DIFFERENT INTENSITIES OF TREADMILL RUNNING EXERCISE DO NOT ALTER MELATONIN LEVELS IN RATS

DIFERENTES INTENSIDADES DE EXERCÍCIO DE CORRIDA EM ESTEIRA NÃO ALTERAM OS NÍVEIS DE MELATONINA EM RATOS

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ABSTRACT

Background: Regular and moderate exercise has been considered an interesting neuroprotective strategy. Our research group demonstrated that a protocol of moderate exercise on a treadmill reduced, while a protocol of high-intensity exercise increased *in vitro* ischemic cell damage in Wistar rats. The molecular mechanisms by which physical exercise exerts neuroprotective effects remain unclear. Accumulating evidence suggests that exercise may have short- and long-term effects on melatonin secretion in humans. Melatonin, the main product of the pineal gland, has been shown to have neuroprotective effects in models of brain and spinal cord injury and cerebral ischemia. A dual modulation of melatonin secretion by physical activity has also been demonstrated. This study aimed to investigate the effect of different exercise intensities, moderate- and high-intensity exercise, on serum melatonin levels in rats.
Methods: Thirty-five adult male Wistar rats were divided into non-exercised (sedentary) and exercised (20- or 60-min sessions) groups. The exercise protocols consisted of two weeks of daily treadmill training. Blood samples were collected approximately 16 hours after the last training session (8:00-10:00) and melatonin levels were assayed by ELISA.
Results: The exercise protocols, two weeks of 20 min/day or 60 min/day of treadmill running, did not affect serum melatonin levels.
Conclusion: Our data demonstrated that melatonin levels may not be directly involved in the exercise-induced, intensity-dependent dual effect on *in vitro* ischemia.

Keywords: *Exercise; melatonin; serum; treadmill; rats*

RESUMO

Introdução: O exercício físico regular e moderado é considerado uma interessante estratégia neuroprotetora. Nosso grupo de pesquisa demonstrou que um protocolo de exercício moderado em esteira reduziu, enquanto um protocolo de intensidade elevada aumentou o dano isquêmico *in vitro* em ratos Wistar. Os mecanismos moleculares pelos quais o exercício físico exerce neuroproteção ainda não estão claramente elucidados. Várias evidências sugerem que o exercício pode ter efeitos a curto e longo prazo sobre a secreção de melatonina em humanos. A melatonina, o principal produto da glândula pineal, tem demonstrado exercer uma atividade neuroprotetora em modelos de trauma cerebral e medular e isquemia cerebral. Foi demonstrada também uma modulação dual da atividade física sobre a secreção de melatonina. O objetivo deste estudo foi estudar o efeito de diferentes intensidades de exercício, moderado e elevado, sobre os níveis séricos de melatonina de ratos.
Métodos: Trinta e cinco ratos Wistar adultos machos foram divididos em não-exercitados (sedentários) ou exercitados (sessões de 20 ou 60 min). Os protocolos de exercício consistiam em duas semanas diárias de treinamento em esteira ergométrica. Os soros foram coletados aproximadamente 16 horas após a última sessão de treino (8:00-10:00) e os níveis de melatonina foram avaliados através da técnica de ELISA.
Resultados: Os protocolos de exercício físico, duas semanas de 20 min/dia ou 60 min/dia de corrida em esteira, não alteraram os níveis séricos de melatonina.
Conclusão: Nossos dados demonstram que os níveis de melatonina parecem não estar diretamente envolvidos com o efeito dual do exercício físico dependente da intensidade na isquemia *in vitro*.

Palavras-chave: *Exercício; melatonina; soro; esteira; ratos*

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Regular physical activity has been indicated as a therapeutic approach to prevent or recover from age-related neurodegenerative diseases (1). The exact molecular mechanisms by which physical exercise affects brain function remain unclear; however, exercise has been suggested to activate cellular and molecular pathways that contribute to neuroprotection. Experimental models have demonstrated neuroprotection in both global and focal ischemia models (2;3), in addition to enhancing neurogenesis, synaptic plasticity, and spatial memory (4).

