ORIGINAL ARTICLE

Tryptophan administration in rats enhances phagocytic function and reduces oxidative metabolism

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Abstract

OBJECTIVES. Since the tryptophan-derived metabolites serotonin and melatonin have been shown to possess reinforcing and/or antioxidant properties in the immune system, this investigation was aimed at determining the possible effect of a 7-day administration of tryptophan (125 mg/kg b.w.), the precursor of both the neurotransmitter and the indole, on the phagocytosis and free radical scavenging of peritoneal macrophages from adult male Wistar rats.

METHODS. 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. Oxidative metabolism was measured by the nitroblue tetrazolium (NBT) reduction test.

RESULTS. In control conditions, PI, PP, and PE significantly increased during the dark period, while the superoxide anion levels underwent a significant reduction. Tryptophan treatment significantly raised the phagocytosis parameters in a general fashion, as well as decreasing the oxidative metabolism with respect to the control values. Also, there was a significant rise in the MESORs of the PI and PE (of around 16% and 12%, respectively), the MESOR of the percentage of NBT reduction was significantly reduced (19%).

CONCLUSION. Orally administered tryptophan enhanced the phagocytic response and detoxification of superoxide anion radicals derived from this immune function in the peritoneal macrophages of rats, very probably through its conversion to the immunoregulatory molecules, serotonin and melatonin.

INTRODUCTION

The involvement of melatonin in the photoperiodic control of several immune parameters was revealed for the first time in the late 1980s when it was observed that the nocturnal peak of melatonin is closely associated with the proliferation peak of progenitor cells for granulocytes and macrophages in mice (Kuci *et al*., 1988). Since then, increasing attention has been devoted to the possible interaction between melatonin and the immune system (Carrillo-Vico *et al*., 2005; 2006). Thus, the literature suggests that there is a close connection between melatonin and immune regulation in both mammals (Barriga *et al*., 2001;2002; Hriscu *et al*., 2005) and birds (Rodríguez *et al*, 1999; Terrón *et al*., 2004; Paredes *et al*., 2007a), showing correlations between diurnal and seasonal changes in the immune system and the synthesis and secretion of the indoleamine (Skwarlo-Sonta, 2002).

The administration of melatonin enhances immune activity. In the particular case of rats, melatonin increases the generation of thymic peptides (Molinero *et al*., 2000) and exerts anti-apoptotic activity on T cells throughout all stages of their development in the thymus (Sainz *et al*., 1995), reinforces the proliferative capacity of rat lymphocytes (Liebmann *et al*., 1996; Martins *et al*., 1998), as well as providing significant protection against the injurious effects of dexamethasone including a decrease of body weight and atrophy of thymus and adrenals (Mori *et al*., 1984; Aoyama *et al*., 1987). In aged rats, exogenous melatonin has been reported to elevate the levels of both IgG and IgM (Akbulut *et al*., 2001).

Despite the numerous actions described for melatonin in the immune system, little is known about the role that tryptophan, the precursor of the pineal indole, may exert on the immunological mechanisms. Nevertheless, in unicellular organisms this amino acid and the related indolic neurotransmitter serotonin, which is also a precursor of melatonin, have been shown to possess the capacity to activate phagocytosis (Csaba *et al*., 1993). This is also the case in birds, where tryptophan enhances phagocyte viability, phagocytic response and detoxification of superoxide anion radicals derived from this immune function (Paredes *et al*., 2007b,c).

In previous investigations in rats, we observed that the administration of tryptophan led to a significant increase in both the circulating levels of melatonin and serotonin (Sánchez *et al*., 2004; 2008). Since both the indole and the neurotransmitter seem to exhibit antioxidative properties (Schuff-Werner and Splettstoesser, 1999; Reiter *et al*., 2004), it may be also of interest to determine whether tryptophan-derived serotonin and melatonin act as immunoregulatory molecules in the phagocytic process. Hence, the aim of the present work was to investigate the effect of tryptophan administration on the phagocytic function and levels of free radical generation of peritoneal macrophages from adult rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (n=240, 10 animals per hour measured) of 14±2 weeks in age were used in the study. The animals were housed individually in cages of 50×23×15 cm, under controlled environmental conditions of light and temperature, fed on Panlab meal and water *ad libitum*. The photoperiod was 12 h light and 12 h dark (dark period from 20:00 to 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).

