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ORIGINAL ARTICLE

Effect of tryptophan administration on circulating levels of melatonin and phagocytic activity

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Summary
Our research group has previously studied the role of melatonin in the immune system of birds and mice, finding that incubation with both pharmacological and physiological doses of melatonin augmented the activity of phagocytes from these animals, and that this activity was lowered in pinealectomized animals. Since melatonin is synthesized from the amino acid tryptophan, the aim of the present work was to determine whether the administration of tryptophan might affect the plasma levels of melatonin and the phagocytic activity of peritoneal macrophages over the course of a circadian cycle. The study animals were 14-week-old male Wistar rats. They were administered tryptophan orally in a daily single dose of 125 mg/kg at 19:00 h for 21 days. Prior to beginning this treatment, the circadian rhythms of plasma melatonin and phagocytic activity were evaluated under basal conditions over a 24-h period, taking blood and cell suspension samples each 2 hours during the light period (08:00−20:00) and each hour during the dark period (20:00−08:00), since it is during this latter period that the secretion of melatonin is maximum. The results showed that, under basal conditions, the rats' plasma melatonin levels and phagocytic activity peaked at 02:00. After the tryptophan administration, there were increases in plasma melatonin levels with respect to basal and control-group values, with a peak at 21:00, and in the phagocytic activity of the peritoneal macrophages, which peaked at 02:00. This suggests that the tryptophan administration stimulated melatonin synthesis, leading to increased and earlier peaking plasma levels of this hormone, and augmented the innate immune response carried out by the peritoneal macrophages as a result of the immunoregulatory action of melatonin.

Keywords: tryptophan – melatonin – immune system – circadian rhythms – rats

INTRODUCTION

Many studies have shown the benefits of the therapeutic use of the hormone melatonin. It helps regulate sleep alterations and jet-lag, enhances the efficacy of cancer treatments by counteracting the adverse effects of chemotherapy (Lissoni et al. 2001), and combats cell-level aging by acting as a free-radical scavenger (Reiter et al. 2000, 2002).

The biosynthesis of melatonin takes place in the pinealocytes present in the pineal gland, beginning with the uptake of the amino acid tryptophan. This is then hydroxylated under the action of the enzyme tryptophan hydroxylase (EC 1.14.16.4) to

5-hydroxytryptophan, which is converted into serotonin by the enzyme aromatic L-amino acid decarboxylase ((EC 4.1.1.28, AADC), then into N-acetylserotonin by N-acetyltransferase (EC 2.3.1.87). It is this enzyme which constitutes the limiting step in melatonin synthesis and which presents a marked circadian rhythm in all species that have been studied (Arendt 1995). The last step is the O-methylation of N-acetylserotonin to melatonin by means of the enzyme hydroxyindole-O-methyltransferase (EC 2.1.1.4). The process is regulated by light signals following a circadian rhythm with maximum levels during darkness and minimum during the light period (Arendt 1995).

The immune system is a physiological process that oscillates following a circadian rhythm (Berger 1988), whose function is defence against the damage caused by microorganisms, foreign molecules, or cancer cells. Melatonin is one of the main factors involved in regulating the circadian rhythm of the immune function, maintaining it with a period of 24 h so as to optimally match changes in the organism's environment (Skwarlo-Sonta 1996, Rodríguez et al.1999, Barriga et al. 2001).

In birds, melatonin has been shown to modulate various immune functions, including antibody production, lymphocyte proliferation, NK cell cytotoxicity, and the release of cytokines (Skwarlo-Sonta 2002). In this line, Rodríguez et al., (1994) studied the effect of pinealectomy on the non-specific immune response after the *in vitro* incubation of heterophils isolated from the blood of ring doves with pharmacological doses of melatonin. The results confirmed that the heterophils incubated in the presence of pharmacological doses of melatonin presented a greater ingestion of latex beads, and later a positive correlation was observed between the circadian fluctuations in melatonin and the phagocytic activity of the immune system (Rodriguez et al. 1997). Given the confirmation in these studies of the involvement of melatonin in regulating the immune system function, the aim of the present work was to determine whether the administration of tryptophan – the precursor of melatonin – might likewise modulate immune system activity by raising the circulating levels of the hormone.

