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Altered circadian rhythms of corticosterone, melatonin, and phagocytic activity in response to stress in rats

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Abstract

Corticosterone is thought to be the main glucocorticoid secreted in response to stressful exercise, while melatonin buffers the adverse immunological effects of stress. The present work was aimed to evaluate whether swimming-exercise-induced stress leads to changes in the chronobiology parameters of the circadian rhythms of melatonin and corticosterone, and in the number and phagocytosis of peritoneal macrophages in 3-month-old male Wistar rats. The animals were subjected to a physical activity trial consisting of 2 h of free swimming. Radioimmunoassay was used to determine the plasma levels of melatonin and corticosterone. Phagocytosis was measured by the latex-bead phagocytosis index (PI), i.e., the number of latex beads ingested by 100 macrophages, the phagocytosis percentage (PP), i.e., the percentage of cells that had phagocytosed at least one latex bead, and the phagocytosis efficiency (PE), i.e., the ratio PI:PP which indicates how effectively the phagocytes ingested the particles. Stress significantly decreased the MESOR and amplitude of the melatonin rhythm, and significantly increased the MESOR of the corticosterone rhythm. The control animals' peritoneal macrophage number and PI showed a circadian rhythm with maxima at 02:00 and 03:00, respectively. The stressed group displayed higher values of PI than the controls at most hours of the night, but the number of cells in the peritoneal cavity was practically the same at all hours studied. These data confirm that melatonin and corticosterone act as modulators of the innate immune response, and that the circadian rhythm of the two hormones are altered in situations of stress.

ORIGINAL ARTICLE

INTRODUCTION

A retino-hypothalamic-sympathetic pathway drives the nocturnal surge of pineal melatonin production that determines the synchronization of pineal function with the environmental light/dark cycle. The increase in the circulating level of nocturnal melatonin is the hormonal signal that it is night. In this way, melatonin regulates numerous physiological processes, including being intimately connected with the immune system [7]. The immune system interacts with the hypothalamic-pituitary-adrenal axis via the so-called glucocorticoid increasing factors, which are produced by the immune system during immune reactions, causing an elevation of systemic glucocorticoid levels that contribute to preservation of the immune reaction specificities [15]. Corticosterone – the principal glucocorticoid secreted in rats [13,8] – and melatonin are true internal chemical pacemakers of different physiological processes, with both hormones having immunoregulatory effects and a circadian rhythm in most vertebrates [28,1]. Melatonin, due to its physiological antioxidant properties, acts as a buffer in stressful situations by quenching the adverse immunological effects of stress by way of its circadian release [17]. In situations of stress, there is a change in the secretion rates of the two hormones. This leads to alterations in others of the organism's circadian rhythms, causing serious disorders for health [35,4]. There is much speculation about the relationship between melatonin's role in the activity of the hypothalamic-pituitary-adrenal system and adrenocortical glucocorticoid secretion, with some workers finding no evidence for a physiological coupling between melatonin and glucocorticoids [14], and others suggesting that melatonin may act as a major regulator of adrenal function [6,30]. It is common belief that, in order to understand the possible mechanisms underlying the immune response to exercise, the role of neuroendocrine factors must be studied, since the plasma concentration of many immunomodulatory hormones rises during exercise. Thus, the aim of the present work was to look deeper into the connections between the rhythms of melatonin, corticosterone, and the non-specific immune response, as well as to study the alterations that affect these connections in conditions of stress. To this end, we determined in Wistar rats – a nocturnally active animal – the circadian rhythms of melatonin and corticosterone in the plasma, and the number and phagocytic activity of peritoneal macrophages. The latter are cells that play an important role in the defence against infection, probably preventing the entry and maintenance of the antigen in situations where the specific immune response seems to be depressed [22], both in control individuals and in those subjected to a situation of stress by means of a model of physical activity consisting of an episode of forced swimming [34,22,35,2].

MATERIALS AND METHODS

Subjects

The trials were performed on male (340 animals) Wistar rats (*Rattus norvegicus*), aged 12±2 weeks, weighing 450±50 g, purchased from the University of Extremadura Animal Service, maintained at a constant temperature (20±2 °C), and fed on "Panlab" meal and water *ad libitum*. The animals were housed, two to a cage, in 500×250×150 mm "Panlab" cages in a 2.86×3.80×2.85 m room, with artificial lighting, indirect ventilation, and at 50% relative humidity. They were kept under a 12/12h light/dark cycle (lights out at 20:00). They were examined before the trials, and only those with no signs of pathology were used.

