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Expression of MicroRNAs miR-126, miR-133b and miR-221 Associated with Apoptosis in Rats Submitted to Focal Cerebral Ischemia and Physical Exercise

Expresión de MicroRNAs miR-126, miR-133b y miR-221 Asociados a Apoptosis en Ratas Sometidas a Isquemia Cerebral Focal y Ejercicio Físico

Rafael Zimak Figueiredo[1]; Mucio Luiz de Assis Cirino[1]; Lucas Barbosa Porsani[1]; Fermino Sanches Lizarte Neto[1]; Paulo Cezar Novais[1,2]; Maria Julia Nunes[1]; Janaína Regina Lellis[1]; Jairo Pinheiro da Silva[1]; Maria de Fátima Galli Sorita Tazima[1]; Marina de Toledo Durand[3]; Valeria Paula Sassoli Fazan[1]; Benedicto Oscar Colli[1]; Daniela Pretti da Cunha Tirapelli[1] & Luis Fernando Tirapelli[1]

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SUMMARY: Stroke is one of the main causes of death and disability worldwide. The great impact on the quality of life of the population and on the health system justifies that we seek relevant alternatives to reduce the incidence and improve the treatment and recovery of patients affected by this disease. Physical exercise appears as an important tool in this scenario, being already pointed out as a possible therapeutic approach for the prevention of non-contagious chronic diseases. In this context, biomarkers such as miRNAs that respond to physical exercise and are directly related to several epigenetic mechanisms appear. Therefore, explaining the molecular mechanisms involved during physical exercise will lead to a better understanding of each stimulus and the dose to be used to better respond to each situation, thus being a promising approach for the evolution of prescription and control of training and processes recovery from various diseases, including stroke. Forty-eight Wistar rats were used, divided into four experimental groups: control group, ischemia group, physical exercise group and exercise + ischemia group. Real-time PCR methodology was used to analyze the expression of miRNAs: miR-126, miR-133b and miR-221. In our study we observed a significant difference in the expression of miR-221 between the control group and the others groups. However, microRNAs: miR-126 and miR-133b do not show significant differences in expression between groups.

KEY WORDS: Cerebral ischemia; Physical exercise; MicroRNAs; Apoptosis.

INTRODUCTION

According to the World Health Organization (WHO) in 2019, stroke is the second leading cause of death and the third leading cause of disability in worldwide (GBD 2016 Stroke Collaborators, 2016). The stroke is the interruption of the blood flow that supplies the brain. This interruption can occur due to a blockage in the blood vessel, ischemic stroke, which is more common, occurring in almost 80 % of cases, and hemorrhagic stroke when there is a rupture of the blood vessel and consequent leakage of blood into the brain (Mirzaei *et al*., 2018). In cerebral ischemia, necrosis is the main route of cell death, predominantly affecting the nucleus of the ischemic focus. In the penumbra region, apoptosis can cause cell death for hours and days after stroke (Hu & Song, 2017).

Previous studies have observed that pre-stroke exercise can produce protective effects on brain damage caused by ischemia/reperfusion lesion, reducing the stroke

[1] Department of Surgery and Anatomy, Medical School of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP 14049-900, Brazil.
[2] Postgraduate Program in Structural and Functional Interations in Rehabilitation, University of Marilia, Marilia, SP 17525-902, Brazil.
[3] University of Ribeirão Preto, Ribeirão Preto, SP 14096-900, Brazil.
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severity and neurovascular injury and improving functional outcomes (Hafez *et al*., 2021; Zhu *et al*., 2022). To elucidate and find efficient mechanisms for using physical activity properly as a therapeutic approach, we can use molecular markers, such as microRNAs (miRNAs).

The miRNAs are small RNA molecules (18-25 nucleotides), non-coding that regulate, at a post-transcriptional level, gene expression (Denham & Prestes, 2016). They are important epigenetic regulatory factors, regulating stem cell proliferation, differentiation and survival, and they are also related in the modulation of several cellular processes, apoptosis, cerebrovascular diseases and cancer (Tirapelli *et al*., 2011; Shi *et al*., 2016).

Therefore, identifying exercise-regulated miRNAs that target anti- and pro-apoptotic proteins could provide the development of new therapeutic approaches in the twilight region of stroke, where cell death occurs predominantly by apoptosis. The resistance of miRNAs and their strong relationship to several mechanisms of the body combined with direct responses to physical training qualify them as a way to understand how physical exercise can collaborate to reduce the affected area in a stroke or even help in recovery, reducing dependence or the incapacity caused to the patient.

The aim of our work was to evaluate the expression profile of miRNAs, miR-126, miR-133b and miR-221 by real time PCR, in the nervous tissue of rats submitted to an experimental model of focal cerebral ischemia associated with physical exercise.

