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The role of exercise in the expression of caspase 3 and microRNAs miR-138 and miR-155 associated with apoptosis in the cerebellum of rats submitted to cerebral ischemia

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ABSTRACT. Cerebral ischemia is among the main causes of death in the world, being the third cause of death after cardiovascular diseases and cancer, in addition, it is one of the biggest causes of permanent sequelae capable of generating disability. In recent decades, experimental studies have demonstrated beneficial effects associating physical exercise with cerebral ischemia. Several molecular mechanisms are involved in the pathophysiology of cerebral ischemia, among which apoptosis stands out. Current research also highlights the role of microRNAs in cerebral ischemia as well as in the mechanism of apoptosis. Therefore, analyzing the expression of anti- and pro-apoptotic members and microRNAs associated with cerebral ischemia, as well as the role of the benefits promoted by physical exercise, may contribute to the elucidation of possible molecular pathways with neuroprotective effects. This study aimed to analyze the gene expression of caspase 3 and microRNAs miR-138 and miR-155 in rats submitted to experimental cerebral ischemia associated with physical exercise. Twenty-two adult male Wistar rats (Rattus norvegicus) were used, divided into four experimental groups: control, subjected to cerebral ischemia, subjected

to physical exercise and subjected to physical exercise associated with cerebral ischemia. Real-time PCR methodology was performed to analyze the expression of caspase 3 gene and microRNAs miR-138 and miR-155 in the cerebellum. We observed a statistically significant difference in the expression levels of miRNAs miR-138 and miR-155 between the studied groups. With greater expression in the ischemia and ischemia associated with physical exercise groups. Despite no significant statistical difference, we observed increased apoptosis due to high levels of CASPASE-3 expression in the ischemic group. We can suggest that the modulation of miRNAs miR-138 and miR-155 may be involved in the repercussions of the ischemic event. More studies are needed to understand the differences in the variables associated with ischemia models, duration of the ischemic event as well as the duration of reperfusion.

Key words: Cerebral ischemia; MicroRNA; Physical exercise; Apoptosis; Cerebellum.

INTRODUCTION

Most cases of stroke are ischemic, that is, caused by interruption of blood flow to a certain region of the brain (Rosamond et al., 2008). Hemorrhagic strokes are less common when compared to ischemic strokes. They occur through the total or partial rupture of a vessel, causing blood leakage to the brain and represent approximately 15% of all strokes. However, 40% of all stroke deaths are attributed to hemorrhagic strokes (Yang et al., 2017). The most common cause of ischemia is the unexpected obstruction of a blood vessel, causing an almost immediate loss of oxygen and glucose in the brain tissue. This deprivation causes a series of events, such as excitotoxicity, oxidative stress and inflammation, thus causing irreversible neuronal damage (Jablonska, 2011). Several studies also demonstrate the effect and interaction of focal ischemia in non-ischemic areas such as the cerebellum, inferior parietal cortex and superior frontoparietal cortex after occlusion of the middle cerebral artery. Identifying the reset of excitatory/inhibitory balance, identifying changes in metabolic activity and neurotransmitters in non-ischemic regions. (Håberg AK, 2009).

Programmed and controlled cell death, or simply apoptosis, is commonly observed in the ischemic brain. (Yang JL et al., 2018). Brain cells have diverse functions, and many cellular transformations occur in the moments after some damage to the brain tissue, such as ischemic injury, the activation of cell death by apoptosis has been proven to be involved in the impairment of tissues in the region of the brain affected by the stroke. (Cheon et al., 2018).

Some current studies aim to elucidate the effectiveness of the possible neuroprotection promoted by the practice of physical exercises associated with cerebral ischemia, with obstruction of the middle cerebral artery being the most prevalent experimental model in the literature (Zhang Z et al., 2019). Molecular mechanisms of cell death have been explored in investigations into the role of physical exercise associated with stroke. The practice of physical exercises is being used in models of experimental cerebral ischemia as a neuroprotective potential, thus promoting the concentration of multiple cerebral neuronal impulses, enabling plastic changes that favor brain structures damaged by the ischemic injury (Damázio et al., 2015).