The animals were divided into two experimental groups: control group (not subjected to any physical activity, but allowed exercise voluntarily performed in their cages), and stress group (subjected to forced swimming physical activity stress). The experimental protocol was carried out under the guidelines of the Ethical Committee of the University of Extremadura (Spain) and was in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the European Community Council directives (86/609/EEC).

Acute physical activity

A classical model of stress induced by physical activity was employed: forced prolonged swimming [34,22,35,2] to which the rats were subjected once. Rats were put individually into a 50×60×60 cm tank full of clean water at 37 °C and made to swim continuously for 2 h. At that point, they were immediately removed from the tank. Control rats were maintained under similar environmental conditions to the previous group but were not subjected to exercise stress. In the nocturnal period, the experiments were conducted under dim red light, perceived as darkness by albino rats [26,12].

Table 1. Cosinor parameters for the plasma corticosterone and melatonin concentrations of the control group and the group subjected to forced swimming physical activity stress.

	Amplitude		MESOR		Acrophase (hours)	
	Corticosterone (ng/mL)	Melatonin (pg/mL)	Corticosterone (ng/mL)	Melatonin (pg/mL)	Corticosterone	Melatonin
Control	59.5±32	46.41±10.2	103.5±17.8	70.67±5.7	22.2±2.2	5.4±0.8
Stress	75.0±46.5	17.7±7.4*	593.9±26.0*	53.6±4.1*	16.0±2.6	7.7±1.6

Each value represents the mean ± SD of 10 determinations. (*) $p<0.05$ with respect to the values obtained in the control.

Plasma collection

After the stressing physical episode, the animals were killed by decapitation and blood from the neck blood vessels was collected in EDTA tubes and centrifugated at room temperature for 15 min at 1100 × g in order to separate the plasma, which was stored frozen (20 °C) until assay. In both groups, the plasma collection was performed at 1-h intervals in the periods from 22:00 to 06:00 and from 16:00 to 18:00, and at 2-h intervals for the rest of the 24-h cycle. The more exhaustive analysis of the first two periods was designed to establish precisely the natural endogenous secretion patterns of the two hormones being studied [23]. In both the control and the stress group the number of animals used for each time point was 10 (n=10).

Determination of plasma corticosterone

Plasma corticosterone levels were determined by means of a radioimmunoassay (RIA kit, DRG Diagnostics International Inc., USA), using ^{125}I-corticosterone (<3 μCi). The results are expressed in ng/mL.

Determination of plasma melatonin

Plasma melatonin levels were determined by means of an RIA kit (IBL Immuno-Biological Laboratories, Hamburg, Germany), using ^{125}I-melatonin (5.5 mL=140 kBq). The results are expressed in pg/mL.

Chronobiological data analysis

For the chronobiological study of melatonin and serotonin in the animals, a mean population cosinor analysis was performed, using the integrated computer software package El Temps (© Antoni Díez-Noguera, University of Barcelona, Spain). Amplitudes (measure of the extent of a rhythmic change in a cycle estimated by the cosine function that approximate the rhythm), MESORs (Midline-Estimating Statistic Of Rhythm – the mean value about which the oscillation occurs, equal to the arithmetic mean of equidistant data covering a whole number of cycles), and acrophases (a phase angle, measure of the timing of the peak activity, expressed as the lag from a reference time – in our study lights off – to the crest time of the cosine function best approximating the data) were calculated for the control and stress conditions.

Collection of peritoneal exudate cells (PECs)

Immediately after recovering the blood for plasma determinations, the peritoneal cavity macrophages were taken for comparison with samples from control animals (the same conditions as the stressed individuals but in the absence of stressor). The abdomen was cleansed with 70% ethanol, the abdominal skin was carefully dissected without opening the peritoneum, and 4 mL of Hank's solution (Sigma Chemical Company, St. Louis, MO, USA) adjusted to a pH 7.4 was injected intraperitoneally. The abdomen was massaged and the peritoneal exudate cells (PECs) removed, with recovery of 90–95% of the injected volume of fluid. The cells were counted and then adjusted to a final concentration of 5×10^5 macrophages/mL in Hank's solution in order to evaluate the different parameters of the phagocytic process. Cell viability was 98±1% as measured by the trypan blue exclusion method.

