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Analysis of the expression of microRNA-34c and BCL-2 gene related to apoptosis in the cerebellum of rats submitted to focal cerebral ischemia associated or not with a physical exercise model

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ABSTRACT. Cerebrovascular accident (CVA) is a major public health problem and is considered one of the leading causes of death and disability worldwide, which, if left untreated, can result in neuronal damage. In addition, apoptotic processes occur far from the ischemic focus, such as in the cerebellum. Due to the disability caused by the stroke in the individual, rehabilitation is necessary to avoid further damage to the patient's health. Therefore, the use of physical exercise can be an alternative to this problem, involving passive or active exercises, progressive resistance, balance and postural exercises. As physical exercise has a systemic action, it can influence the expression of biomarkers, such as microRNAs, which are small non-coding RNA molecules that act in transcription and post-

transcription to regulate gene expression. To evaluate the expression profile of microRNA-34c and BCL-2 gene related to apoptosis in cerebellar tissue after experimental induction of temporary focal cerebral ischemia, associated or not with a model of pre-ischemic physical exercise. A total of twenty-two animals were used, subdivided into 4 experimental groups: control group, physical exercise group, ischemic group, ischemic group + physical exercise, were submitted to a treadmill training protocol for four weeks, and the training lasted 30 min a day at a speed of 18m/min. Cerebellar tissue samples were collected for the analysis of apoptosis from the expression of microRNA-34c and the BCL-2 gene, using the real-time PCR methodology. We observed a significant difference in the expression of the BCL-2 gene in the control group in relation to the groups: physical exercise, ischemic and physical exercise + ischemic. We did not observe a significant difference in the expression of microRNA-34c.

Key words: Stroke; microRNAs; Apoptosis; Physical Exercise.

INTRODUCTION

Stroke is considered one of the most common causes of death and disability worldwide (Caprio FZ, 2019), as well as being one of the main causes of hospitalizations, being a public health problem and having a high prevalence in the population (Schmidt et al., 2019). In addition to all this incidence and harm caused, another difficulty is in relation to post-treatment, since the costs are high (Rajsic et al, 2019).

Stroke occurs due to an occlusion of a cerebral blood vessel, causing an interruption in blood flow and impairing oxygenation and nutrition in the affected region. This interruption of blood flow can be caused by ischemic origin, with a lack of oxygenation in the area, or hemorrhagic origin, when the vessel ruptures, but ischemic is more common. Cerebral ischemia is among the most serious cerebrovascular diseases, involving morbidity and mortality worldwide (Siqueira Abrahão et al., 2020; Zimak Figueiredo et al., 2022).

The interrupted flow can be returned, with the process of blood reperfusion, however, this can cause greater damage than ischemia itself, and oxidative events such as oxidative stress, inflammatory events and brain damage can occur (Porsani et al., 2019). Cell apoptosis is a process present in ischemic stroke and is present during ischemia and after the entire ischemic process, as well as being accentuated during reperfusion (Porsani et al., 2019; Silva et al., 2019).

When ischemic stroke occurs, various substances are released due to the damage, and the neurovascular unit is compromised after the process. As a result, studies have shown that physical exercise can be an alternative for neuroprotective issues related to the lesions that occur because of the ischemic process (Zhang H, 2022). Preconditioning to cerebral ischemia promotes cerebral neuroprotection in rats and, among the related mechanisms, there is a decrease in the inflammatory response, a decrease in cerebral edema, an increase in the synthesis of brain-derived neurotrophic factor and neuronal growth factors. All these processes are neuroprotective against the damage caused by cerebral ischemia (Shamsaei et al., 2015; Damázio et al., 2015).

Rodents, for this type of study, both for physical exercise protocols and for ischemia, are the most used for experiments related to brain function. In relation to physical activity, we can mention voluntary and forced activity exercises, obtaining results equivalent to aerobic metabolism training (Aguiar JR, 2007).

Therefore, physical exercise is an intervention that helps in the prevention and rehabilitation of people with ischemia, playing a protective role and reducing the brain damage caused, promoting processes such as angiogenesis and neuroplasticity and reducing the apoptotic process. However, there are many challenges to be fully understood in relation to all the processes involving exercise intervention on stroke, being necessary to understand the ideal duration for physical activity, the start time, the intensity of the exercise, to understand the different responses developed by exercise in different brain regions, in addition to understanding the issue of gender and age (Zhang H, 2022).