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Interestingly, our previous results showed that a moderate-intensity exercise training protocol (two weeks of daily 20-min training sessions) reduced, while a protocol of high-intensity exercise (two weeks of daily 60-min training sessions) increased *in vitro* cell damage in the hippocampus of animals forced to run on a treadmill (5).

Recently, we have demonstrated that brain-derived neurotrophic factor (BDNF) content and oxidative status may not be directly involved in the mechanisms of neuroprotection induced by two weeks of daily 20-min treadmill training (6). In addition, this protocol significantly reduced adenosine diphosphate (ADP) hydrolysis, with a trend to reduce adenosine triphosphate (ATP) hydrolysis in both hippocampal synaptosomes and blood serum of rats (7). It is therefore reasonable to investigate other possible biochemical mechanisms involved in exercise-induced neuroprotection.

Melatonin levels have also been shown to be modulated by physical activity (8,9). Accumulating evidence suggests that exercise may have short- and long-term effects on melatonin secretion in humans. Melatonin is a neurohormone produced mainly by the pineal gland that has diverse effects on a wide range of physiological functions, such as regulation of circadian and seasonal rhythms (e.g. sleep-wake cycle), acting as an immunostimulator and a cytoprotective agent (10,11). Several studies have demonstrated neuroprotective activity of melatonin in hyperbaric hyperoxia, traumatic brain injury, and ischemia/reperfusion models, among others (12,13). Furthermore, the pineal hormone melatonin is reported to have free radical scavenging properties, including scavenging activity against hydroxyl radicals (14-16), and to reduce lipid peroxidation related to the integrity of cell membranes (17).

Current evidence supports an exercise-induced modulation of cellular oxidative status. Although controversy exists regarding the effects of exercise on oxidative stress, such as the fact that exercise-induced free radical generation may be detrimental to cell function, regular exercise has been suggested to induce adaptations of the cellular antioxidant defense system (18,19). It is possible that this modulation may be related at least in part to pineal melatonin secretion.

A dual modulation of melatonin secretion by physical activity has been demonstrated, since high-intensity training has been shown to reduce the secretion of pineal melatonin (20). Moreover, our previous results showed intensity-dependent effects of exercise on *in vitro* ischemic injury (5).

Although current evidence indicates that melatonin secretion is modulated by exercise and that melatonin has neuroprotective properties, studies have not investigated the involvement of melatonin levels in exercise-induced neuroprotection. In order to expand our previous results, this study aimed to investigate the effect of different intensities of treadmill running exercise on serum melatonin levels in rats.

METHODS

Thirty-five male Wistar rats aged 2-3 months were used in the experiment. The animals were housed in groups of five in Plexiglass home cages (65 x 25 x 15 cm) under standard conditions, a 12-hour light-dark cycle (LD 12:12) [lights on at 07:00, Zeitgeber time (ZT) 0, and lights off at 19:00, ZT12], in a temperature controlled environment (22±2°C), with rat chow and water available *ad libitum*. All animal experiments were approved by the Animal Care and Use Committee of the institution (GPPG No. 08-155).

Training

Rats were familiarized with the treadmill apparatus to minimize novelty stress and then randomly divided into two groups: non-exercised or sedentary (SED) and exercised (EXE) groups. The EXE group was subjected to treadmill running once a day for 20 min (EXE 20; N =12) or 60 min (EXE 60; N =9) during two weeks. The SED (N =14) group was handled exactly as were the experimental animals, except that they were maintained on the turned-off treadmill for 5 min (5).

Exercise training consisted of running sessions on an adapted motorized rodent treadmill at 60% of maximal oxygen uptake (21). Peak oxygen uptake (VO_2) was indirectly measured in all animals before training considering exhaustion. All rats ran on the treadmill at a low initial speed followed by 5 m/min increases in speed every 3 min until the point of exhaustion (i.e., failure of the rats to continue running). Time to fatigue (min) and workload (m/min) were used as exercise capacity indexes, expressed as VO_2 max (21,22). Selected animals that initially refused to run were encouraged by gently tapping their backs. Neither electric shock nor physical prodding was used in this study. Rats were scheduled to run once daily during the light cycle between 15:00 and 18:00 (ZT8 and ZT11, respectively) for two weeks. During the first days, the 20-min group adapted to the treadmill by running at a speed of 6.7 m/min for the first 2 min, 10 m/min for 4 min, 12 m/min for 8 min, 10 m/min for 4 min, and 6.7 m/min for the last 2 min. Finally, animals were running at 6.7 m/min for the first 4 min, 12 m/min for 12 min, and 6.7 m/min for the last 4 min. The 60-min group adapted to the treadmill by running at 6.7 m/min for the first 6 min, 10 m/min for 12 min, 12 m/min for 24 min, 10 m/min for 12 min, and 6.7 m/min

for the last 6 min. Finally, animals were running at 6.7 m/min for the first 12 min, 12 m/min for 36 min, and 6.7 m/min for the last 12 min.