Phagocytosis assay

The latex phagocytosis assay was carried out following a method described previously (12). Aliquots of 200 μl of the suspension of PEC were incubated (37 °C and 5% CO_2 atmosphere) on culture plates for 30 min, and the adhered monolayer was washed with Hank's solution at 37 °C. Then, 20 μl of latex beads (Sigma, 1.02 mm, diluted to 0.1% in Hank's solution) and 200 μl Hank's solution were added, followed by another 30 min of oven incubation under the same conditions as before. Finally, the samples were fixed and stained with DiffQuick containing methanol (5 min), eosin (five passes), and haematoxylin (five passes). The plaques were rinsed with tap water and dried, followed by counting under oil-immersion phase-contrast microscopy at 100×. The number of particles ingested per 100 macrophages was expressed as the latex-bead phagocytosis index (PI). The percentage of cells that had phagocytosed at least one latex bead was expressed as the phagocytosis percentage (PP). The ratio PI/PP was calculated, giving the phagocytosis efficiency (PE).

Statistical analysis

Data are expressed as mean (X)±standard deviation of the number of determinations carried out in duplicate. The results were analyzed by using the following non-parametric tests: the Friedman two-way ANOVA on ranks (paired samples) and the Kruskal-Wallis one-way ANOVA on ranks (unpaired samples) for multiple comparisons. For the correlation studies, a multiple regression test was performed, taking as statistically significant those data whose coefficient of determination (R^2) was greater than 0.5.

RESULTS

Table 1 lists the cosinor parameters – amplitude, MESOR, and acrophase – for the plasma corticosterone and melatonin concentrations of the control and stress groups. In general, there was a significant decrease ($p<0.05$) in the amplitudes and MESORs of the plasma melatonin levels of the animals subjected to stress, with the amplitude and MESOR of the stressed group being approximately 50% and 25% less than the control values ($p<0.05$). On the other hand, the MESOR of the plasma corticosterone concentration increased roughly five-fold with respect to the control value ($p<0.05$). There were no significant differences between the two groups in the corticosterone amplitude or in the acrophases of either hormone.

