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Circadian urinary citrate excretion in a rat model of exercise

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ABSTRACT

Aims: Circadian rhythms are the approximate 24 h biological cycles that function to prepare an organism for daily environmental changes. Circadian rhythms unquestionably play critical roles in metabolic homeostasis and the exercise has emerged as a strong non-photic time cue or zeitgeber in animal models and humans. Numerous studies about the effects of exercise on the citrate synthase activity have been published. Citrate is used to assess energy production or expenditure because it is a substrate of the Krebs Cycle, a cycle for oxidative energy production.
Main methods: We tested the existence of a rhythmic urinary citrate excretion in a rat model that is made to exercise at six different points during the day.
Key findings: The data obtained by the enzyme assays were fitted to a mathematical model (Fourier series), showing for the first time, the existence of a distinct ultradian rhythm in the urinary citrate excretion. The aerobic exercise led to the increase in the period length of the ultradian rhythm and raised the acrophase value of the urinary citrate excretion. Therefore, the urinary citrate excretion pattern changed after exercise, showing a clear circadian rhythm fitted to the mathematical model.
Significance: The citrate urine samples could provide accurate data for ranking an individual's metabolic status. Using exercise to maintain the circadian clock at an appropriate phase and amplitude might be effective to prevent cardiometabolic disease development.

1. Introduction

Rhythms in metabolism are obvious at several stages, likely enhancing performance and homeostasis during daily fluctuations in energetic demands [1]. Circadian rhythm entrainment has long been assumed to depend absolutely on periodic cues in the external environment. However, new data proposes that appropriately timed activity may also phase shift the circadian clock. Rodent and human studies have established whether levels of exercise/activity associated with spontaneous behavior provided sufficient feedback to phase shift or synchronize circadian rhythms [2]. Physical exercise training has been shown to promote anti-diabetic and anti-atherogenic responses, but the role of the circadian clock-machinery remains unknown [3].

Citrate is one of the intermediates of the Krebs Cycle, an essential cycle for oxidative energy production. Citrate is synthesised from oxaloacetate and acetyl CoA, the reaction of which is catalyzed by the citrate synthase (CS) and inhibited allosterically by adenosine triphosphate (ATP) [4]. The oxidative CS activity is a marker of mitochondrial density [5], and exercise training has been shown to increase CS activity, which since then has been reproduced several times with various types of training [6]. An increased CS activity results in more citrate synthesis and hence increased urinary citrate excretion [7,8].

The purpose of this study was to test the urinary citrate excretion rhythm, as well as the exercise's influence on it, using a rat model. The disruption of circadian rhythms was correlated to an augmented incidence of metabolic disease, subsequently the citrate urine samples could provide accurate data for ranking an individual's metabolic status. Using exercise to maintain the circadian clock at an appropriate phase and amplitude might be effective for preventing a cardiometabolic disease development.

2. Methods

2.1. Animals

Adult male Wistar rats (350–400 g, aged 3 months, $n = 10$) were housed individually in metabolism cages on a 12:12 light:dark cycle (light on 23:00 to 11:00) under standard conditions. The metabolic cages are used to completely separate the faeces and urine, with minimal contact and minimal evaporation of urine due to the proximity of the storage vessel and the lack of exogenous air currents. They had free access to water and food (Teklad Global 14%, Harlan). All rats

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were housed under laboratory conditions for at least 15 days. All experimental procedures followed the guidelines set forth in the Care and Use of Animals and The University of Oviedo's Experimental Animal Use Committee approved all experimental procedures.

2.2. Training program

Rats were trained to run on a motor-driven wheel. The animals were familiarized with the wheel for at least 10 days. The chosen experimental aerobic exercise workload was based on data from a previous study [9]. The protocol consisted of a 30 min graded running trial in which each rat initially ran at a speed of 6.6 m/min and increased progressively up to 15.4 m/min. This physical exercise of low to moderate intensity depends primarily on the aerobic energy-generating process. At the completion of the last running trial, all rats were returned to their individual clear cages outfitted to separately collect urine. The exercise stimulus was performed at the end of the light phase, at this time the animals are more disposed to do exercise as the mechanisms involved in the onset of activity. The circadian patterns were assessed 24 h before to start the training program and 24 h immediately after the last trial.

2.3. Urine collection and citric acid determination

Urines were collected at 4-h intervals (11:00–15:00, 15:00–19:00, 19:00–23:00, 23:00–03:00, 03:00–07:00, 07:00–11:00). Each rat served as its own control (before exercise versus after exercise). Samples were centrifuged for purification and frozen until analysis. Citric acid was determined using Boerhinger Mannheim commercial kits. The method described [4] is based on the absorbance determination of NADH at the wavelength of 340 nm at two different times. With precision in a double determination using one sample solution, a difference of 0.005–0.010 absorbance units may occur.

2.4. Mathematical model

Fourier series [10] were used to study citrate urinary excretion rhythm. A Fourier series of a function $f(x)$ is defined in general as:

$$f(x) \approx \frac{a_0}{2} + \sum_{k=1}^{\infty} (a_k \cos(kwx) + b_k \sin(kwx))$$

The coefficients a_0, a_k and b_k ($k \geq 1$) are called Fourier coefficients, w is the frequency. The periodic functions are obtained by Curve fitting toolbox provided by Matlab R2013 [11]. The goodness of the fitted functions is measured in terms of SSE and R-Square. A SSE value closer to 0 indicates that the model has a smaller random error component, so the fit will be more useful for prediction. R-Square can take on any value between 0 and 1, values closer to 1 indicate that the model accounts for a greater proportion of variance.

