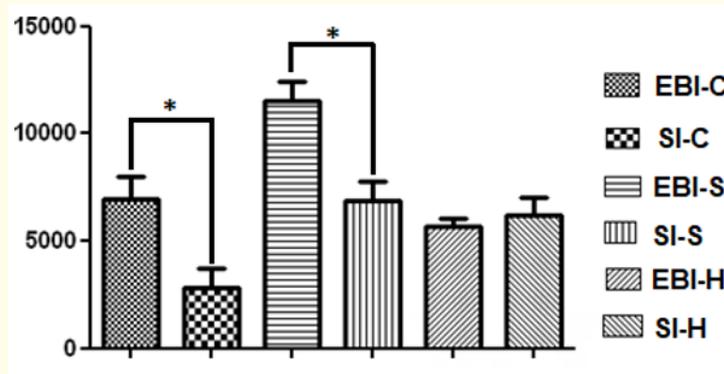


Figure 4: Means of the neuronal volumes, in μm^3 , in the cerebral cortex, striatum, and hippocampus of the right cerebral hemisphere. EBI-C represents the group of slides of the cerebral cortex of rats that exercised before ischemia; SI-C, slides of the cerebral cortex of sedentary rats that were submitted to ischemia; EBI-S, slides of the cerebral striatum of rats that exercised before ischemia; SI-S, slides of the cerebral striatum of sedentary rats that were submitted to ischemia; EBI-H, slides of the cerebral hippocampus of rats that exercised before ischemia; SI-H, slides of the cerebral hippocampus of sedentary rats that were submitted to ischemia.

The mean values of the hippocampal neuron volumes did not show significant differences when comparing the rats that underwent exercise prior to cerebral ischemia and those who were sedentary before the procedure. Such a fact refers to both the right hemisphere ($5672 \mu\text{m}^3$; $6232 \mu\text{m}^3$; $p > 0.05$) and the left hemisphere ($5714 \mu\text{m}^3$; $4380 \mu\text{m}^3$; $p > 0.05$).

Discussion

Physical activity performed before cerebral ischemia promotes neuroprotection through the decrease in the area of cerebral infarction [23] and the release of neurotrophic factors [24-26]. The expression of these factors plays a prominent role in the proliferation, maturation, and survival of neuronal cells [23,27]. In the present study, such an effect was observed by analyzing neuronal cell volume and calculating the number of general cells and AgNORs present in the motor cortex, striatum, and hippocampal regions of the brain of animals that underwent MCAO, comparing the data obtained from the animals that were submitted to exercise with the sedentary ones.

The AgNOR technique enables the visualization of nucleolar organizing regions (NORs) through silver impregnation [28]. NORs are DNA segments where the genes that encode ribosomal RNA (rRNA) are found [29] and the amount of these structures is directly related to the rate of cell proliferation [30]. The data obtained herein demonstrated a higher number of AgNORs in the hippocampal region of the animals exercised before ischemia than the sedentary groups, indicating greater cellular proliferation and confirming the neuroprotective effect of physical activity.

Several studies have shown that physical exercise induces an increment in neurogenesis in the dentate gyrus (DG) in young, adult, and old animals, exerting a pro-proliferative effect by first acting on the neural progenitors of the hippocampus. Rats that run 5 km per day over a period of 7 to 12 days may have the generation of three times as many newborn neurons in the DG as sedentary animals. The formation of new neurons induced by physical activity contributes to the increase in volume of the granular layer of the DG and also helps in the processes of learning and memory [31]. In the present study, exercise prior to cerebral ischemia also promoted an increase in the number of AgNORs in the hippocampus of the trained animals, evidencing the beneficial impacts of such activity before ischemic events. The results also showed that, in the hippocampus of the cerebral hemisphere contralateral to the lesion, greater proliferation was observed, demonstrating the beneficial effects of neuronal plasticity in the hemisphere without the injury.