MATERIAL AND METHOD

Animals and study design. 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. 48 adults male Wistar rats (Rattus norvegicus) weighing 280-310g were used. The animals were randomly divided into four experimental groups: control (C): 12 animals sacrificed without undergoing the surgical procedure; ischemic (I): 12 animals submitted to the transient middle cerebral artery (MCAO) model for 60 minutes, followed by reperfusion for 24 hours and sacrificed; physical exercise (PE): 12 animals submitted to physical exercise; and ischemic and physical exercise (PE + I): 12 animals submitted to the same treatment as the PE group and focal cerebral ischemia for 60 minutes, followed by reperfusion for 24 hours. The weekly measurements of the animals' weight were carried out in the different study groups.

Training protocol. All the animals went through 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 in order 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 hours 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 suture 2.5 cm long and an end thickened with silicone by an extension of 5 mm (Carlotti Jr. *et al*., 2001). The suture 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 the moment of RNA extraction.

The total RNA was extracted with the Trizol reagent (Applied Biossystems, 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 subjected to a spectrophotometer that provides the concentration of RNA in a sample of 1 to 2ml. 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 10ml, using 5ml of the specific reagent Taqman Master Mix, 0.5ml of each specific probe and 4.5ml 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 DCT 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 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

The graphs show the mean expression of the studied miRNAs in the different groups. Statistically significant difference was found in mir-221 expression between the control group and the other groups (Fig. 1). No statistically significant difference was observed in the expression of mir-126 and mir-133b between the groups (Figs. 2 and 3).

No statistically difference was found in the comparison of the expression levels between the miRNAs in each group (Fig. 4).

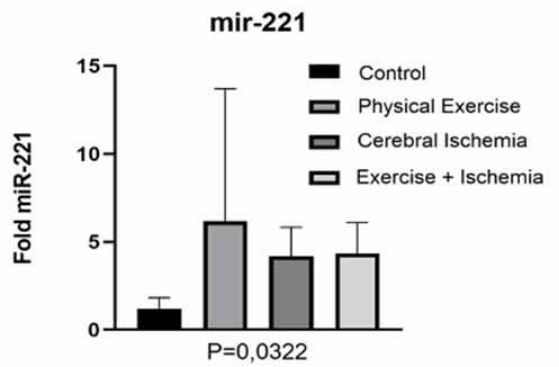

Fig. 1. Representation of mean values (± standard error) of microRNA-221 expression between the groups studied.

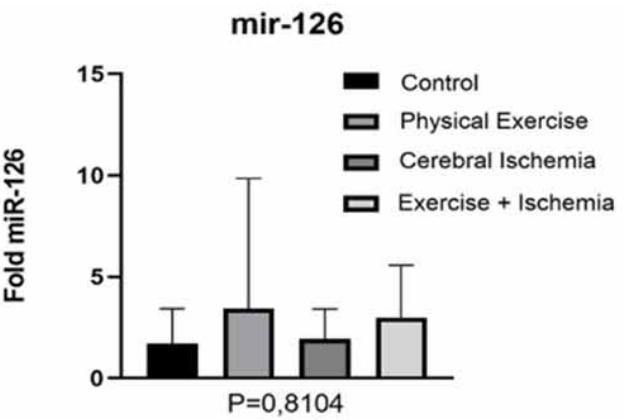

Fig. 2. Representation of mean values (± standard error) of microRNA-126 expression between the groups studied.

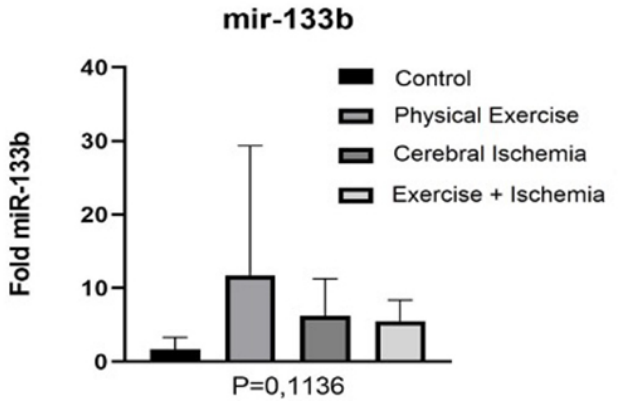

Fig. 3. Representation of mean values (± standard error) of microRNA-133b expression between the groups studied.