Animal treatment

Animals were treated for 7 consecutive days with a single daily oral dose (125 mg / 1 ml NaCl saline solution per animal / day) of L-tryptophan (Sigma, St. Louis, MO, USA) at 19:00 using a gavage needle (Tormo *et al*., 2004). Control rats received only 1 ml of saline solution with the same schedule as the tryptophan-treated animals.

Collection of peritoneal exudate cells (PECs)

After the rats were killed by decapitation, the skin was carefully removed from the abdomen and an intraperitoneal injection of 4 ml of Hank's solution (Sigma, St. Louis, MO, USA) adjusted to pH 7.4 was given. The abdomen was gently massaged and a peritoneal suspension (macrophages and lymphocytes) was withdrawn with a syringe. The recovery was approximately 90–95% of the injected volume of the Hank's solution. 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. Cell viability was 98±1% as measured by the trypan blue exclusion method. Trials were performed at 1-h intervals in the dark period, and at 4-h intervals for the remainder of the 24-h cycle.

Phagocytosis of latex beads

The latex phagocytosis assay was carried out following a method described previously (Paredes *et al*., 2007d). Aliquots of 200 µl of the suspension of phagocytes were put into the wells of plastic macrophage migration-inhibition factor (MIF) type plates and, after 30 min of incubation at 37 °C in an oven with 5% CO_2 atmosphere, the adhered monolayer was washed with PBS at 37 °C.

Then 20 µl of latex beads [1.09-mm diameter particle size (Sigma, St. Louis, MO, USA) diluted to 1% in PBS] and 200 µl PBS were added, followed by another 30-min incubation under the same conditions as previously. Finally, the samples were fixed and stained with Diff-Quick containing methanol (5 min), eosin (five passes) and haematoxylin (five passes). The plates were washed with tap water and dried, followed by counting under oil-immersion phase-contrast microscopy at a magnification of 100×. The number of particles ingested per 100 macrophages expressed the latex-bead phagocytosis index (PI). The percentage of cells that had phagocytosed at least one latex bead expressed the phagocytosis percentage (PP). The ratio PI:PP gave the phagocytosis efficiency (PE).

Quantitative nitroblue tetrazolium (NBT) test

The trials were performed following a previously described technique (Paredes *et al*., 2007a,b). An aliquot of 250 µl of macrophages suspension (1×10^6 cells/ml) was incubated for 60 min with an equal volume of NBT (Sigma, 1 mg/ml in PBS solution) in the presence of 50 µl of latex beads (1.09-mm diameter particle size, diluted to 1% in PBS; Sigma, St. Louis, MO, USA) (stimulated samples). Aliquots of the macrophage suspension incubated in the absence of latex beads were used as non-stimulated samples. In all cases, after shaken incubation (60 min) at 37 °C, the reaction was stopped with 2.5 ml of 0.5 N hydrochloric acid. The tubes were centrifuged for 30 min at 600×g and 4 °C, the supernatant was discarded, and the reduced NBT (blue formazan) extracted from the cell pellet with 1 ml of dioxan. The tubes were then centrifuged for 30 min at 600×g, and the absorbance of the supernatant was determined in a spectrophotometer at 525 nm using dioxan as the blank control. The percentage stimulation of the NBT reduction was then determined, 100 being the value given to the absorbance obtained in the tubes without latex beads.

Data analysis

For the chronobiological study of the phagocytosis and NBT studies, a mean population cosinor was computed, using the integrated computer software package CSR 3.0.2 (C) Panlab S.L. Barcelona (Antoni Díez Noguera, University of Barcelona, Spain). From this the amplitudes were calculated (measure of the extent of a rhythmic change in a cycle as estimated by the cosine function that best fits 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 measuring the timing of the peak activity, expressed as the lag from a reference time (in the present study 00:00 h) to the crest of the cosine function best approximating the data].