Sample preparation

Rats were killed by decapitation approximately 16 hours after the last treadmill running session. This protocol was based on our previous finding that exercise reduces hippocampal susceptibility to *in vitro* ischemia (5;6). Trunk blood was drawn in the morning between 08:00 (ZT1) and 10:00 (ZT3). Considering that rats are nocturnal rodents, ZT0 corresponds approximately to the beginning of their resting phase, whereas ZT12 corresponds to the beginning of the active phase. Blood samples were centrifuged in plastic tubes at 5000 xg for 5 min at room temperature. Serum was obtained and frozen at −70 °C until assayed.

Serum melatonin assays

Serum melatonin was assayed by ELISA, using commercial kits purchased from MP Biomedicals, Inc (Irvine, California, USA). The procedure followed the basic principle of competitive immunoassay. In brief, 50 µL aliquots of samples, controls, and standards were micropipetted into their corresponding plate wells, followed by adding 50 µL of melatonin-biotin, then 50 µL melatonin antiserum, and overnight incubation at 2-8°C. Next day, three washes with buffer were followed by incubation with 150 µL of enzyme conjugate for 120 min at room temperature, then three washing cycles with buffer and incubation with 200 µL of PNPP substrate for 20-40 min at room temperature, and terminated by adding stop solution. ELISA analyzer absorbance readings were taken at 405 nm. Sample melatonin concentrations were calculated using their average optical density based on the kit standard curves. The detection limit of the assay was 300 pg/mL.

Statistical analysis

Data were expressed as mean ± standard deviation. Comparisons between groups were analyzed by one-way ANOVA followed by Tukey's test. SPSS 17.0 for Windows was used for statistical analysis. Significance level was set at $P<0.05$.

RESULTS

One-way ANOVA revealed no significant differences in serum melatonin levels between exercised (two weeks of daily 20- or 60-min treadmill running) and sedentary groups (Figure 1).

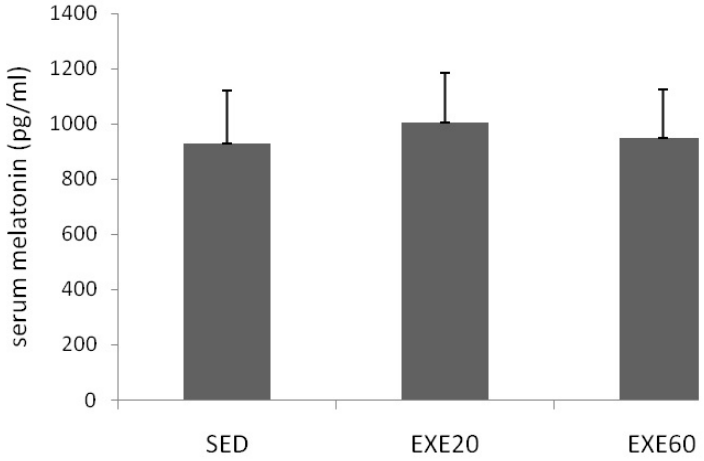

Figure 1 - Effects of daily 20- or 60-min treadmill running sessions on serum melatonin levels. Columns represent the mean ± SD of 35 animals (pg/mL for melatonin). Number of animals/group: 20-min exercise group (EXE 20, N=12); 60-min exercise group (EXE 60, N=9); sedentary group (SED, N=14). There were no differences between groups; one-way ANOVA, P >0.05.

DISCUSSION

The present study did not corroborate the hypothesis that physical exercise affects melatonin levels. Moderate-intensity exercise (two weeks of 20 min/day) and high-intensity exercise (two weeks of 60 min/day), which had shown, respectively, neuroprotective effects and exacerbation of *in vitro* ischemic damage (5), did not alter serum melatonin levels in rats.