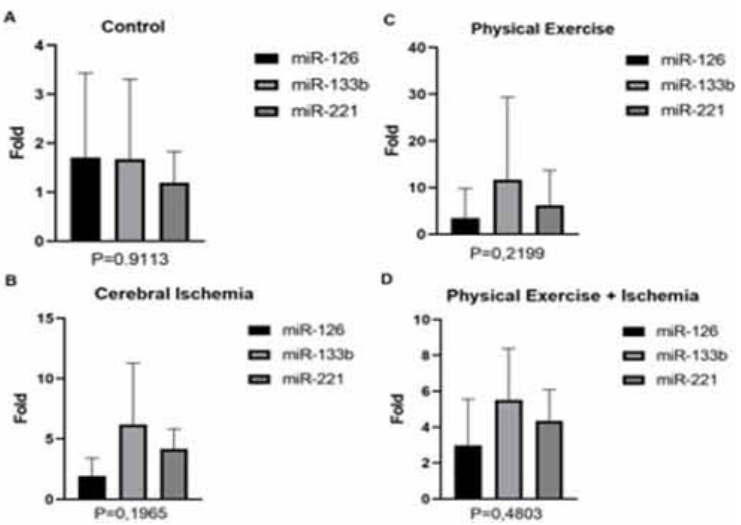

Fig. 4. Representation of the mean values (± standard error) of the expression of microRNA-126, microRNA-133b, miRNA-221 in the groups: control (A), cerebral ischemia (B), physical exercise (C), physical exercise + ischemia (D).

DISCUSSION

MicroRNA-126 is presented in the literature as an important factor in the pathogenesis of stroke and cardiovascular diseases in general, promoting vascular remodeling and reducing fibrosis. The expression and short-term prognostic value of miR-126 expression in patients with acute stroke was investigated in a recent study, Patients with a mild condition or good prognosis had greater expression of miR-126 than patients with a severe condition or poor prognosis (Qi *et al*., 2020).

A previous study by our research group analyzed the cerebellum of rats undergoing occlusion of the distal middle cerebral artery for 90 minutes, followed by reperfusion for 48 hours in an alcoholism model and concluded that miR-126 showed increased expression in the isolated ischemic group and correlation with cellular apoptosis in ischemic rats associated with the chronic alcohol model. Therefore, cell damage occurred even in areas far from the ischemic focus (Silva *et al*., 2019).

Analysis performed with rats trained with moderate exercise has protective effects when experimentally submitted to middle cerebral artery occlusion in the acute and chronic stages, which occurs through the release of miRNA-126 via exosomes, with miRNA-126 levels being negatively correlated with infarct volume and cellular apoptosis, while it was positively correlated with microvessel density (Wang *et al*., 2020).

In our study, we did not observe a statistically significant difference in miR-126 when comparing the studied groups, but we could observe that it was under expressed in the control group and in the ischemic group when compared to the other groups, which may suggest a possible involvement of this miRNA with physical exercise, which corroborates many studies in the literature (Ma?czyn´ska *et al*., 2019). The low expression levels of miR-126 observed in our study in the ischemic group corroborated the findings of Chen *et al*., who also found hypoexpression of this miRNA in serum and heart in an experimental model of ischemia (Chen *et al*., 2016). Based on the study by Chen *et al*. (2016) as well as the findings of our previous study by Silva *et al*. (2019) we can suggest that miR-126 presents an expression pattern not directly associated with the ischemic focus but with the nervous tissue generally and even systemically.

MiR-133b is considered a skeletal muscle-specific miRNA, and its expression is altered according to the type and intensity of physical training, as well as other myomiRNAs (Banzet *et al*., 2013; Silva *et al*., 2017). Dai *et al*. (2021) found miR-133b underexpressed in an in vitro model of neuronal cells from the HT22 mouse hippocampus with oxygen and glucose depletion, and in an in vivo model of C57BL/6J mice submitted to middle cerebral artery occlusion compared to the sham group. Our results, despite not showing a significant statistical difference, do not corroborate the work of Dai *et al*. (2021) because it was possible to observe an increase in the expression of miR-133b in the physical exercise, ischemic and physical exercise groups associated with ischemia compared to the control group. Therefore, we can suggest that possibly the difference in the animal model used, may influence and that further studies need to be carried out in order to investigate the potential of miR-133b triggered by physical exercise associated with cerebral ischemia as proposed by Huang *et al*. (2017).

Clinical data demonstrate that miR-221 levels are reduced in patients with cerebral ischemia, indicating that this miRNA is a promising biomarker and a possible therapeutic target in stroke patients (Tsai *et al*., 2013; Sørensen *et al*., 2014; Peng *et al*., 2020; Shan *et al*., 2021). In the animal model of MCAO, Shan *et al*. (2021) also observed reduced levels of miR-221 expression in mice. On the other hand, in this same study it was shown that pretreatment with a miR-221 mimic, injected intracerebroventricularly, reduced the volume of cerebral infarction and improved behavioral deficits in this animal model (Shan *et al*., 2021).