MicroRNAs (miRNAs or miRs) are single-stranded RNA molecules consisting of 18 to 25 nucleotides, specifically explicit, regulated by development and act directly in the negative regulation of protein expression in several eukaryotic organisms (Mendell J.T, 2005). In recent decades, miRNAs have emerged as potential regulators of several biological processes in the brain, regulating various mechanisms and physiological brain activities, such as: proliferation, differentiation, apoptosis, synaptic plasticity and memory (Fernandes et al., 2017). Studies based on bioinformatics analyzes prove that miRNAs are deregulated in injuries caused by cerebral ischemia and indicate that many signaling pathways are involved in this process (Liu XS et al., 2013).

Recent research shows the relationship between physical exercise and cerebral ischemia, and seek to elucidate the involvement of miRNAs in an attempt to elucidate the molecular mechanisms involved in the neuroprotection conferred by the practice of physical activities. (Porsani L.B, 2020). Experimental studies have demonstrated beneficial effects of physical exercise associated with cerebral ischemia. Several molecular mechanisms are involved in the pathophysiology of cerebral ischemia, including changes in the expression profiles of miRNAs. Real-time PCR methodology was used to analyze the expression of miRNAs related to neurotransmitters. Greater expression of the miRNAs miR-15b and miR222 was observed in rats subjected to a model of focal cerebral ischemia with the association of ischemia and physical exercise. (De Assis Cirino M.L, 2019)

Figueiredo R.Z. and colleagues (2022) demonstrated the effect of physical exercise in rats subjected to cerebral ischemia due to occlusion of the middle cerebral artery and analyzed the expression of the microRNAs miR-126, miR-133b and miR-221 by real-time PCR. Wistar rats were used, divided into four experimental groups: control group, ischemia group, physical exercise group and exercise/ischemia group. The authors observed a significant difference in the expression of miR-221 between the control group and the other groups.

The elucidation of the modulation provided by miRNAs in the brain during cerebral ischemia may contribute to the development of new therapeutic approaches that could be used in clinical practice in the future (Zhang et al., 2012). Studies are necessary to clarify the regulation of apoptosis promoted by physical exercise with the aim of understanding the neuroprotective action promoted by physical exercise.

MATERIAL AND METHODS

General procedures

The experiments were carried out according to the Ethical Principles for Experimental Animals (COBAO) and the study was approved by the Animal Experimentation Committee (CETEA) of the Medical School of Ribeirão Preto - University of São Paulo. Twenty-two adult male rats (Rattus norvegicus) of the Wistar lineage, weighing between 280 and 310 grams, were used in the study. The animals were provided by the Central Animal Facility of the Ribeirão Preto Campus of the University of São Paulo.

The animals were divided into four experimental groups:

Control Group (C): consisted of 7 animals that, after anesthesia and stabilization of biological variables, were euthanized without performing the surgical procedure.

Cerebral Ischemia Group (I): consisted of 4 animals subjected to focal ischemia by occlusion of the MCA for 60 minutes, followed by 24-hour reperfusion, and subsequently subjected to euthanasia.

Physical Exercise Group (PE): consisted of 7 animals that, after physical training, were euthanized without undergoing the surgical procedure.

Physical Exercise + Cerebral Ischemia Group (PE+I): consisted of 4 animals that, after completing physical training for a period of four weeks, were subjected to focal ischemia by occlusion of the MCA for 60 minutes, reperfusion for 24 hours and subsequently subjected to euthanasia.

Weekly measurements of the weight of the animals were held in the different study groups.

All animals were partially anesthetized by halothane inhalation and intubated with an orotracheal cannula. At two occasions during the ischemic period, arterial blood samples were collected for the determination of glycemia, paCO2, paO2 and pH. MCA occlusion was carried out through the external carotid artery (ECA), which was ligated cranially and sectioned for the retrograde introduction of a 2.5 cm long obstructive 4-0 mononylon suture with one end thickened with silicone over an extension of 5 mm. The suture was introduced until reaching the common carotid artery (CCA) and then cranially progressed through the internal carotid artery (ICA) to reach and obstruct the MCA. After the period of ischemia, we proceeded to the removal of the obstructing thread, replacing the temporary clamp in the CCA to prevent the flow of blood and in the ICA to prevent the blood reflux. The proximal stump of the ACE was permanently connected and the temporary clamps were removed. Then, the skin and subcutaneous tissues were closed in animals from groups I and PE/I.