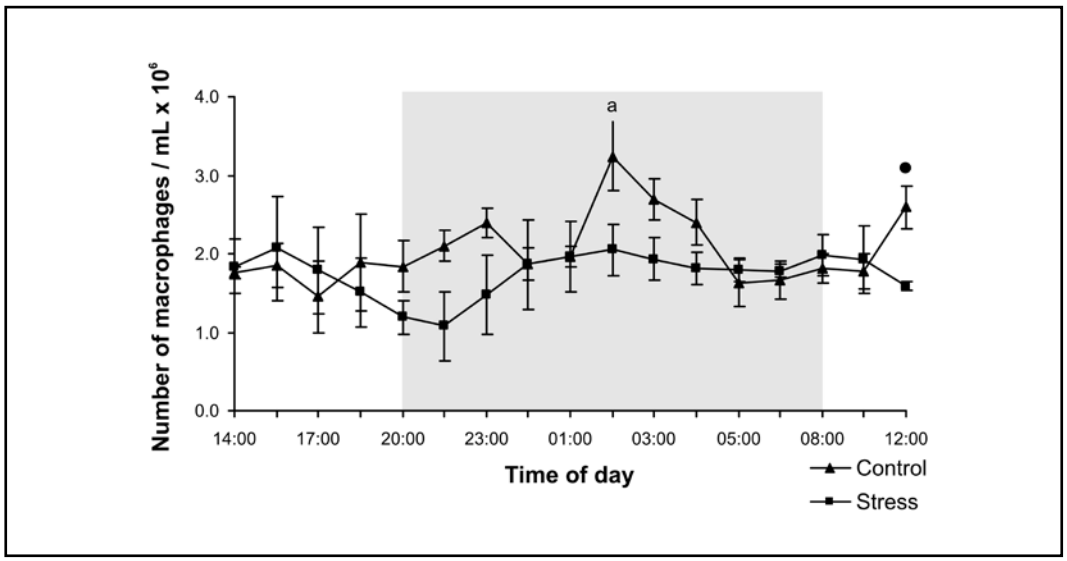

Figure 1. Plot of the number of peritoneal macrophages per millilitre from 3-month-old male Wistar rats – one group of controls and another subjected to stress. Each value represents the mean ± standard deviation of six determinations performed in duplicate. The shaded band represents the determinations made during the period of darkness. (a) $p<0.05$ with respect to the values obtained at 05:00, 06:00, 10:00, 14:00, and 17:00. (•) $p<0.05$ with respect to the values obtained at the same hour in the control group.

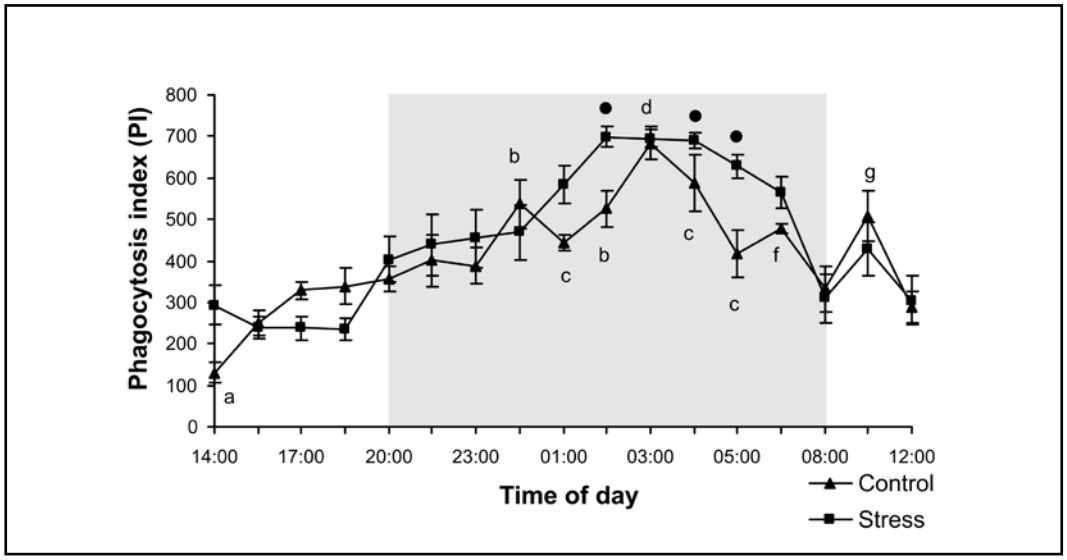

Figure 2. Plot of the phagocytosis index of peritoneal macrophages from 3-month-old male Wistar rats – one group of controls and another subjected to stress. Each value represents the mean ± standard deviation of six determinations performed in duplicate. The shaded band represents the determinations made during the period of darkness. (a) $p<0.05$ with respect to the values obtained at 08:00, 17:00, 18:00, 20:00, 22:00, and 23:00. (b) $p<0.05$ with respect to the values obtained at 08:00, 12:00, 14:00, 16:00, 17:00, 18:00, and 20:00. (c) $p<0.05$ with respect to the values obtained at 14:00 and 16:00. (d) $P<0.05$ with respect to the values obtained at 01:00, 05:00, 06:00, 08:00, 10:00, 12:00, 14:00, 16:00, 17:00, 18:00, 20:00, 22:00, and 23:00. (e) $p<0.05$ with respect to the values obtained at 05:00, 06:00, 12:00, 14:00, 16:00, 17:00, 18:00, 20:00, 22:00 and 23:00. (f) $p<0.05$ with respect to the values obtained at 12:00, 14:00, and 16:00. (g) $p<0.05$ with respect to the values obtained at 08:00, 12:00, 14:00, 16:00, 17:00, and 18:00. (•) $p<0.05$ with respect to the values obtained at the same hour in the controll group.

Figure 1 shows the number of macrophages per millilitre in the peritoneal fluid from the control group and the group subjected to stress. There was a peak ($p<0.05$) in the control animals at 02:00 ($3.24\pm0.44\times10^6$ macrophages/mL). The animals subjected to stress showed practically equal values throughout the day.