Recent studies have shown possible markers for various pathologies, including brain pathologies (Novais et al., 2020). These biomarkers are called microRNAs, which are small non-coding RNA molecules, with 19 to 25 nucleotide pairs, with the function of modulating the transcriptional expression of the gene, playing roles in various cellular events, such as regulation of biological processes, regulation of growth, cell differentiation, cell proliferation, apoptosis, neurogenesis and other processes (Croce CM, 2005; Todoran et al., 2023). Thus, microRNA is a biomarker of great potential in serum and tissue to understand the molecular mechanisms involved in cerebral ischemia (Novais et al., 2020).

Because of their role in regulating a variety of targets, microRNAs are increasingly being studied in research, as they have shown potential in the early detection of diseases, which can be used in risk assessment, pathology prognosis and the development of new therapeutic targets (De Smaele E, 2010).

The microRNA-34 family has the following members: miR-34a, -34b and -34c, which are regulated by p53 (Hermeking et al., 2010; Lv et al., 2019). The expression of microRNA-34c was observed in the hippocampus of rats subjected to chronic injury by lead neurotoxicity, in which this microRNA, along with microRNA-34b and others, was overexpressed in the hippocampus due to this toxicity. This microRNA regulates the BCL-2 gene in different ways, interfering with its expression (Hermeking et al., 2010; An et al., 2014).

The effects of physical exercise on the body can act in various ways, and some biomarkers can be used for research associated with various molecular mechanisms, such as the apoptotic process, with the possibility of associating microRNAs, which regulate this mechanism and can be positively or negatively regulated.

The aim of our study was to evaluate the expression levels of microRNAs miR-34c and BCL-2 genes by real time PCR, previously associated with in rats submitted to an experimental model of focal cerebellar ischemic associated with physical exercise.

MATERIAL AND METHODS

The experiments were carried out in accordance with the Ethical Principles for Experimental Animals (COBAO) and the study was approved by the Animal Experimentation Committee (CETEA) of Ribeirao Preto Medical School - University of São Paulo. 22 adults male Wistar rats (Rattus norvegicus) weighing 280-310g were used. The animals were randomly divided into four

experimental groups: control (C): 7 animals sacrificed without undergoing the surgical procedure; ischemic (I): 4 animals submitted to the transient middle cerebral artery (MCAO) model for 60 minutes, followed by reperfusion for 24 hours and sacrificed; physical exercise (PE): 7 animals submitted to physical exercise; and ischemic and physical exercise (PE + I): 4 animals submitted to the same treatment as the PE group and focal cerebral ischemia for 60 minutes, followed by reperfusion for 24 hours. During the weekly, the animals weight were recorded.

Training Protocol

The animals in the exercise and exercise + ischemic group underwent a period of acclimatization for five days with speeds (5 to 18m / min) and progressive durations (5 to 15 min). The purpose of the adaptation period was to reduce the stress levels presented during the manipulation and use of the treadmill. The protocol consisted of a total period of 4 weeks. The warm-up stage consisted of 2 minutes at a speed of 5 m / min and was gradually increased until reaching a speed of 18 m / min, in which the animals remained for 30 minutes with 0 degrees of inclination. The animals that showed signs of fatigue or poor adaptation to the use of the treadmill, the exercise was interrupted. During training, electric shocks were not used on the animals, only small touches with the hands to stimulate them. If an animal was unable to resume, they were removed from the treadmill to rest and recover. The training was only resumed when the animals were fully recovered.

Induction of Cerebral Ischemia

The animals were sacrificed at the end of the experimental procedures, 36 h after the last training session or after the same period for sedentary groups. All animals were partially anesthetized by halothane inhalation and intubated with an orotracheal cannula. Occlusion of the middle cerebral artery (MCAO) was performed through the external carotid artery, which was cranially connected and sectioned for retrograde introduction of a 4 cm mononylon obstructive wire 2.5 cm long with an end thickened with silicone by an extension of 5 mm (Carlotti Jr. et al., 2001). The wire was introduced until it reached the common carotid artery and then progressed cranially through the internal carotid artery until reaching and obstructing the MCA.

RNA extraction and cDNA synthesis

After removal of the brain, the left cerebral hemisphere cortex was isolated and a sample measuring 7 mm in diameter was drilled for a biopsy centered along the MCA. The samples were placed in cryovials and stored in liquid nitrogen at -196°C until they were used for RNA extraction.

The total RNA was extracted with the Trizol reagent (Applied Biosystems, USA) according to the manufacturer's instructions. To verify the integrity of the RNA obtained, each sample was subjected to electrophoresis on 1% agarose gel and subjected to a spectrophotometer that provides the concentration of RNA in a sample of 1 to 2μL. In addition to concentration, this device provides values for a ratio related to the integrity of the samples (ratio 260/280). The ideal range to be obtained is 1.7 to 1.9.