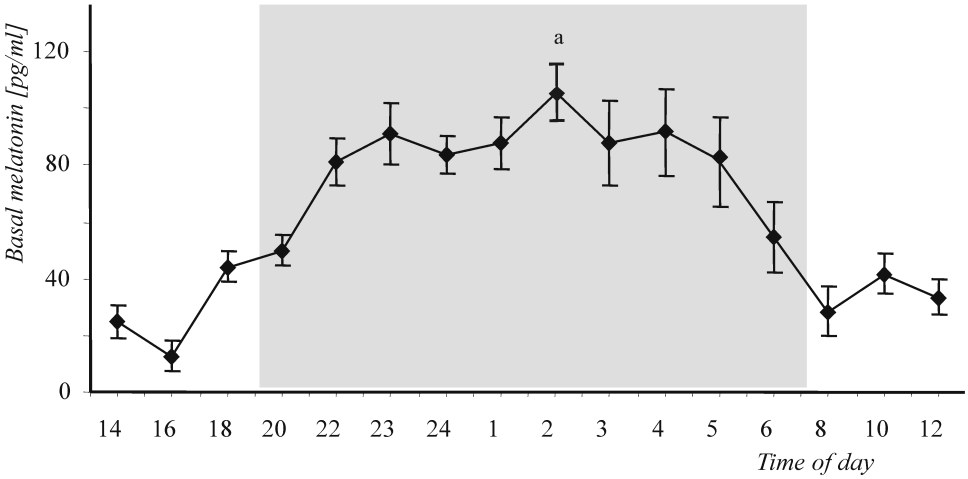

Fig. 1. **Basal melatonin levels in rats over a 24-h period**. Each value is the mean ± SD of 10 determinations performed in duplicate. (a) $p<0.05$ with respect to the other times of day. The shaded band corresponds to the hours of darkness

MATERIAL AND METHODS

Animals

The animals used were male Wistar-strain rats (*Rattus norvegicus*) of 14±2 weeks in age, whose weight was 455±50 g. The number of animals used per experimental group was 10; they were housed in cages (Panlab) of 50×23×15 cm, 2 rats per cage, in a room at a temperature of 20±5°C and relative humidity of 50–60%. They were allowed "UAR" feed (Panlab) and water *ad libitum*. The photoperiod was 12 h light and 12 h dark (dark period 20:00–08:00). All handling during the dark period was done under dim red light (<2 lux). 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's Council directives (86/609/EEC).