With respect to the phagocytic process, Figure 2 shows the capacity of the macrophages to ingest antigens (latex beads) as quantified by the phagocytosis index (PI). In the control animals, there was a peak in antigen ingestion (PI=684±40) at 03:00. In the animals subjected to stress, the PI values were greater during the dark period than during the light period, with differences ($p<0.05$) in the values at 02:00 (PI=698±25) and 04:00 (PI=691±19) with respect to the other hours of the day and with respect to their corresponding control group values. Figure 3 shows the values of the phagocytosis percentage (PP). For the control animal group, there

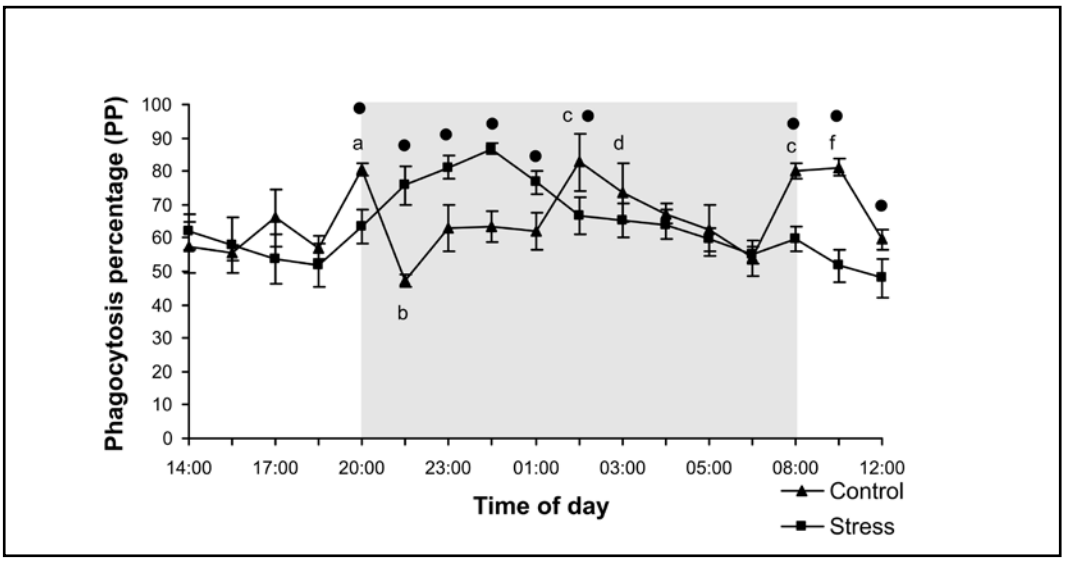

Figure 3. Plot of the phagocytic percentage of peritoneal macrophages from 3-month-old male Wistar rats – one group of controls and another subjected to stress. Each value represents the mean ± standard deviation of six determinations performed in duplicate. The shaded band represents the determinations made during the period of darkness. (a) $p<0.05$ with respect to the values obtained at 06:00, 12:00, 14:00, 16:00, 18:00, and 20:00. (b) $p<0.05$ with respect to the values obtained at 04:00 and 17:00. (c) $p<0.05$ with respect to the values obtained at 00:00, 01:00, 05:00, 06:00, 12:00, 14:00, 16:00, 18:00, 22:00, and 23:00. (d) $p<0.05$ with respect to the values obtained at 06:00 and 22:00. (e) $p<0.05$ with respect to the values obtained at 06:00, 12:00, 14:00, 16:00, 18:00, and 22:00. (f) $p<0.05$ with respect to the values obtained at 01:00, 05:00, 06:00, 12:00, 14:00, 16:00, 18:00, and 22:00. (•) $p<0.05$ with respect to the values obtained at the same hour in the control group.