In a previous study, animals subjected to a single session of restraint stress and assessed immediately after stress showed a phase delay of the melatonin rhythm immediately after stress,

surpassing peak melatonin levels to be observed at ZT0 (early morning), and a loss of rhythmic pattern 6 and 24 hours after stress (Souza, personal communication). Thus, the stress component may be considered a potential confounding factor in the animal exercise model used here, since animals were forced to run on a motorized treadmill. However, it is worth noting that, although forced exercise is more similar to human exercise performance, most studies on physical training employ voluntary exercise as a model (23).

Human studies have shown that melatonin is also modulated by physical activity, but the results are controversial. Increased plasma melatonin levels were reported after a bout of physical activity, returning to pre-exercise levels 1 hour after physical exertion (9). After an 8-week physical training program, an increase in plasma melatonin levels was observed before and after exercise when compared to levels at training onset (8). Similarly, high daytime baseline plasma melatonin levels were observed in adolescents with an average of 3–5 years of training, when compared with baseline levels of non-athletic adolescents (24). However, some reports have not confirmed the above findings (25,26). Furthermore, vigorous exercise training has been suggested to reduce plasma melatonin levels (20,27). Despite conflicting results in the literature, in general, data show long-term changes in exercise-induced melatonin secretion.

Interestingly, both exercise and melatonin have dual effects, since moderate-intensity exercise and low-dose melatonin administration appear to exert neuroprotective effects, while training at a higher intensity or high-dose melatonin may exacerbate cell damage (5,28).

Our finding that physical exercise does not alter melatonin levels fails to corroborate data from other studies, indicating that melatonin secretion may not be related to the effects of moderate- and high-intensity exercise. Therefore, other molecular mechanisms cannot be ruled out.

One study showed that swimming induced a significant reduction in the melatonin content in the rat pineal gland; however, the activity of N-acetyltransferase, the supposed rate limiting enzyme in the melatonin production, was not changed. Similar results were observed under light exposure, since melatonin levels in the pineal and serum of light exposed rats decreased, although this drop was accompanied by a suppression of N-acetyltransferase activity (26).

The physiological role of melatonin in both circadian and seasonal rhythms may be related to our findings, since some reports have demonstrated circadian phase-dependent effects of exercise on melatonin secretion in humans (25,29). Also, in humans, short episodes of high-intensity exercise late in the evening appear to be associated with a blunting of the melatonin nocturnal rise, persisting until the early morning hours (30). Conversely, the presence and nature of both acute and delayed effects appear to be dependent on the duration, intensity, and type of exercise. Regardless of intensity, exercise near the offset of melatonin secretion or during the daytime has no consistent acute effect on melatonin secretion (31).

Several limitations warrant caution in interpreting our findings. First, although the aim of the study was to investigate the effect on melatonin of different intensities of a 2-week treadmill running protocol that had previously shown neuroprotective effects (20-min/day treadmill training) and exacerbation (60-min/day treadmill training) of *in vitro* ischemic damage, it would be more appropriate to investigate the parameters at different running speeds (light, moderate, intense) to better explore the effect of exercise intensity on melatonin. Second, since melatonin was only measured at one time point (16 hours after the last exercise session), measurements at several time points could be suggested as a better strategy, although our experimental design was similar to that described in a previous study (32), when we demonstrated that, at the exact moment of the ischemic episode, neither oxidative stress markers nor BDNF levels were affected by moderate-intensity neuroprotective treadmill exercise. In addition, conflicting interpretations may arise from the fact that melatonin levels are influenced by the circadian rhythm, and our study was conducted during daytime, i.e., animals were killed early in the morning, while training occurred in the afternoon. These limitations highlight the need for future studies.

In conclusion, considering that our exercise protocols, two weeks of daily 20- or 60-min treadmill running, have been implicated in neuroprotection and exacerbation of ischemic damage, we may assume that melatonin levels may not be directly involved in the mechanisms of exercise-induced dual effect on *in vitro* ischemia.

Conflict of Interest

No author or related institution has received any financial benefit from research in this study.

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