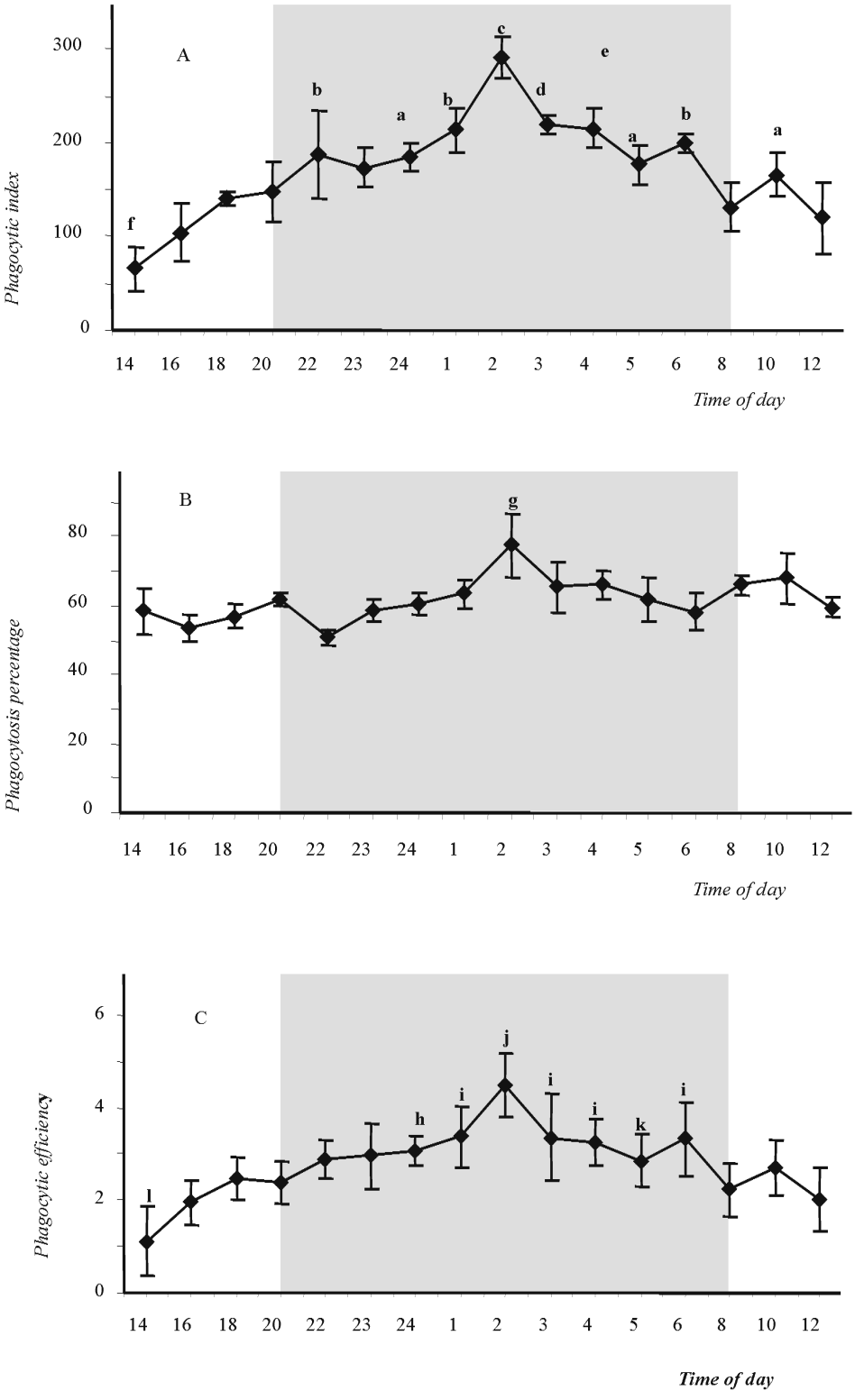

Fig. 2. **Plot of the basal phagocytic activity in Wistar rats over a 24-h period**. Each value is the mean±SD of 10 determinations performed in duplicate. The shaded band corresponds to the hours of darkness. *A Phagocytic index*. (a) $p<0.05$ with respect to the values obtained at 14:00 and 16:00; (b) $p<0.05$ with respect to the values obtained at 12:00, 14:00, and 16:00; (c) $p<0.05$ with respect to the values obtained at other times of day; (d) $p<0.05$ with respect to the values obtained at 08:00, 12:00, 14:00, 16:00, 18:00, and 20:00; (e) $p<0.05$ with respect to the values obtained at 08:00, 12:00, 14:00, 16:00, and 18:00; (f) $p<0.05$ with respect to the values obtained at 08:00, 18:00, 20:00, and 23:00. *B. Phagocytosis percentage*. (g) $p<0.05$ with respect to the values obtained at other times of day. *C. Phagocytic efficiency*. (h) $p<0.05$ with respect to the values obtained at 08:00, 14:00, and 16:00; (i) $p<0.05$ with respect to the values obtained at 08:00, 12:00, 14:00, 16:00, and 20:00; (j) $p<0.05$ with respect to the values obtained at 00:00, 01:00, 05:00, 06:00, 08:00, 10:00, 12:00, 14:00, 16:00, 18:00, 20:00, 22:00, and 23:00; (k) $p<0.05$ with respect to the values obtained at 14:00 and 16:00; (l) $p<0.05$ with respect to the values obtained at 10:00, 18:00, 22:00, and 23:00

Experimental design

The animals were divided into three groups:

(i) Basal animals. These rats were subjected to no kind of treatment during their lifetime. They were used to determine the circadian rhythm of the melatonin levels and phagocytic activity of peritoneal macrophages, taking samples during a 24-h period each 2 hours during the light period and each hour during the dark period, since it is during this latter period that melatonin is synthesized and hence its levels are at a maximum.