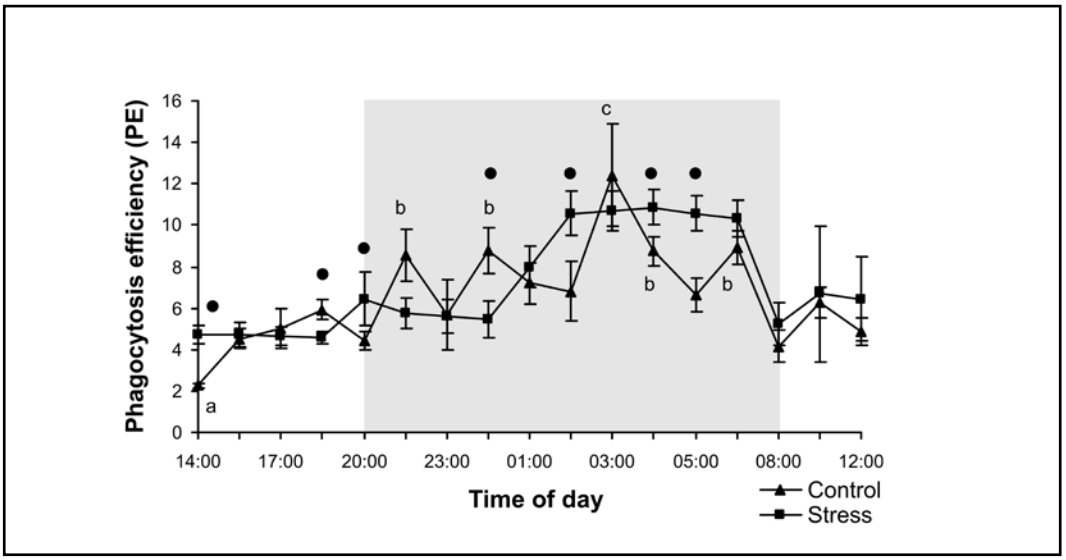

Figure 4. Plot of the phagocytic efficiency of peritoneal macrophages from 3-month-old male Wistar rats – one group of controls and another subjected to stress. Each value represents the mean ± standard deviation of six determinations performed in duplicate. The shaded band represents the determinations made during the period of darkness. (a) $p<0.05$ with respect to the values obtained at 01:00, 2:00, 05:00, 10:00, 18:00, and 23:00. (b) $p<0.05$ with respect to the values obtained at 08:00, 12:00, 14:00, 16:00, 17:00, and 20:00. (c) $p<0.05$ with respect to the values obtained at 00:00, 01:00, 02:00, 04:00, 05:00, 08:00, 10:00, 12:00, 14:00, 16:00, 17:00, 18:00, 20:00, 22:00, and 23:00. (•) $p<0.05$ with respect to the values obtained at the same hour in the control group.

was no clear circadian rhythm in this parameter. For the animals subjected to stress, there was a peak at 00:00 (PP=87±3), the difference being statistically significant with respect to the rest of the values. Finally, Figure 4 shows the values of the phagocytosis efficiency, PE. The highest value corresponded to the control group at 03:00 (PE=11.8±2.5; $p<0.05$).

In the study of the possible correlations between the circadian levels of melatonin or corticosterone and the phagocytosis parameters (number of macrophages and phagocytosis indices), the only significant correlation found was in the control group between the plasma melatonin levels and PI (positive correlation, $R^2=0.54$; see Figure 5).

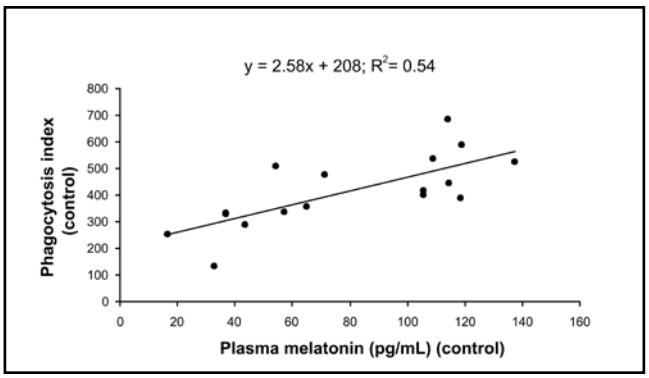

Figure 5. Correlation between the plasma levels of melatonin (pg/mL) and the phagocytosis index for the control animals.

DISCUSSION

Sleep and wakefulness, heart beat, messages between neurons, and body temperature are some of the organism's innumerable rhythmic physiological routines that are under the control of an internal clock. Each of the body's reactions is regulated to occur at the appropriate time in anticipation of foreseeable disturbances.

