Journal of
APPLIED
BIOMEDICINE

J. Appl. Biomed.
6: 177–186, 2008
ISSN 1214-0287

ORIGINAL ARTICLE

The effect of tryptophan administration on the circadian rhythms of melatonin in plasma and the pineal gland of rats

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Received 4th September 2008.
Revised 6th November 2008.
Published online 28th November 2008.

Summary
The most physiological processes that take place in the body have a circadian rhythm which is controlled by an internal biological clock located in the suprachiasmatic nucleus. The indole melatonin synthesized in the pineal gland, acts to synchronize these biological rhythms, and also it is synthesized and released following a circadian rhythm. The present study analyzed the levels of melatonin over a 24-hour period in Wistar rats in both basal and control conditions and after the oral administration of 125 mg/kg tryptophan, the amino acid that is the precursor of this indole, for 7 days. The levels of melatonin in the plasma and the pineal gland were measured by radioimmunoassay every hour during the night, and every 4 hours during the day. The results indicated that the tryptophan administration provoked raised levels of melatonin at all hours studied in both plasma and pineal. Of the chronobiological parameters studied, there were also increases in the values of the melatonin MESOR with respect to the values obtained in the basal and control groups (the respective increases being 45% and 52% in plasma, and 46% and 47% in the pineal), as well as an advanced acrophase with respect to the basal and control groups. In summary, our findings confirm that tryptophan intake one hour before lights-off increases melatonin levels in plasma and pineal over a 24-hour period, as well as advancing the peak of its synthesis.

Keywords: Circadian rhythms – melatonin – pineal gland – plasma – rat – tryptophan

INTRODUCTION

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Circadian rhythm studies have utilized as markers of the circadian clock: the onset phases of such rhythmic outputs as the immune system function, (Berger 1983, 1988), locomotor activities of laboratory rodents (Pittendrigh et al. 1976), temperature, and melatonin secretion (Liu et al. 2005a, b). The reliability and consistency of these circadian output markers are essential for accurate pacemaker analysis across multiple circadian cycles of the same individual; between different individuals of the same animal strain; between

different strains of the same animal species, or between individuals of different species (Daan et al. 2003).

The indole melatonin is considered to be the principal component of the internal biological clock located in the suprachiasmatic nucleus (SCN). It is now clear that melatonin production is subject to circadian rhythms with high levels at night and low by day, and also to seasonal variations throughout the year (Kennaway et al. 2002). This indole is synthesized at night mainly in the pinealocytes present in the pineal gland (Wurtman et al. 1964) in response to an endogenous internal clock regulated by environmental photoperiodic stimuli (Sugden et al. 1989, Reiter et al. 1991, Klein et al. 1997). The synthesis of melatonin takes place not only in the pineal gland but also in the retinal cells and the enterochromaffin cells of the gut. The pineal gland of all vertebrates produces melatonin from the amino acid tryptophan, which is converted into serotonin in response to the norepinephrine released from the superior cervical ganglion (SCG) (Klein et al. 1992, Borjigin et al. 1999). The serotonin is then converted into melatonin by the enzyme aralkylamine N-acetyltransferase (AA-NAT), which constitutes the limiting step in melatonin synthesis. This enzyme presents a marked circadian rhythm in all species that have been studied (Arendt 1995), and is generally considered to be the most important factor for rhythmicity (Klein et al. 1997) since it is rapidly induced via adrenergic stimulation, and rapidly inactivated, most likely by proteosomes (Gastel et al. 1998). Because of the close association of pineal melatonin release with the clock's activity, melatonin is regarded as an accurate marker of the circadian pacemaker in both human and animal circadian rhythm studies (Honma et al. 1997, Benloucif et al. 2005, Arendt 2006).