Unlike previous studies cited, in our protocol we used the ischemia-reperfusion model with 60-minute occlusion and 24-hour reperfusion of the MCA (Tsai *et al*., 2013; Sørensen *et al*., 2014; Peng *et al*., 2020; Shan *et al*., 2021). Thus, it is possible that reperfusion was the factor responsible for the elevation of miR-221 expression, in order to promote a neuroprotective effect through the suppression of the inflammatory response. Similarly, physical exercise promoted a significant increase in miR-221 levels when compared to the control group, but no additional increase was detected in the ischemic group associated with exercise compared to the ischemic group. These findings support our hypothesis that reperfusion was the factor that promoted the highest expression of miR-221, as well as physical exercise. Recently, Peng *et al*. (2020) demonstrated that miR-221 is involved in modulating endothelial cell function. The authors demonstrated that the inhibition of miR-221 reduced the viability, migration and invasiveness and promoted apoptosis of human endothelial cells in the umbilical vein, while the increase in miR-221 promoted opposite effects through the regulation of the PTEN/PI3K/ pathway. AKT. Knowing this, it is possible to infer that the function of endothelial cells

may also play a regulatory role in the expression of miR-221. The increase in blood flow during cerebral reperfusion and physical exercise, by promoting the stretching of endothelial cells caused by blood cell friction (shear stress) may have been a factor that stimulated the increase in miR-221 levels observed in our study (Peng *et al*., 2020). In fact, other studies have already shown that physical exercise modulates the expression of mir-221, increasing its levels in various pathological situations, such as cardiovascular diseases (Souza *et al*., 2015; Barber *et al*., 2019).

In the analysis comparing the expression levels between the miRNAs, we also did not observe statistically significant differences, but miR-126 was more expressed in the control than miR-133b and miR-221, which was not observed in the other groups and we can suggest a pattern of hypoexpression of this miRNA as observed in the literature.

Although we did not observe any statistical difference, there was greater expression of miR-221 miRNA in the physical exercise group, which can also be observed slightly in the physical exercise group associated with cerebral ischemia, which leads us to suggest the revealing involvement of the modulation of this miRNA associated with physical exercise. The same expression pattern associated with the exercise groups was also observed for miR-126 also without statistical difference. Which leads us to suggest a possible specificity of these miRNAs with physical exercise.

CONCLUSION

No statistically significant difference in miR-126 and miR-133b was found between the groups studied; however, miR-126 presented hypoexpression in the control group and in the ischemic group when compared to the other groups, which may suggest a possible involvement of this miRNA with physical exercise. MiR-221 showed statistically significant differences between the control group and the other groups. Our hypothesis is that reperfusion was the factor that promoted the highest expression of miR-221, as well as physical exercise. Nevertheless, further studies are needed to identify microRNAs with potential use in clinical practice.

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RESUMEN: El ictus es una de las principales causas de muerte y discapacidad en todo el mundo. El gran impacto en la calidad de vida de la población y en el sistema de salud justifica buscar alternativas pertinentes para reducir la incidencia y mejorar el tratamiento y recuperación de los pacientes afectados por esta enfermedad. El ejercicio físico aparece como una herramienta importante en este escenario, siendo ya señalado como un posible abordaje terapéutico para la prevención de enfermedades crónicas no contagiosas. En este contexto, aparecen biomarcadores como los miRNAs que responden al ejercicio físico y están directamente relacionados con varios mecanismos epigenéticos. Por lo tanto, explicar los mecanismos moleculares involucrados durante el ejercicio físico conducirá a una mejor comprensión de cada estímulo y la dosis a utilizar para responder mejor a cada situación, siendo así un enfoque prometedor para la evolución de la prescripción, el control del entrenamiento y los procesos de recuperación de diversas enfermedades, incluido el accidente cerebrovascular. Se utilizaron cuarenta y ocho ratas Wistar, divididas en cuatro grupos experimentales: grupo control, grupo isquemia, grupo ejercicio físico y grupo ejercicio + isquemia. Se utilizó la metodología de PCR en tiempo real para analizar la expresión de miRNAs: miR-126, miR-133b y miR-221. En nuestro estudio observamos una diferencia significativa en la expresión de miR-221 entre el grupo control y los demás grupos. Sin embargo, los microARN: miR-126 y miR-133b no mostraron diferencias significativas en la expresión entre grupos.

PALABRAS CLAVE: Isquemia cerebral; Ejercicio físico; microARN; Apoptosis.

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Corresponding author:
Rafael Zimak Figueiredo
Ribeirao Preto Medical School
University of Sao Paulo
Department of Surgery and Anatomy
Av. Bandeirantes, 3900
Monte Alegre
Ribeirao Preto
São Paulo 14048-900
BRAZIL

E-mail: lab.biomol.cirurgia@fmrp.usp.br