The animals went through a period of acclimatization for five days with velocities (5 to 18m min) and durations (5 to 15 min), increasing progressively. The objective of the adaptation period was to reduce the stress levels presented during the manipulation and the use of the treadmill. The protocol was composed of a total period of 4 weeks. The session consisted of 2 min at a speed of 5 m/min and this was gradually increased until reaching the speed of 18 m/min in which the animals stayed for 30 min always with 0 degrees of inclination. When the animals showed any signs of fatigue or maladaptation to the use of the treadmill, the exercise was interrupted. During the training, no electric shocks were used on the animals, only slight touches with the hands to stimulate them. In a persistence situation, the animal was removed so that it could rest and recover. The training only resumed when the animal was fully recovered.

Analysis of caspase 3 and microRNAs miR-138 and miR-155

For the analysis of gene expression, all animals were used per group. A fragment was obtained from each animal of the four groups. Total RNA was extracted with 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 agarose gel 1% RNA and put through the spectrophotometer that provides the RNA concentration in a sample of 1 to 2µL. In addition to the concentration, this device provides us with values of a reason relating to the integrity of the samples (260/280 ratio). The ideal range to be obtained is 1.7 to 1.9. To prepare the real-time polymerase chain reaction (PCR), reverse transcription of RNA samples was performed using the High-Capacity cDNA kit (Applied Biosystems, USA). The cDNA was amplified with quantitative Real Time Polymerase Chain Reaction (q-PCR) using TaqMan Master Mix (Applied Biosystems) for reactions. U6 was used as endogenous control for the reaction of the microRNA

and the gene, respectively. All reactions were carried out in duplicate and analyzed with the 7500 Sequence Detection System apparatus (Applied Biosystems).

STATISTICAL ANALYSIS

Data concerning the gene and microRNAs in the various groups were analyzed statistically by Kruskal-Wallis test followed by the Dunns post-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

Expression of miR-138 in cerebellum samples from the studied groups (Figures 1-6).

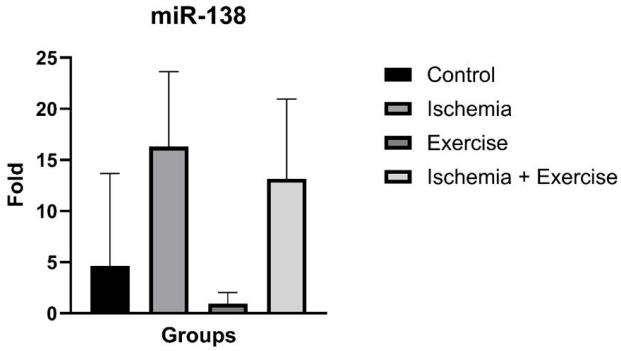

Figure 1. Expression of miR-138 in cerebellum sa1mples from the studied groups.

Representation of the mean (± standard deviation) of microRNA-138 expression in the studied groups. There was no statistically significant difference between the groups p=0.0463, Kruskal-Wallis test.

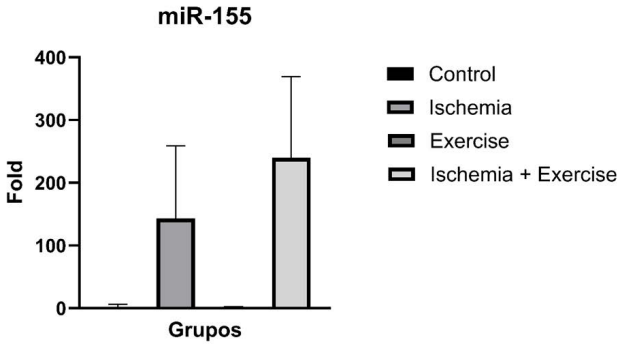

Figure 2. Expression of miR-155 in cerebellum samples from the studied groups.

Representation of the mean (± standard deviation) of microRNA-155 expression between the studied groups p=0.0300, Kruskal-Wallis test. There was a statistically significant difference in the groups: Ischemia group when compared to the Exercise group (p=0.0365) and Exercise group when compared to the Exercise + Ischemia group (p-0.0205), Dunns post-test.