Statistical analysis

Data are expressed as mean ± standard deviation of the number of determinations carried out in duplicate. The results were analysed using the non-parametric one-way ANOVA test followed by post-hoc Newman-Keuls test for multiple comparisons within-group. Two-way ANOVA test by Bonferroni test was used for comparisons between groups. Only values with $p<0.05$ were accepted as significant.

RESULTS

Figure 1 shows the variations in the capacity of the macrophages to ingest latex beads (PI) under control conditions and after 7 days of tryptophan treatment. There was a significant rise ($p<0.05$) in the control group in the nocturnal PI values with respect to those values during the light period, with the levels of PI at all the night hours studied being significantly higher ($p<0.05$) than those obtained at 09:00, 13:00, and 17:00 h. In the animals given tryptophan, the PI values were also greater during the dark period than during the light period, with statistically significant differences

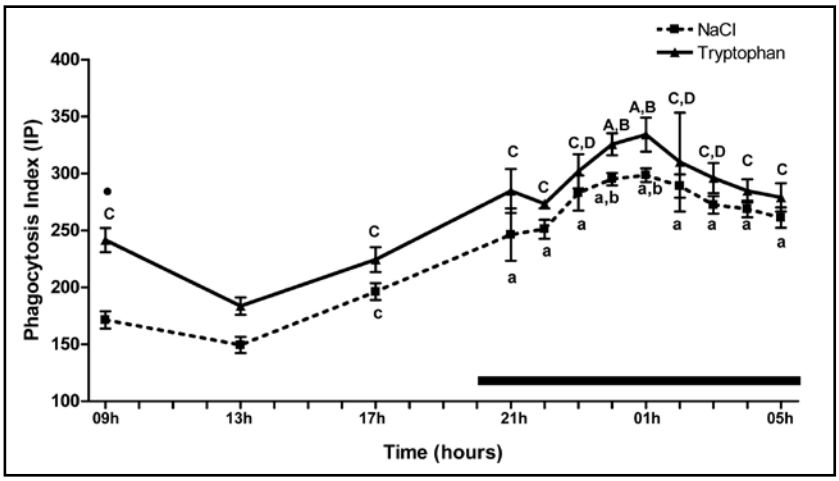

Figure 1. Phagocytosis index (PI) of control and tryptophan-treated Wistar rats over a period of 24 h. Each value is the mean ± SD of 10 determinations performed in duplicate. The black bar represents the period of darkness. (•) $p<0.05$ with respect to the values obtained in the control group at the same time of day. (a) $p<0.05$ with respect to the values obtained at 09:00, 13:00, and 17:00 h; (b) $p<0.05$ with respect to the values obtained at 21:00 and 22:00 h; (c) $p<0.05$ with respect to the values obtained at 13:00 h; (A) $p<0.05$ with respect to the values obtained at 13:00 and 17:00 h; (B) $p<0.05$ with respect to the values obtained at 09:00 h; (C) $p<0.05$ with respect to the values obtained at 13:00 h; (D) $p<0.05$ with respect to the values obtained at 17:00 hours.