Melatonin, a hormone secreted mainly by the pineal gland, represents the signal carried by the blood stream that is responsible for coordinating the perfect adjustment of physiological processes [32]. Its circulating levels rise during the night, contrary to the glucocorticoids which peak after dawn in diurnal animals [23] or at dusk in nocturnal animals [35]. Melatonin and corticosterone (cortisol in man) act as circadian pacemakers, presenting an immunoregulatory activity [11,24,29,18].

The parameters of the immune system also present a circadian activity [19,24]. In the present work, we observed that in the control animals the number of macrophages peaked between 02:00 and 04:00, consistent with the results reported by McNulty et al. [19]. According to the hypothesis proposed by Nevid [20], the circadian rhythm of immunity could have an adaptive significance, because an animal is more likely to encounter pathogens when it is moving about the environment in which it lives. Thus, according to this hypothesis, in our nocturnal animal model the greatest number of macrophages and the maximum activation of the phagocytic process would occur at night, and indeed we observed the peak ingestion of antigens (latex beads) to occur at 03:00. In the controls, there was a positive correlation between the peritoneal macrophages' antigen ingesting capacity and plasma melatonin concentrations, with both parameters peaking during the hours of darkness. The maxima of the nocturnal melatonin levels and of the phagocytic activity did not, however, completely coincide in time – in the controls, the former was found to peak at 02:00 and the latter (the number of phagocytosed particles and the PE) at 03:00. This could be because the recently characterized melatonin receptors in the phagocytes [10] do not acquire their maximum expression until some time after their maximum stimulation by the hormone [35].

Situations of stress cause alterations in the rhythm of melatonin and corticosterone secretion, which will in the long term lead to physiological disorders [5]. The particular objective of the present work was to determine how the stress caused by forced swimming may affect the circadian rhythms of melatonin, corticosterone, and phagocytosis in a nocturnal animal model (Wistar rats).

There are many forms of provoking stressing episodes in organisms. Among them, physical exercise is considered to be a good model [31]. In the present work, we used a classical model of physical activity stress – prolonged forced swimming [13,8,21,9]. Corticosterone, the main glucocorticoid in murines [8,31], has a major influence on immune cells [22]. Unlike the case of melatonin whose maximum secretion occurs during the period of darkness in both diurnal animals and our nocturnal animal model, plasma glucocorticoid concentrations rise in anticipation of the onset of the period of motor activity, i.e., during the afternoon in nocturnal species and during the day in diurnal species [24]. This coincides with our determination of the acrophase at approximately 22.2 h after lights out. In the animals subjected to stress, the corticosterone levels presented a loss of rhythmicity, replaced by a generalized increase in the secretion of the hormone at all the times evaluated relative to the control group. Indeed, the corresponding MESOR was five times greater than that of the unstressed animals (Table 1). The phagocytic activity corresponding to the stressed group was also enhanced at most of the hours of darkness, suggesting that the stress-provoked increase in corticosterone levels is accompanied by an increase in the phagocytosis carried out by the peritoneal macrophages [21,9,2]. On the other hand, we observed that the circulating levels of melatonin in situations of stress were lower than in the control situation – the MESOR was lower by a quarter than that of the control group, and the amplitude of the cycle by a half (Table 1). This decrease could be due to different circumstances: that corticosterone inhibits melatonin synthesis [16,27]; that the circulating levels of L-tryptophan decline due to glucocorticoid activation of the enzyme tryptophan 2,3-dioxygenase which catalyzes the opening of the penta-atomic ring of the amino acid to form N-formylkynurenine, and hence lessens the pinealocytes' capacity to form serotonin, the precursor of melatonin [3]; and that the melatonin is used as a buffer to neutralize the free radicals produced by the glucocorticoids in the situation of stress [17]. Indeed, once inside a cell, the neurohormone melatonin can have manifold actions as a scavenger of different reactive oxygen species [25].

In sum, the circadian clock affects the daily course of many physiological processes, creating optimal periods throughout the day for certain activities to be carried out [33]. In normal conditions, our organism reacts to be in harmony with its environment to safeguard its integrity and hence its survival. Situations of stress, however, break

this harmony in the non-specific immune response and in the secretion of key pacemaker hormones, and trigger alterations in homeostasis which in the long term lead to severe damage to the individual's health.

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