(ii) Control animals. These rats were administered saline solution (NaCl) by buccopharyngeal cannula (1 ml/rat) at 19:00 (1 h before lights out) every day for 21 days.

(iii) Animals administered tryptophan. These rats were administered tryptophan (125 mg/kg) dissolved in saline solution (1 ml/rat) for 21 days (Tormo et al. 2004). The administration method was the same as that for the control group.

In the control and tryptophan administration groups, samples were taken punctually three times a day: at 02:00, the time at which maximum values of plasma melatonin and phagocytic activity were obtained under basal conditions; at 09:00, one hour after lights on; and at 21:00, one hour after lights out.

(i) Plasma collection. At the beginning of treatment and halfway through it, plasma was obtained from blood drawn from the tail and collected in tubes containing plasma separating gel (EDTA). At the end of the treatment, and also in the basal group, plasma was obtained from blood collected from neck veins after decapitation of the rat. The blood was centrifuged at 240×*g* at 25°C for 15 min. Aliquots of the resulting plasma were passed into Eppendorf tubes and maintained at -30°C until use.

(ii) Plasma melatonin assay. The melatonin levels of all three experimental groups were determined using a commercial kit (ITISA BIOMÉDICA) with ^{125}I-melatonin (0.54 μCi/ml).

(iii) Collection of peritoneal exudate cells (PECs). Rats were killed by decapitation. An intraperitoneal injection of 4 ml of Hank's saline solution adjusted to pH 7.4 was used. The abdomen was gently massaged and the peritoneal suspension (macrophages and lymphocytes) was extracted. The recovery was approximately 65–75% of the injected volume. The cells of the peritoneal suspension were identified and counted using a Neubauer haemocytometer under a phase-contrast microscope, adjusting the concentration of the suspension to a final value of 5×10^5 macrophages/ml culture medium (Hank's). This cell suspension was used immediately for the phagocytosis assay.

(iv) Phagocytosis assay. The assay of the phagocytosis of latex beads was carried out following a previously described method (Ortega et al. 1996). 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).

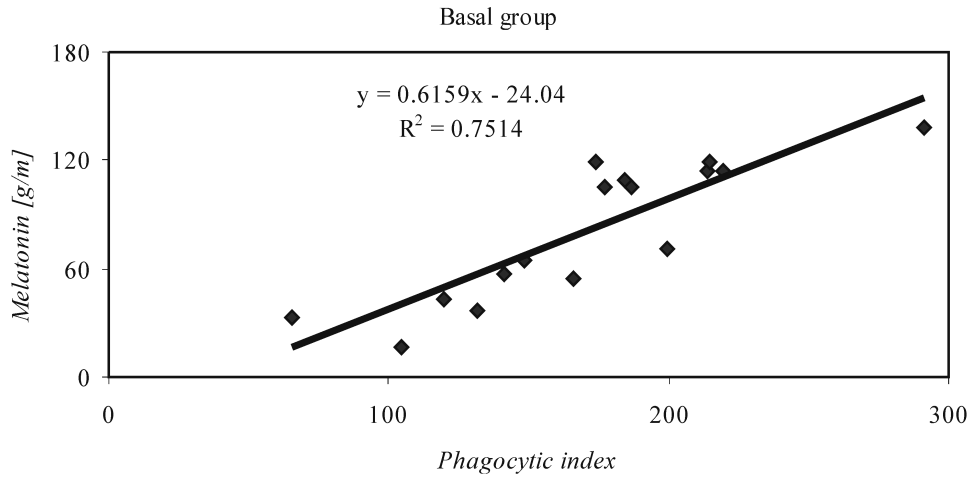

Fig. 3. **Plot of the correlation between the circadian variations of plasma melatonin levels and phagocytic index in basal Wistar rats**

(v) Statistical analysis. All data are expressed as the mean ± SD determined from 10 determinations carried out in duplicate. Variables were tested for normality, and the different groups were compared using the Scheffe ANOVA parametric *F*-test. $P<0.05$ was considered statistically significant. Correlations by multiple regression of the different functional capacities with the melatonin values at different times of the day were taken as significant if $r^2>0.5$.