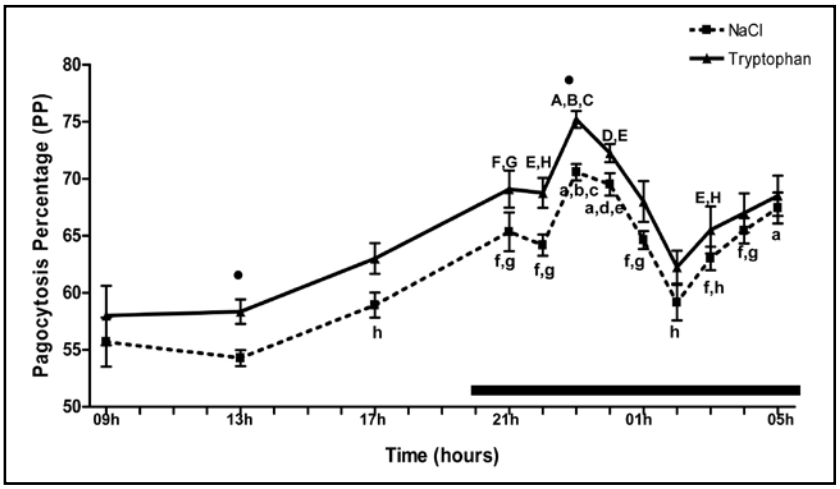

Figure 2. Phagocytosis percentage (PP) of control and tryptophan-treated Wistar rats over a period of 24 h. Each value is the mean ± SD of 10 determinations performed in duplicate. The black bar represents the period of darkness. (•) $p<0.05$ with respect to the values obtained in the control group at the same time of day. (a) $p<0.05$ with respect to the values obtained at 09:00, 13:00, and 02:00 h; (b) $p<0.05$ with respect to the values obtained at 22:00 and 03:00 h; (c) $p<0.05$ with respect to the values obtained at 03:00 h; (d) $p<0.05$ with respect to the values obtained at 21:00, 01:00, and 04:00 h; (e) $p<0.05$ with respect to the values obtained at 22:00 and 01:00 h; (f) $p<0.05$ with respect to the values obtained at 09:00 and 13:00 h; (g) $p<0.05$ with respect to the values obtained at 17:00 h; (A) $p<0.05$ with respect to the values obtained at 09:00, 13:00, 17:00, 02:00, and 03:00 h; (B) $p<0.05$ with respect to the values obtained at 21:00 and 04:00 h; (C) $p<0.05$ with respect to the values obtained at 01:00 and 05:00 h; (D) $p<0.05$ with respect to the values obtained at 09:00, 13:00, 17:00, and 02:00 h; (E) $p<0.05$ with respect to the values obtained at 03:00 h; (F) $p<0.05$ with respect to the values obtained at 09:00 and 13:00 h; (G) $p<0.05$ with respect to the values obtained at 02:00 and 17:00 h; (H) $p<0.05$ with respect to the values obtained at 09:00 h.