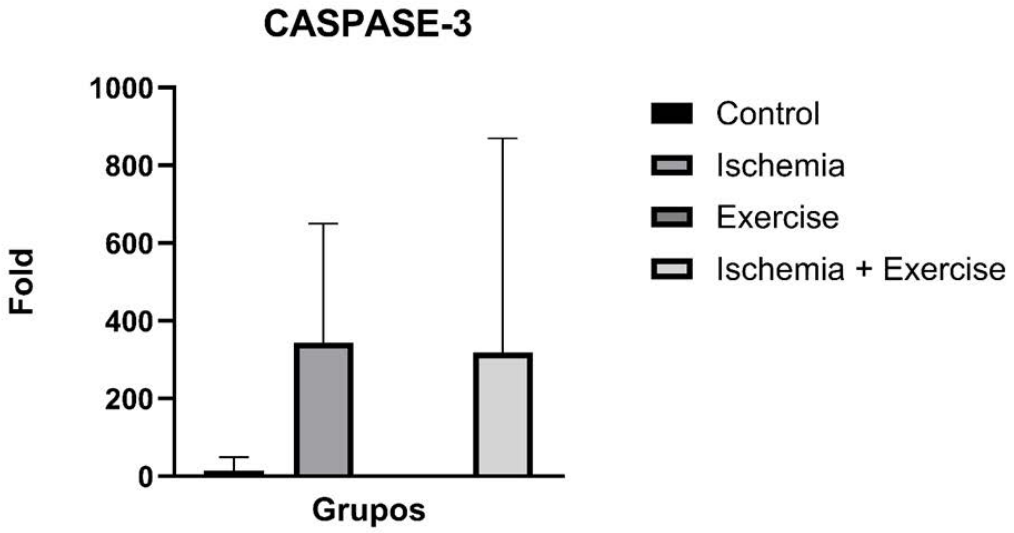

Figure 3. CASPASE-3 expression in cerebellum samples from the studied groups.
Representation of the mean (± standard deviation) of CASPASE-3 gene expression in the studied groups. There was no statistically significant difference between the groups p=0.2060, Kruskal-Wallis test.

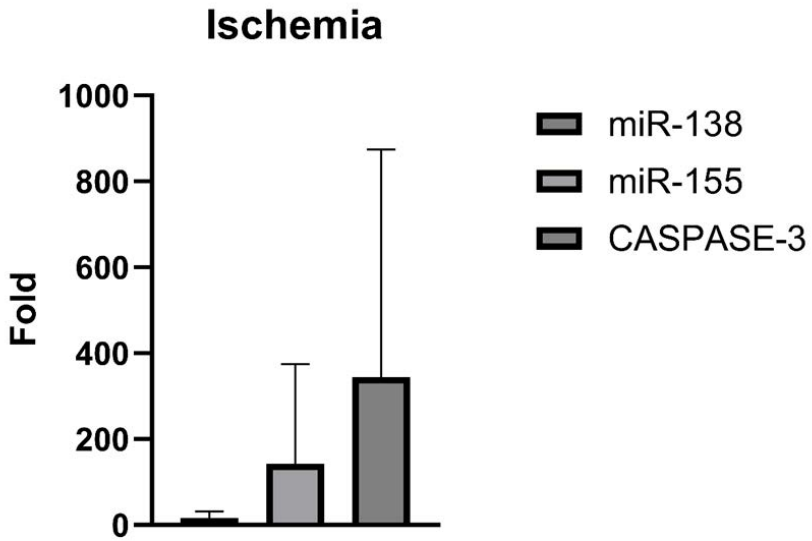

Figure 4. Expression of microRNAs and CASPASE-3 in cerebellum samples from the Ischemia group.
Representation of the mean (± standard deviation) expression of microRNAs: miR-138 and miR-155 and CASPASE-3 in the ischemia group. There was no statistically significant difference between the groups p=0.5816, Kruskal-Wallis test.