Real-time PCR reactions were performed in duplicate. The amplification was performed in a final volume of 10μL, using 5μL of the specific reagent Taqman Master Mix, 0.5μL of each specific probe and 4.5μL of cDNA. A 7500 Real Time PCR System (Applied Biosystems) PCR detection device was used together with the Sequence Detection System software to obtain the CT values. The

data were then exported to Excel spreadsheets to calculate the ΔCT values. The GraphPad Prism 4.0 software (GraphPad Prism, Inc, San Diego, CA, USA) was used to generate the graphs and calculate the statistical significance.

U6 was used as an endogenous control for the microRNA and gene reactions, respectively. All reactions were performed in duplicate and analyzed with the 7500 Sequence Detection System (Applied Biosystems).

Statistical analysis

Data concerning the microRNA and gene in the various groups were analyzed statistically by Kruskal-Wallis test followed by the Dunn's multiple comparisons test using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The level of significance was set at $p < 0.05$ for two-tailed tests.

RESULTS

We did observe a significant difference in the expression of BCL-2 gene (P = 0.0027) between the control group and the physical exercise, ischemic and physical exercise in the associated ischemic cerebral and not observe any significant differences in the expression of microRNA-34c (P = 0.1729) between the groups (Figure 1 and 2)

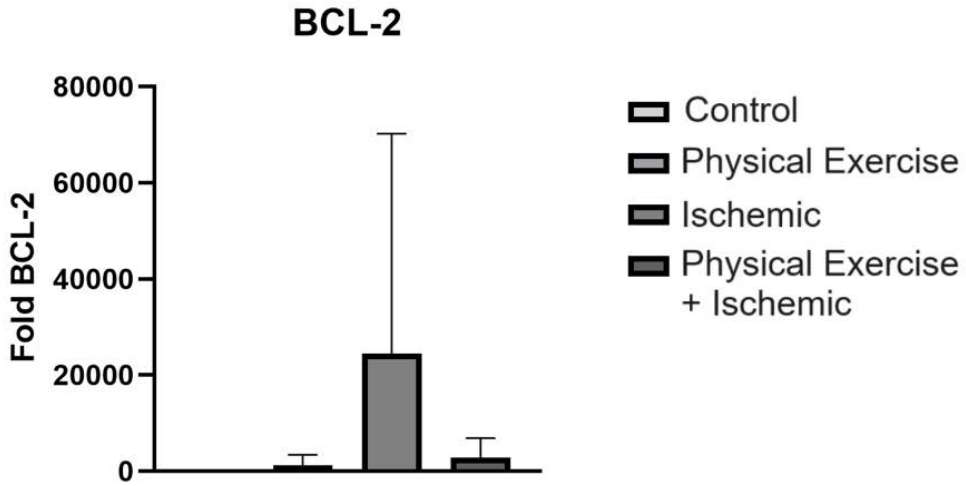

Figure 1. Representation of mean values (± standard error) of BCL-2 gene between the groups studies of cerebellar tissue of rats (P = 0.0027, Kruskall Wallis test, Dunn´s post-test).

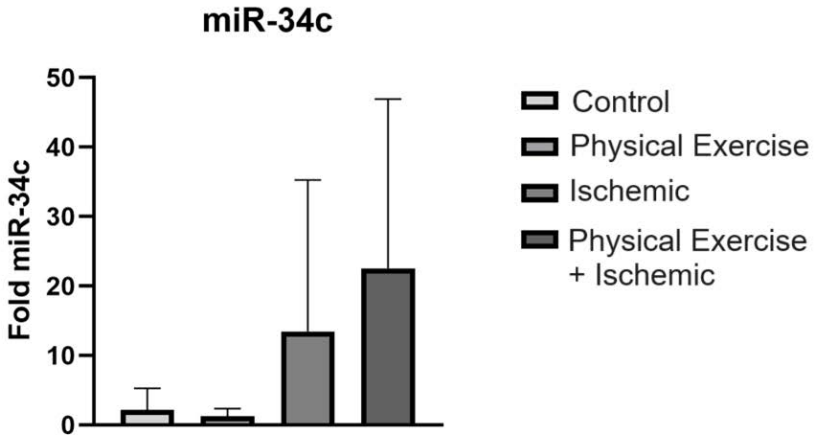

Figure 2. Representation of mean values (± standard error) of microRNA-34c between the groups studies of cerebellar tissue of rats (P = 0.1720, Kruskall Wallis test, Dunn´s post-test).

DISCUSSION

The aim of this study was to evaluate the expression of microRNA-34c and the BCL-2 gene, which are related to the apoptosis mechanism, being pro-apoptotic (microRNA-34c) and anti-apoptotic (BCL-2), in research using animals submitted to cerebellar tissue evaluation, based on a cerebral ischaemia model, after occlusion of the middle cerebral artery, associated or not with physical exercise, in order to observe whether there was any statistically significant difference between the groups studied.