RESULTS

The results showed that Wistar rats presents a circadian rhythm of melatonin under basal conditions, with the plasma melatonin levels peaking during darkness at 02:00 (Fig. 1). There was also a circadian rhythm of the phagocytic activity of the peritoneal macrophages of these animals under basal conditions, with the greatest values again being found during darkness at 02:00 both for PI (Fig. 2a) and PE (Fig. 2c). In the case of the PP index, however, while this peaked at 02:00 there was practically no circadian rhythm at the other times of day (Fig. 2b).

The correlation between phagocytic activity and melatonin levels in basal conditions is shown in Figure 3. There was a positive correlation (r2=0.7514) between the parameters of phagocytic index and plasma melatonin levels during a period of 24 hours.

For the tryptophan-treated animals, the plasma melatonin levels were higher at 21:00 and 09:00 than in the control and basal groups. There were no significant differences between these last two groups which presented the maximum levels of melatonin again at 02:00, while in the tryptophan-treated group the maximum was at 21:00 both halfway through the treatment (Fig. 4a) and at the end of the treatment (Fig. 4b).

In all three groups at the end of the treatment period, the maximum phagocytic activity was observed at 02:00 for all three parameters – PI (Fig. 5a), PP (Fig. 5b), and PE (Fig. 5c). The tryptophan-treated group showed increased values of this activity with respect to the basal and control groups in all three parameters.

Figure 6 shows the correlation between the melatonin levels and the phagocytosis percentage in tryoptophan-treated animals. A positive correlation can be observed between both parameters (r2=0.8837).