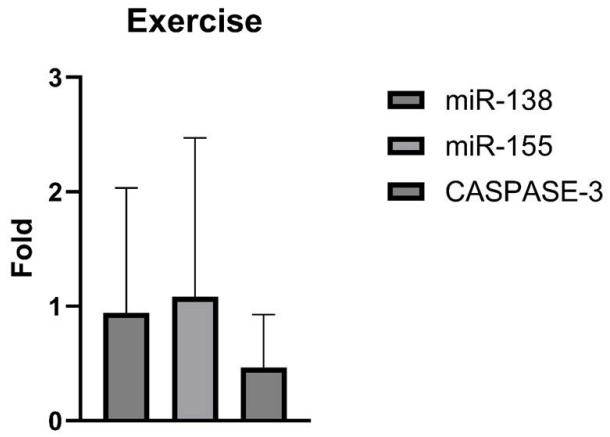

Figure 5. Expression of microRNAs and CASPASE-3 in cerebellum samples from the Exercise group.

Representation of the mean (± standard deviation) expression of microRNAs: miR-138 and miR-155 and CASPASE-3 in the exercise group. There was no statistically significant difference between the groups p=0.6462, Kruskal-Wallis test.

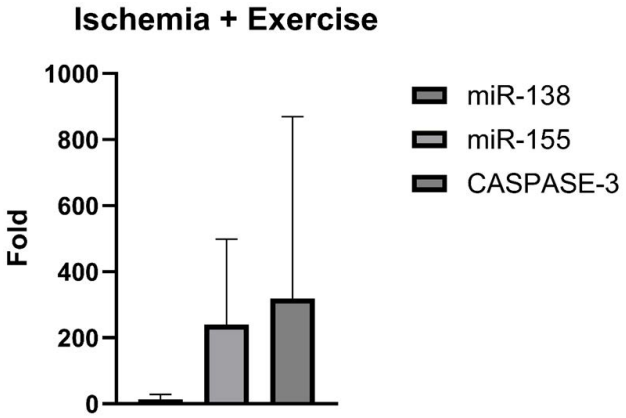

Figure 6. Expression of microRNAs and CASPASE-3 in cerebellum samples from the Ischemia group associated with the Exercise group.

Representation of the mean (± standard deviation) expression of microRNAs: miR-138 and miR-155 and CASPASE-3 in the ischemia+exercise group. There was no statistically significant difference between the groups p=0.3536, Kruskal-Wallis test.

DISCUSSION

In the literature, there is an increasing number of scientific articles that have evaluated the neuroprotective role promoted by physical exercise, both in clinical studies and in studies that use experimental models. Many studies also demonstrate the benefits of exercise interventions before and after a stroke, that is, in the prevention and treatment of the disease, including the chronic motor sequelae of the stroke, which improves the quality of life and physical independence of patients (Wang et al., 2014; Pin-Barre, 2015).

The reduction in infarct volume in the first weeks after a stroke through physical rehabilitation with a reduction in chronic impairments in motor and sensory function as well as the effects of chronic physical activity present much evidence in the literature. However, acute physical exercise also proves to be a promising approach for functional motor recovery, promoting neuroplasticity at different cellular and molecular levels, with mechanisms such as inflammation, angiogenesis and apoptosis being directly involved (Pin-Barre, 2015).

In this context, it is worth highlighting the importance of understanding the repercussions of stroke in regions distant from the ischemic focus, such as the cerebellum, as its main function is related to somatic motor skills, being responsible for motor coordination, control of muscle tone and balance. In previous studies by our group, Carvalho et al., (2016) and Silva et al., (2019) analyzed the role of apoptosis in the cerebellum in an experimental model of cerebral ischemia associated with experimental chronic alcoholism. Therefore, in this study we evaluated the possible neuroprotective role of physical exercise by regulating the apoptosis mechanism in the cerebellum of rats subjected to a model of focal cerebral ischemia by occlusion of the MCA for 60 minutes, followed by reperfusion for 24 hours.

Martin, Sieber and Traystman (2000), using an experimental model of global cerebral ischemia, observed cellular degeneration in two distinct ways: the first being necrosis in pyramidal neurons and the second apoptosis in granule neurons in the dentate gyrus and cerebellum. The authors highlight that the elucidation of the contributions of cell death mechanisms in the adult central nervous system after ischemia due to apoptosis and necrosis can make relevant contributions to the development of new therapeutic strategies (Martin, 2000).