In the analysis of neuronal volume and cell density, no significant difference was observed in the hippocampus of the animals that underwent physical exercise before cerebral ischemia, in comparison with the sedentary groups. This outcome may be related to the depth of the structure in the rat brain and its irrigation, performed by the hippocampal arteries, which are divided into three branches: anterior, middle, and posterior. In addition, the results did not differ from the literature, which correlates lesions by MCAO to the cortex and striatum, with a relatively low association with the hippocampus [32]. Therefore, it is possible to note that the physical training benefited the generation of new cells in the hippocampus, although new neurons or glial cells were not necessarily formed since the total number of cells did not alter. It is important to highlight that the present study did not analyze the survival of these new cells, thus invalidating the assumption of whether or not they would die in the process.

The data show that a larger volume of neurons was found in the motor cortex and striatum of the rats that exercised before cerebral ischemia when compared to the sedentary animals. In contrast, no difference was observed in the number of AgNORs and cell density, indicating that no new cells were formed in these regions. Nonetheless, the neuroprotective effect of the physical exercise occurred prior to the ischemic event, as corroborated by previous studies, demonstrating the neuroprotective effect of pre-ischemia exercise when analyzing motor behavior and the increased expression of the brain-derived neurotrophic factor (BDNF). In addition to its neuroprotective character and abundance in the hippocampus, cerebral cortex, cerebellum, striatum, and amygdala, BDNF is also involved in synaptic plasticity and efficiency and neuronal connectivity, thus contributing to brain function [33].

The striatum is considered in the literature as a structure susceptible to ischemia caused by MCAO, probably because its irrigation originates mainly from branches of the middle cerebral artery [34]. This fact may explain the data found in the present study, such as the difference between the non-ischemic ($11513 \mu\text{m}^3$) and the ischemic sides ($9148 \mu\text{m}^3$), in rats exercised before MCAO.

In the right cerebral hemisphere, significant changes were observed in neuronal volume in the cerebral cortex, striatum, and hippocampus, when compared to the left hemisphere. Such a result is in agreement with the process of cerebral neuroplasticity, where the uninjured hemisphere establishes synapses with neurons of the injured hemisphere, increasing connections and neuronal morphology. This fact was confirmed in studies that investigated the association between neurotrophic release, ischemia, and exercise, in which rats trained post-ischemia presented an increase in Neurotrophin-4 mainly in the contralateral hemisphere of the injury, denoting a significant difference when compared to the production of the substance in the ipsilateral hemisphere. These results corroborate the hypothesis of cerebral neuroplasticity since the neurotrophin is a protective factor of neural function [35]. Additionally, the release of BDNF adds to the role of Neurotrophin-4, aiding in neuroplasticity.

Several studies state that exercise acts as a protective factor, being considered a way to prevent damage, although it is important to stress its duality as prevention-treatment. Many surveys have shown that post-ischemia exercise is also capable of helping the organism recover from damage caused by brain injury, such as reducing the volume of cerebral infarction [12]. Therefore, it is crucial to stimulate the practice of physical activity to avoid ischemic conditions, but also for the recovery of individuals who have sustained this form of injury [36].

Some results reported in the literature corroborate with those obtained herein, such as the fact that physical exercise promotes cerebral neuroprotection. Other studies indicate an increase in glutamate receptor antagonists and, thus, reduction of brain damage caused by ischemia [34], as well as increased expression of genes that regulate mitochondrial biogenesis in the brain, which promotes rehabilitation after ischemia [12]. Another aspect is that exercise is indicated in some surveys as being crucial for motor recovery, since it regulates motor function and BDNF expression, and inhibits the access of harmful substances in the post-ischemic brain, such as glutamate and calpain [33,34,37].

Conclusion

Exercise performed before cerebral ischemia acts as a neuroprotector since the animals that underwent training exhibited greater cell proliferation in the hippocampus and higher mean neuronal volume in the sensorimotor cortex and the striatum. Such an increase occurred more expressively in the contralateral hemisphere of the lesion, demonstrating the capacity of physical exercise to promote cerebral neuroplasticity.

Conflicts of Interest

The authors declare no conflicts of interest.

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