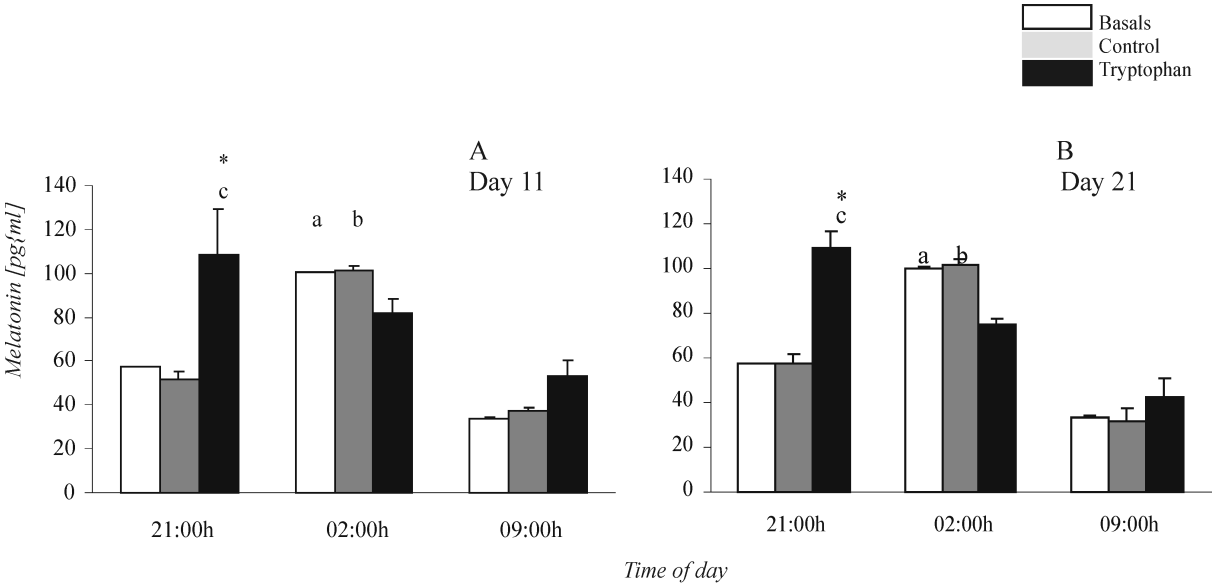

Fig. **4. Plasma melatonin levels in basal, control and tryptophan-treated Wistar rats.** Each value is the mean±SD of 10 determinations performed in duplicate. (a) $p<0.05$ with respect to the values obtained at the other times of day in the basal group; (b) $p<0.05$ with respect to the values obtained at the other times of day in the control group; (c) $p<0.05$ with respect to the values obtained at the other times of day in the tryptophan-treated group; (*) $p<0.05$ with respect to the values obtained at the same time of day for the basal and control groups.
A, plasma melatonin levels halfway through the treatment (day 11); *B,* plasma melatonin levels at the end of the treatment (day 21)