2.5. Statistical analysis

All data shown are expressed as mean (±S.E.M.). ANOVA and the Tukey tests were used for a post analyzed of variance and paired Student's *t*-tests were used to calculate the difference within each before-and-after exercise pair of measurements. A statistical package (SPSS version 19.0, IBM Inc., Chicago, Illinois, USA) was used to perform all statistical analyses and $p < 0.05$ was considered significant for all of the tests performed.

3. Results

The circadian patterns were assessed 24 h before (Fig. 1) and immediately after the last trial (Fig. 2) at six different points during the day. Total 24 h post-exercise urine citrate concentration (9.08 ± 1.23 mg/L/24 h) was significantly higher ($p < 0.05$) than total 24 h pre-exercise urine citrate concentration (5.94 ± 0.58 mg/L/24 h). However, if we compare pre- versus post-exercise data, post-exercise levels only were significantly higher ($p < 0.05$) than pre-exercise levels during the sixth interval (07–11 h). The urine citrate peak (acrophase) occurred between 23 and 03 h (acrophase), and the minimum values (batiphase) occurred between 07 and 11 h (pre-exercise) and 11–15 h (post-exercise). The urinary citrate excretion before exercise was similar for all times (Fig. 1) but after exercise (Fig. 2), urine citrate values were significantly lower ($p < 0.01$) at first interval (11–15 h) than those at second (15–19 h), third (19–23 h), fourth (23 h − 03 h) and fifth (03–07 h) periods. In Figs. 1 and 2, the data fitted to the theoretical curves show a SEE of 0.1327 ($R^2 = 0.9862$) and a SEE of 1.497 ($R^2 = 0.9753$) respectively, therefore its predictability is valid. Thus, the proposed model is able to explain the 100% of the variance of the pre- and post-exercise data.

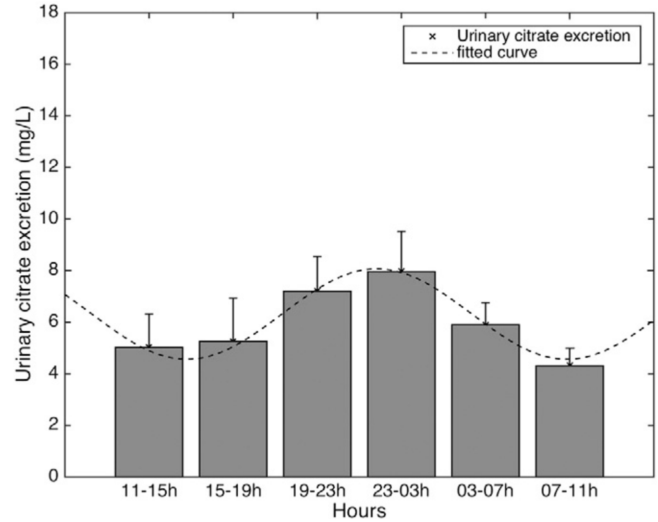

Fig. 1. The histogram shows the analysis of the urinary citrate excretion levels (mg/L) before exercise (control) at 4-h intervals (11–15 h, 15–19 h, 19–23 h, 23–03 h, 03–07 h, 07–11 h) of the day. The curve shows the rhythm expression of urinary citrate excretion fitted by Fourier analysis. $n = 10$ animals. Results are expressed as the mean ± S.E.M.

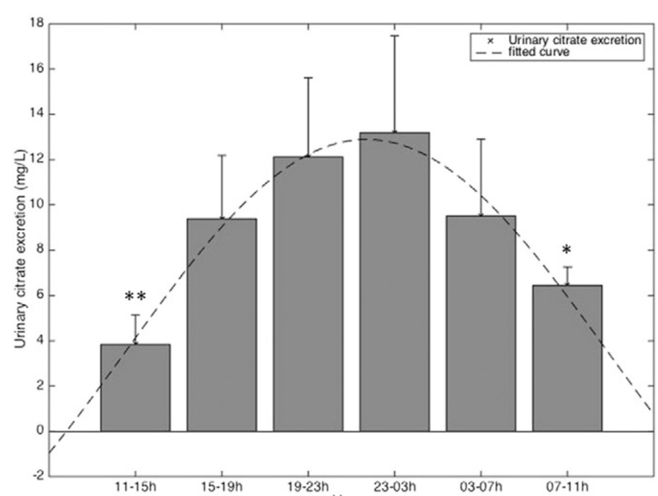

Fig. 2. The histogram shows the analysis of the urinary citrate excretion levels (mg/L) after exercise (post-exercise) at 4-h intervals (11–15 h, 15–19 h, 19–23 h, 23–03 h, 03–07 h, 07–11 h) of the day. The curve shows the rhythm expression of urinary citrate excretion fitted by Fourier analysis. $n = 10$ animals. Results are expressed as the mean ± S.E.M. *$P < 0.05$ post-exercise vs. pre-exercise data (Fig. 1) at the sixth interval (07–11 h). **$P < 0.01$, first interval (11–15 h) vs. second (15–19 h), third (19–23 h), fourth (23 h–03 h) and fifth (03–07 h) intervals.