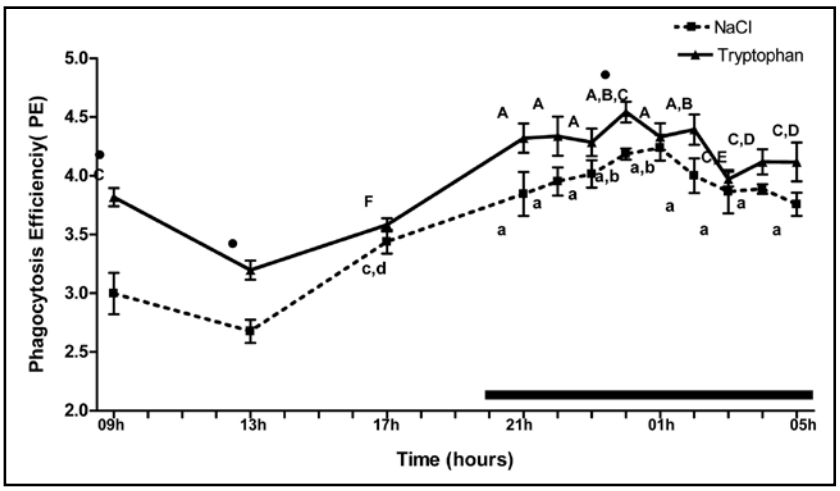

Figure 3. Phagocytosis efficiency (PP) of control and tryptophan-treated Wistar rats over a period of 24 h. Each value is the mean ± SD of 10 determinations performed in duplicate. The black bar represents the period of darkness. (•) $p<0.05$ with respect to the values obtained in the control group at the same time of day. (a) $p<0.05$ with respect to the values obtained at 09:00 and 13:00 h; (b) $p<0.05$ with respect to the values obtained at the 17:00 h; (c) $p<0.05$ with respect to the values obtained at 13:00 h; (d) $p<0.05$ with respect to the values obtained at 09:00 h; (A) $p<0.05$ with respect to the values obtained at 13:00 and 17:00 h; (B) $p<0.05$ with respect to the values obtained at 09:00 h; (C) $p<0.05$ with respect to the values obtained at 03:00 h; (D) $p<0.05$ with respect to the values obtained at 09:00 and 13:00 h; (E) $p<0.05$ with respect to the values obtained at 17:00 h; (F) $p<0.05$ with respect to the values obtained at 13:00 h.

($p<0.05$) in the values at 00:00 h (PI=325.6) and 01:00 h (PI=334.1) with respect to the daytime values. When the control and tryptophan-treated values were compared, there was a general increase in the PI of the latter, with significant differences ($p<0.05$) between both groups at 09:00 h.

The percentages of activated macrophages (PP) are displayed in Figure 2. In a general fashion and similarly to the case of the PI parameter, the values obtained during the dark period were significantly higher ($p<0.05$) with respect to the daytime values in both groups of animals, and were also higher in the tryptophan-treated group than in the control rats; the differences between these values were significant ($p<0.05$) at 13:00 and 23:00 h.

Figure 3 illustrates the number of latex beads ingested by each activated macrophage (PE). As noted for the PI and PP parameters, the highest values were reached in the dark period ($p<0.05$). Thus, the values obtained in the control animals from 22:00 to 02:00 h were 3.95, 4.01, 4.18, 4.24, 4.00 compared to a PE of only 2.68 at 13:00 h. The nocturnal tryptophan-treated values were higher ($p<0.05$) than those in the diurnal period, and also higher ($p<0.05$) than the controls, with significant differences at 09:00, 13:00, and 00:00 h.

The variations in oxidative metabolism (as measured by NBT reduction) are shown in Figure 4. In both

Table 1. Cosinor parameters for the Phagocytosis Index (PI; number of particles ingested per 100 macrophages), Phagocytosis Percentage (PP; the percentage of cells that had phagocytosed at least one latex bead), Phagocytosis Efficiency (ratio PI:PP), and the percentage of nitroblue tetrazolium (NBT) reduction in rats without (control) or with tryptophan administration.

	Amplitude		MESOR		Acrophase (time)	
	Control	Tryptophan	Control	Tryptophan	Control	Tryptophan
Phagocytosis Index (PI)	70.9±13.1	60.0±18.0	218.5±8.0	252.8±10.9*	24:57±0.45	01:09±1.13
Phagocytosis Percentage (PP)	6.0±4.5	6.3±4.2	60.7±2.7	63.9±2.5	24:05±3.59	23:05±2.84
Phagocytosis Efficiency (PE)	0.7±0.1	0.5±0.2	3.5±0.1	3.9±0.1*	24:13±0.91	24:29±1.84
% NBT Reduction (S/NS x 100)	98.4±36.8	100.1±26.0	235.6±21.2	191.0±15.1*	17:37±1.25	17:11±0.99