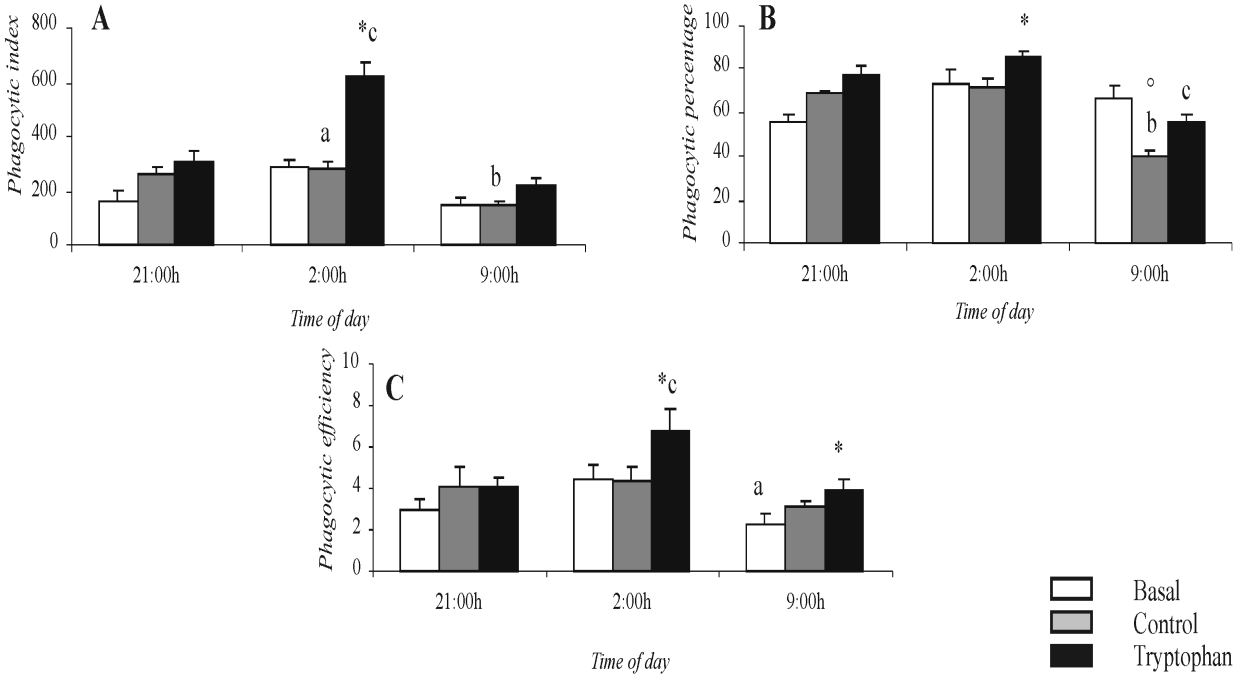

Fig. 5. **Measures of the phagocytic activity performed by macrophages at the end of the treatment in basal, control, and tryptophan-treated Wistar rats.** Each value is the mean ± SD of 10 determinations performed in duplicate. (a) $p<0.05$ with respect to the values obtained at the other times of day in the basal group; (b) $p<0.05$ with respect to the values obtained at the other times of day in the control group; (c) $p<0.05$ with respect to the values obtained at the other times of day in the tryptophan-treated group; (*) $p<0.05$ with respect to the values obtained at the same time of day for the basal and control groups; (•) $p<0.05$ with respect to the values obtained at the same time of day for the basal and tryptophan-treated groups. *A,* phagocytic index*; B,* phagocytosis percentage; *C,* phagocytic efficiency

DISCUSSION

It is essential for an organism to interpret the message of melatonin in the body in order to adapt its physiological functions to its needs and facilitate its survival. The immune system is one of the principal physiological processes responsible for that survival. It acts in perfect synchrony with the other systems of the body to maintain homeostasis and the correct functioning of the organism by following a circadian rhythm (Berger 1988) that varies according to different environmental factors (Berger 1983). The present work studied the variation of the immune system over a day/night cycle as reflected in the phagocytic activity of Wistar rat peritoneal macrophages. The activity was evaluated by the parameters: phagocytosis index (PI), phagocytosis percentage (PP), and phagocytosis efficiency (PE). The values of all three parameters were maximum at 02:00 and minimum at 14:00. There were differences between day and night in PI and PE, indicating a greater efficacy of phagocytosis and more activated macrophages during darkness, in coherence with earlier work by our research group on ring doves (Rodríguez et al. 1999) and mice (Barriga et al. 2001). The results also confirmed the findings of Berger and Slapničková (2003) of an increased phagocytic activity in rats during the night and early morning. Since rats are nocturnal animals, this lends support to the opinion that changes in phagocytic characteristics may be part of the circadian rhythm of the rat's immune system.

The synthesis of the hormone melatonin follows a circadian rhythm, with the levels being higher during darkness due to increased activity of N-acetyltransferase (EC 2.3.1.87), inter al. Melatonin secretion peaks about halfway through the dark period (Urbansky 2000), and is at lower levels during

the light period (Crespo 1997). This situation is reflected in the present Wistar rat model in which the melatonin levels rose during darkness to peak at 02:00, just halfway through the dark period between 20:00 and 08:00.

The existence of a relationship between the immune system and melatonin has been described by many workers. For instance, Rodríguez et al. (1994) showed that pinealectomized ring doves presented alterations in the blood heterophil phagocytic process compared with non-pinealectomized animals. The same research group later evaluated the changes in the heterophil phagocytic process after incubation in vitro with pharmacological doses of melatonin, they observed that the heterophils ingested more latex beads after melatonin treatment and that there was a decrease in the oxidative metabolism (Rodríguez et al. 1998). A positive correlation has also been observed between the circadian levels of melatonin and the phagocytic capacity of heterophils. In the present work we have also observed a positive correlation between both parameters in basal conditions, as well as after the administration of tryptophan.

The principal goal of the present work was to determine how the administration of the amino acid tryptophan – a precursor of melatonin (a minor fate of tryptophan is metabolized towards melatonin) – might influence the immune system. In particular, the objective was to study the phagocytic activity and the plasma levels of melatonin. Studies performed by Young and Anderson in 1982 found an increase in melatonin levels following an intraperitoneal injection of tryptophan to rats, independently of whether the administration was at night or in the morning. Similar results were reported by Brzozowsky et al. (1997) who found increased plasma melatonin levels following the intragastric administration of tryptophan (25–200 mg/kg) to Wistar rats. The present results are coherent with those earlier findings. We found that a daily single tryptophan dose (125 mg/kg) also led to increases in plasma melatonin levels both halfway and at the end of the treatment with respect to the control group. Nevertheless, in the tryptophan-treated group the highest melatonin levels were found at 21:00, and not at 02:00 as was the case in both the basal and the control groups. Indeed, there was even a decline in levels in the tryptophan-treated group at 02:00. This leads us to hypothesize that tryptophan administration caused the melatonin synthesis to peak earlier. Studies by Moreno-Madrid et al. (1999), lend support to this hypothesis, since, after administering tryptophan orally to a group of children between 2 and 11 years old, they found not only that the melatonin levels rose but also that the peak levels of the synthesis of the hormone occurred two to three hours following the administration of the amino acid, a time period coherent with the results of the present study. On the other hand, Herichova et al. (1998) administered tryptophan (150 mg/kg) orally to chickens and found no changes in plasma melatonin levels 1 h afterwards, although after 3 h there were significant changes in the levels of the hormone in the pineal (but still none in the circulating levels in the plasma). Nonetheless, it must be borne in mind that variations in melatonin levels following tryptophan administration depend on several factors, including the amount administered, the time of administration, the number of doses, and the type of animal (Moreno-Madrid et al. 1999).