In our results, despite not finding a significant statistical difference, we observed an increase in apoptosis due to high levels of CASPASE-3 expression when we analyzed the ischemic group, which corroborates the literature.

Mizutani and colleagues (2010) evaluated an experimental model of physical exercise with treadmill training and compared it with an experimental model of cerebral ischemia after 2.5 hours of transient occlusion of the middle cerebral artery. The findings demonstrate that the 25 kDa synaptosome-associated protein and glial fibrillary acidic protein may be related to the improvement in motor coordination and exercise-induced angiogenesis observed by the remodeling of synaptic connections and proliferation of astroglial cells (Mizutani et al., 2010).

In our results, we did not observe a statistically significant difference in CASPASE-3 expression levels when we analyzed the target studied only in the physical exercise group.

LEE and colleagues (2019) analyzed the cerebellum of rats subjected to a model of cerebral ischemia by bilateral occlusion of the common carotid arteries (BCCAO) and compared it with BCCAO associated with physical exercise. Among the evaluations carried out by the authors, the expression of CASPASE-3 was evaluated by immunohistochemistry. A decrease in apoptosis rates in the cerebellar vermis was observed and the authors conclude that physical exercise has a neuroprotective role.

In our results, we did not observe a statistically significant difference in CASPASE-3 expression levels when we analyzed only the cerebral ischemia group associated with physical exercise, which could perhaps be attributed to differences in the variables associated with the ischemia model, duration of the ischemic event, as well as as reperfusion time.

In the search for a better understanding of the involvement of the apoptosis mechanism in the possible neuroprotection promoted by physical exercise in the cerebellum of rats subjected to cerebral ischemia, two microRNAs associated with apoptosis regulating CASPASE-3 were selected, namely: miR-138 and miR -155.

Sha and colleagues (2017) describe that miR-138 acts in the extrinsic apoptosis pathway by regulating the expression of CASPASE-8. The authors also describe the role of this microRNA in the intrinsic apoptosis pathway, decreasing the expression of BCL-2 and resulting in the activation of CASPASE-3 and consequently causing cell death by apoptosis (Sha et al., 2017).

Mao, Luan and Qi (2022) analyzed the expression of miR-138-5p in the serum of stroke patients 90 days after treatment. ROC curve analyzes demonstrated the diagnostic potential of serum miR-138-5p to distinguish stroke patients from control individuals. Decreased expression of miR-138-5p was detected in stroke patients who had a poor prognosis. The authors conclude that the decrease in miR-138-5p is identified as a risk factor for the occurrence of stroke and is associated with worse patient outcomes (Mao, 2022).

Miao and colleagues (2015) evaluated the global expression profile of microRNAs by microarray technique in the cerebral cortex of mice pre-trained on a running wheel (RW) and found that some miRNAs such as miR-21, miR-92a, miR- 874, miR-138, let-7c and miR-124 may be involved in the neuroprotection provided by exercise (Miao et al., 2015).

In our results, we observed a statistically significant difference in miR-138 expression levels between the groups studied. However, the expression was higher in the ischemia and ischemia groups associated with physical exercise, which allows us to suggest that the modulation of this microRNA may be involved in the repercussions of the ischemic process.

Guo and colleagues (2019) describe that miRNA-155 regulates the expression of anti-apoptotic targets, such as Bcl-2 and XIAP in several disease models, which corroborates the study by Farooqi and colleagues who demonstrated that transfection of miR-155 inhibitors in MCF7 cells, induced apoptosis (Farooqi et al., 2014; Guo et al., 2019).

Jiang and colleagues (2023) used a cerebral ischemia and reperfusion (I/R) injury model in rats established by middle cerebral artery occlusion (MCAO) followed by reperfusion. Colorimetric quantification analyzes were performed and the expression of the m6A methyltransferases METTL3, METTL14 and WTAP, and the demethylases FTO and ALKBH5 were determined using qPCR and Western Blot analyses. FTO was overexpressed in brain tissues through intracerebroventricular injection of adenoviruses encoding FTO. MeRIP assays were used to detect the impact of FTO overexpression on the m6A modification of pri-miR-155. Overexpression of FTO increased the m6A modification of pri-miR-155 and enhanced its maturation to form miR-155. The increase in miR-155 expression levels provided the protective effect of FTO against cerebral I/R injury (Jiang et al., 2023).