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

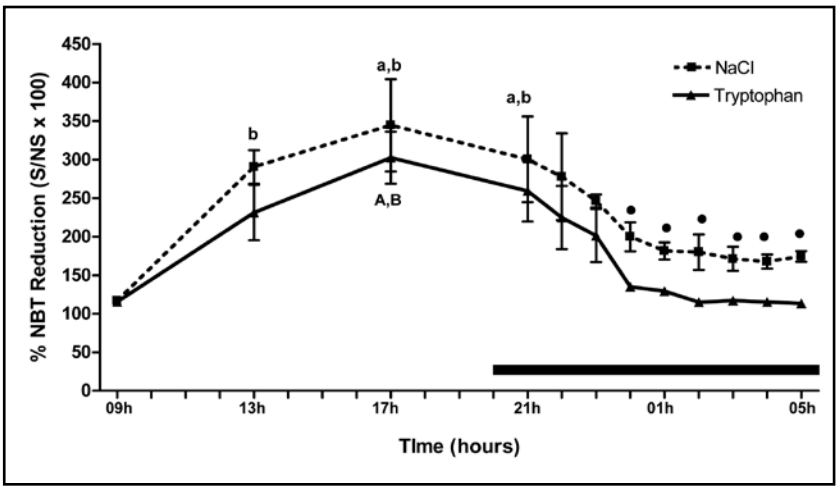

Figure 4. Plot of the circadian rhythms of the reduction of nitroblue tetrazolium (NBT) in control and tryptophan-treated Wistar rats over period of 24 h. Each value is the mean ± SD of 10 determinations performed in duplicate. The black bar represents the period of darkness. (•) $p<0.05$ with respect to the values obtained in the control group at the same time of day. (a) $p<0.05$ with respect to the values obtained at 09:00 h; (b) $p<0.05$ with respect to the values obtained at 01:00, 02:00, 03:00, 04:00, and 05:00 h; (A) $p<0.05$ with respect to the values obtained at 09:00 h; (B) $p<0.05$ with respect to the values obtained at 00:00, 01:00, 02:00, 03:00, 04:00, and 05:00 h.

groups, there was a reduction ($p<0.05$) in the nocturnal NBT levels with respect to the diurnal values. This reduction was greater ($p<0.05$) in the tryptophan-treated rats, whose diurnal and nocturnal levels were additionally lower ($p<0.05$) with respect to their corresponding controls at most hours analyzed during the dark period.

Table 1 lists the cosinor parameters (amplitude, MESOR, and acrophase) for the PI, PP, PE, and NBT of the control and tryptophan-treated rats. In general, there was a significant rise ($p<0.05$) in the MESORs of the PI and PE, with the MESOR of the percentage of NBT reduction being statistically significantly lower ($p<0.05$). In particular, it was found that the MESORs for the PI and PE increased roughly 16% and 12%, respectively. On the other hand, the MESOR of the NBT reduction was roughly 19% less than the control value ($p<0.05$). There were no significant differences between the two groups in the amplitudes or the acrophases for any of the parameters analyzed. The maximal values for the PI, PP, and PE under control conditions were reached respectively at 24:57, 24:05, and 24:13 h. In the tryptophan-treated groups, these parameters peaked between 23:00 and 01:00 h. Peak values were obtained at 17:37 and 17:11 h for the control and tryptophan-treated animals, respectively, in the NBT reduction study.

DISCUSSION

Phagocytosis is an important component in non-specific immunity and is fundamental for the host's defense against infection, providing an efficient means for the removal and destruction of invading pathogens (Abbas *et al*., 2003). Moreover, phagocytosis is an immune parameter that exhibits a circadian rhythm function (Berger and Slapnickova, 2003). Experimental findings in recent years have supported a functional connection between the pineal gland and the immune system in both mammals and birds (Carrillo-Vico *et al*., 2005), with the pineal secretory product, melatonin, acting as an immunomodulator of the phagocytic process in a variety of species (Barriga *et al*., 2001; 2002; Nelson *et al*., 2002; Terrón *et al*., 2004; Paredes *et al*., 2007a). In fact, exogenous melatonin increases circulating levels of melatonin as well as the phagocytic activity of both nocturnally and diurnally active species. Additionally, melatonin detoxifies free radicals produced as a consequence of the metabolic burst triggered by phagocytic cells once an antigen has been ingested (Hriscu, 2005; Terrón *et al*., 2005; Paredes *et al*., 2007a,c).