In a study using the same model as ours, they observed pre-training neuroprotection before the cerebral ischaemic process, analysing the BAX, BCL-3 and CASPASE-3 genes by Western blotting. In this work, they showed a decrease in the BAX gene and CASPASE-3, pro-apoptotic genes, and an increase in BCL-2 expression with physical exercise (ZHANG et al., 2019).

In our results, we observed a statistically significant difference between the Control Group and the Trained, Ischaemic and Trained + Ischaemic Groups in relation to the BCL-2 gene, with higher expression in the ischaemic group and lower expression in relation to physical exercise, not correlating with the neuroprotective effect, since the BCL-2 gene is anti-apoptotic, given that the increase in the ischaemic group may be related to a control effect to try to prevent the apoptotic process in the affected cerebellar region.

Further studies associating the BCL-2 gene with the mechanism of apoptosis in cerebral ischaemia related to physical exercise were described in a study by our group, where no difference was observed in the expression of BCL-2 in the groups studied (Porsani et al., 2019).

Analyses of apoptotic and non-apoptotic genes can demonstrate the neuroprotective effects of exercise before the ischaemic process. In a study of physical exercise in Sprague-Dawley rats subjected to middle cerebral artery occlusion for 2 hours, the authors observed the effects of apoptosis by studying the expression of the BAX, CASPASE-3 and BCL-2 genes. BCL-2 levels showed no significant difference in the effect of physical exercise in the ischaemic brain region, unlike the BAX

and CASPASE-3 genes, which increased when the animals were subjected to cerebral ischaemia (LI et al., 2017).

These results for the BCL-2 gene did not corroborate our results, since we obtained a significant difference between the control group and the trained + ischaemic group, and this difference must be related to the affected region, since the studies cited show the affected brain region and in our study, we observed the affected cerebellar region (far from the ischaemic focus), with a difference in relation to the expression of the BCL-2 gene.

One of the pathways activated when cardiac ischaemia occurs is the pi3-kinase/Akt signalling pathway, which induces the expression of the BCL-2 gene in cardiomyocytes to activate IGF-1, which is a neuroprotector and its loss can cause myocardial damage. The study by Qin et al., (2020) showed that BCL-2 expression levels are decreased in cardiomyocyte injury under ischaemic conditions and after reoxygenation. With this, even though it was in the cardiac region and not the cerebellar region, the mechanism was the same, relating to an ischaemic process; however, the results did not corroborate ours, as we obtained an increase in BCL-2 in these conditions.

In human umbilical cord vein endothelial cell lines and in murine models of mouse hind limb ischaemia, microRNA-34c-5p was used to observe cell viability and the process of autophagy, which is a mechanism of cell degradation. Under conditions of hypoxia in the region, microRNA-34c was overexpressed in the cell line. In addition to the microRNA, the BCL-2 gene was used, as it is regulated by this microRNA, showing that microRNA-34c suppressed the BCL-2 response (KIM et al., 2022).

Regarding microRNA-34c, in our study, there was no significant difference between the groups studied; however, it is possible to observe an increase in the ischaemic group and in the trained + ischaemic group, which was higher in the latter compared to the others. This result corroborates the previous study, which showed an increase in microRNA-34c in the process of hypoxia in the cell line, and this process occurs in cerebellar ischaemia. Furthermore, it showed that even with physical exercise, there was no decrease.

The up-regulation of microRNA-34c-5p promotes the process of apoptosis; it is therefore considered a tumour suppressor. In a study related to cerebral ischaemia, performing middle cerebral artery occlusion showed a reduction in the expression of microRNA-34c-5p after the ischaemic and reperfusion process, as well as inhibiting the expression of TNF-alpha, IL-6, iNOS and COX-2 and increasing the expression of IL-10, showing activation of endogenous anti-inflammatories. In addition, it was observed that the overexpression of microRNA-34c-5p can negatively regulate the increased expression of CASPASE-3 and BAX, and positively regulates the decreased expression of BCL-2. The authors concluded that microRNA reduced cell damage through inflammatory and apoptotic action (Tu Y, 2021).

In our study, the trained + ischaemic group showed an increase in microRNA-34c and a decrease in the BCL-2 gene, showing this regulation of the microRNA in the gene; however, this was not observed in the ischaemic group, as mentioned by the study, not corroborating our results. In addition, the authors commented that there was a reduction in cell damage, but apoptotic action is increased, which could cause more damage.

Therefore, more studies are needed to identify microRNAs and genes that have potential for use in clinical practice, related to the mechanism of apoptosis in cerebral ischaemia associated with physical exercise.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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