Hamid, Hoseini and Rahim, 2024 describe that some microRNAs are crucial in the adaptive response to exercise, including miR-155. However, the authors highlight the need for more studies to better clarify the interactions between miRNAs and their targets involved in adaptive changes depending on the type and duration of training. The authors analyzed 16 patients with a family history of type 2 diabetes, healthy young men (FH+) or no family history of type 2 diabetes (FH-), who received 8 weeks of combined resistance exercise training. The expression of the microRNAs miR-29a, miR-133a, miR-133b and miR-155 was quantified in serum before and after physical

training. There were no differences in miRNA expressions between FH - and FH + . Exercise training did not alter miRNA expressions in FH - or FH +, despite improvements in insulin sensitivity, aerobic capacity and muscle strength. miR-29a and miR-155 were inversely related to fasting blood glucose, and miR-133a and miR-133b were negatively correlated with glucose tolerance; however, no correlations with insulin sensitivity were observed. Circulating miRNAs miR-29a, miR-133a, miR-133b and miR-155 are related to measures of glucose metabolism in healthy, normoglycemic men, but do not reflect peripheral insulin sensitivity or improvements in metabolic health after 8 weeks of combined physical training (Hamid, 2024).

In our results, we observed a statistically significant difference in miR-155 expression levels between the studied groups. However, like miR-138, expression was higher in the ischemia and ischemia groups associated with physical exercise, which allows us to suggest that the modulation of this microRNA may be involved in the repercussions of the ischemic process.

Caspase-3 is essential for normal brain development as well as for the formation of apoptotic bodies (Porter, 1999).

Jie and collaborators (2011) in an experimental model of cerebral ischemia due to occlusion of the middle cerebral artery in rats demonstrate the role of CASPASE-3 in secondary lesions that are not irrigated by the middle cerebral artery. Immunohistochemical analyzes demonstrated that CASPASE-3 played an important role in MCAO-induced apoptosis of cerebellar cortex neural cells. The authors suggest the role of CASPASE-3 in the mechanism of secondary infarct injury in the cerebellar cortex following middle cerebral artery occlusion in rats and may provide a new treatment strategy for individuals with human ischemic stroke (Jie et al., 2011).

Packer and Hoffman-Goetz (2015) used C57BL/6 mice to evaluate the effect of an acute exercise session on hippocampal inflammation in young, middle-aged and older animals. The results demonstrate an increase in TNF-α and caspase-3/7 in the hippocampus in each age group post-exercise, with older mice showing greater expression of TNF-α (main effect of age, $P < 0.05$) in compared to younger animals at the beginning of the study. Young mice demonstrated a greater increase in caspase-7 after acute exercise compared to older mice (Packer, 2015).

Aboutaleb and colleagues (2015) analyzed the effects of pre-ischemic exercise on CA3 neurons. The rats were divided into three groups. Animals in the exercise group were trained 5 days a week for 4 weeks. Ischemia was induced by occlusion of both common carotid arteries (CCAs) for 20 min. The number of TUNEL-positive cells increased significantly in the ischemia group, but pre-ischemic exercise significantly reduced apoptotic cell death. Furthermore, a significant increase in the Bax/Bcl-2 ratio was found in the ischemia group. An increase in the number of active neurons positive for caspase-3 was also observed in the ischemia group (Aboutaleb et al., 2015).

In our results, we did not observe a statistically significant difference in CASPASE-3 expression levels between the studied groups.

In conclusion, we observed a significant increase in the expression levels of the miRNAs miR-138 and miR-155 between the groups studied. With greater expression in the ischemia and ischemia groups associated with physical exercise.

Despite no significant statistical difference, we observed increased apoptosis due to high levels of CASPASE-3 expression in the ischemic group.

We can suggest that the modulation of the miRNAs miR-138 and miR-155 may be involved in the repercussions of the ischemic event.

More studies are needed to understand the differences in variables associated with ischemia models, duration of the ischemic event as well as reperfusion time.

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