In the current study, the focus was on the possible effects of administering the precursor of melatonin, the amino acid tryptophan, on the phagocytic activity (phagocytosis and oxidative metabolism) of peritoneal

macrophages throughout a circadian cycle. The phagocytosis experiments performed herein showed increases in latex ingestion capacity (PI) and in the number of activated cells (PP), which resulted in an augmented PE, when comparing the control nocturnal values to those obtained during the diurnal period. In addition, the oral administration of tryptophan increased the innate immune response in this animal model, with the PI, PP, and PE values reached during the night being significantly higher than those measured during the day. Also the MESORs for the PI and PE were 16% and 12% greater than those of the non-treated animals. This is most probably due to the immuno-enhancing action of melatonin, since it was previously reported that the oral ingestion of tryptophan provokes a significant increase of the levels of the indoleamine (Hajak *et al*., 1991; Huether *et al*., 1992; Paredes *et al*., 2007e), which is also associated with a rise in the phagocytic activity (Sánchez *et al*., 2004; Esteban *et al*., 2004; Cubero *et al*., 2006; Paredes *et al*., 2007b,c). Although in the pineal gland the synthesis of melatonin is inhibited by photic stimulation (Ebihara *et al*., 1997), elevated circulating levels of melatonin have been detected after exogenous tryptophan administration during the day (Sánchez *et al*., 2004; Garau *et al*., 2006; Paredes *et al*., 2007b,c,e). Presumably, the enterochromaffin cells of the gastrointestinal tract may have been the source of newly-synthesized melatonin accounting for the elevated plasma concentrations of the indoleamine seen after tryptophan administration as well as the changes in circulating melatonin levels seen after feeding tryptophan (Huether, 1993). The rise in circulating melatonin may have been the cause of the elevated phagocytosis observed during the light period in the tryptophan-treated rats.

The oxidative metabolism experiments were carried out using the NBT reduction test, which evaluates the respiratory burst as indicated by the levels of superoxide anion radicals in peritoneal macrophages after inert particle phagocytosis. Thus, in the control animals we found lower levels of these radicals at night, the interval that coincides with elevated endogenous circulating melatonin levels in the rat (Paredes *et al*., 2005;2007d). Also, tryptophan administration enhanced the reduction of superoxide anions, with the MESOR for this parameter being 19% greater than that of the control rats. This was likely due to the rise of the circulating serum levels of melatonin normally resulting from tryptophan administration (Sánchez *et al*., 2004; Paredes *et al*., 2007b,e). This is consistent with previous studies showing that melatonin is a powerful direct free radical scavenger and general antioxidant, which reduces oxidative damage at both physiological and pharmacological concentrations (Reiter *et al*., 2003; Tan *et al*., 2007; Mauriz *et al*., 2007). Typically, higher levels of melatonin are accompanied by a reduction in the free radical levels produced by all cells, including peritoneal macrophages (Jou *et al*., 2007; Maldonado *et al*., 2007).

Serotonin receptors exist on leucocytes and the serotonin transporters have been found in macrophages, mononuclear leucocytes and B cells, with serotonin being regarded as a critical player at the interface between the nervous system and the immune system (Mossner and Lesch, 1998). In addition, it has been reported that serotonin also functions as an antioxidant (Schuff-Werner *et al*., 1995). Since tryptophan ingestion has also been described to significantly raise the circulating levels of serotonin in the brain (hypothalamus, hippocampus, striatum, and cerebellum) and plasma in the rat (Sánchez *et al*., 2008), as well as in diurnally active species including the ringdove (Garau *et al*., 2006; Paredes *et al*., 2007b,e), it is then probable that serotonin also aided melatonin in the scavenging process observed in the present study.

In sum, the results show that a tryptophan-supplemented diet enhanced phagocytosis and free radical scavenging in peritoneal macrophages from adult Wistar rats, with effect being exaggerated during the dark period. This information expands knowledge on the interrelationship between the endocrine system and the immune system, which is regarded as of crucial importance in normal physiology. It is suggested that the inclusion of tryptophan-enriched food in the diet may be beneficial for strengthening the innate immune response against antigens. Further investigation is needed to elucidate the various mechanisms involved in the immuno-enhancing effect exerted by the amino acid tryptophan.

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