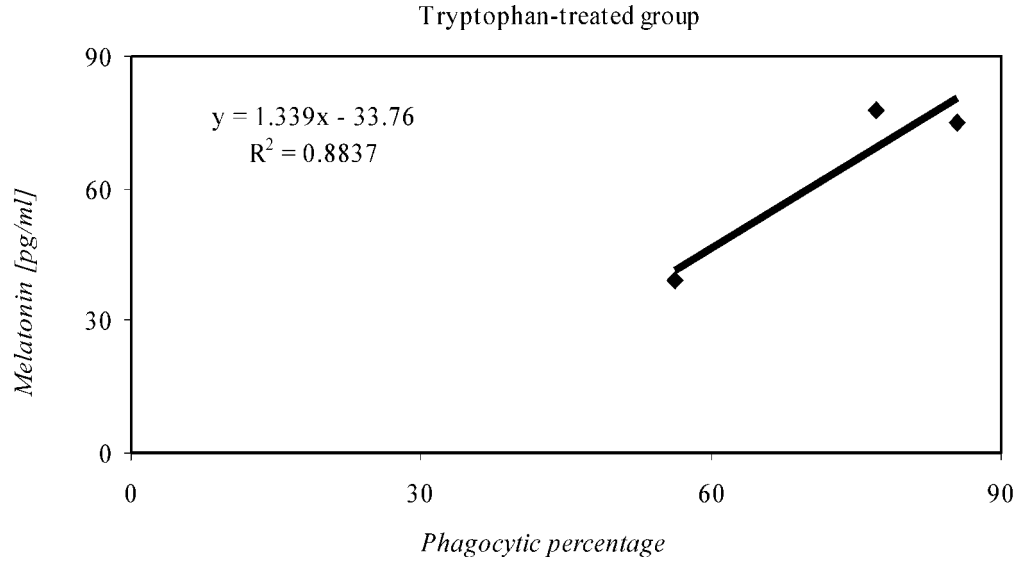

Fig.6. **Plot of the correlation between the circadian variations of plasma melatonin levels and phagocytosis percentage in tryptophan-treated Wistar rats**

With respect to the phagocytic activity of the tryptophan-treated rats, the maximum values at the end of the treatment were again reached at 02:00 as was the case in the control group, both groups therefore repeating the pattern observed in the basal group. The level of the phagocytic activity, however, was significantly higher in the tryptophan-treated group than in the control group. This suggests that, via its transformation into melatonin, tryptophan enhances the capacity of the innate immune system. Findings of Barriga et al. (2001) in mice indicate that melatonin is directly involved in regulating the non-specific immune response by increasing the capacity of macrophages to ingest foreign particles. The same workers (Barriga et al. 2002) later confirmed these results in vitro. This immunoregulatory action of melatonin on the phagocytic capacity may be due to its direct effect on the oxygen-dependent biochemical processes that accompany the respiratory burst during phagocytosis, acting to scavenge the free radicals that are released, as has been shown in the ring dove (Rodríguez et al. 1999, 2001).

In sum, it can be stated that tryptophan administration raises circulating melatonin levels, which in turn could act on the immune system to produce a concomitant increase in the phagocytic activity of peritoneal macrophages.

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