Melatonin is an extremely interesting indole, and its physiological effects and pharmacological properties have been extensively studied. Melatonin regulates sleep cycles and readjusts altered circadian rhythms – hence its use in the treatment of jet-lag (Cajochen et al. 2003) – enhances the efficacy of cancer treatments by counteracting the adverse effects of chemotherapy (Lissoni et al. 2001), and combats cell-level ageing by acting as a free-radical scavenger (Reiter et al. 2000, 2002, Rodriguez et al. 2001, Sánchez et al. 2004, Paredes et al. 2007a). But little is known about the effect of the administration of the precursor in its synthesis – the essential amino acid tryptophan. Tryptophan is the precursor of both the monoamine serotonin and the pineal indole melatonin. It has been observed that oral administration of L-tryptophan in different species of animal increases the availability of serotonin in the brain (Femstrom 1988), and then immediately stimulates the synthesis of melatonin increasing its levels in plasma (Hajak et al. 1991, Huether et al. 1992, Herichova et al. 1998, Cubero et al. 2006). Tryptophan administration or a high plasma ratio between tryptophan and large neutral amino acids can raise brain tryptophan levels, and accelerate melatonin synthesis. Given this context, the aim of the present work was to investigate the effects of the administration of tryptophan for 7 days on the levels of melatonin over a 24-hour period in the plasma and pineal of rats. Studies done so far have considered specific times of day, although from the standpoint of clinical research, experiments based on obtaining a single measurement are of dubious validity when applied to chronotherapeutic patterns of administration. For this reason, we believe it is important to know how a variable changes over a period of 24 hours. In the present case, an analysis of how the levels of melatonin change over a 24-hour period after the administration of tryptophan can help determine the most suitable time of day at which to administer it in order to maximize the increase in melatonin levels and thus optimize the therapeutic effectiveness of this indole in the treatment of various diseases.

MATERIAL AND METHODS

Animals

Male Wistar rats of 14 ± 2 weeks in age were used in the study (n=360, 120 animals in each experimental group, and 10 animals per hour measured). 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).

Experimental design

The animals were divided into three groups:

- Basal animals. These rats were subjected to no

kind of treatment during their lifetime. They were used to determine the circadian rhythm of melatonin levels in plasma and pineal in basal conditions, taking samples during a 24-hour period every 4 hours during the light period (measurements from 09:00 to 17:00) and every hour during the dark period (measurements from 21:00 to 05:00), since it is during this latter period that melatonin is synthesized and hence its levels are at a maximum.

- Control animals. These rats were administered a single daily oral dose of saline solution (NaCl) by gavage needle (1 ml/rat) at 19:00 (1 hour before lights out) for 7 days. Sampling for determining the levels of melatonin was the same as for the basal group.
- Tryptophan-treated group. These rats 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 (Sánchez et al. 2008a). Sampling for determining the levels of melatonin was the same as for the basal group.

Plasma collection

At the mid of treatment, plasma was obtained from blood drawn from the tail and collected in tubes containing plasma separating gel (EDTA). At the end of the treatment, plasma was obtained from blood collected from neck veins after decapitation of the rat. The blood was centrifuged at 4 °C for 5 min at 2300×g. Aliquots of the resulting plasma were frozen (–20 °C) until assay. The samples were taken to cover a 24-hour period, every 4 hours during the light period and every hour during the dark period.

Pineal gland extraction

Pineal melatonin levels were measured only at the end of treatment, when the rat was sacrificed and the pineal gland removed. Once the rats were sacrificed, the brain was quickly removed and dissected on an ice-cold plate. The pineal was stored at –80 °C for their later analysis. This organ was selected because it is the main site of melatonin synthesis. At analysis, the pineal glands were weighed, placed individually in cold tubes containing 1ml of 25mM Tris-HCl, pH 7.4, 1mM EDTA, and 1mM EGTA, and homogenized with an Ultra-Turrax homogenizer (type Tp 18/10). The homogenate was centrifuged at 11000×g for 30 min at 4 °C. The resulting supernatant was filtered through 0.45 µm syringe filters (Spartan-3, Aldrich Chemical, Milwaukee, Wis., USA) and divided into aliquots for melatonin determinations. Tissue extracts were assayed for protein concentrations.

Determination of the protein concentration in pineal tissue

After homogenization as described in the previous section, we determined the concentration of proteins present in the pineal gland, following the method of Bradford (1976). This highly sensitive method for protein assay consists of the formation of a blue-colour absorption compound between the basic amino acid residues of the proteins and the dye Coomassie blue. The absorbance depends on the content of basic and aromatic amino acids, and thus on the amount of protein present in the tissue.

Pineal and plasma melatonin assay

The melatonin levels in pineal and plasma of all three experimental groups were determined using a commercial kit (ITISA BIOMÉDICA, Spain) with ^{125}I-melatonin (0.54 µCi/ml), enzyme, enzyme buffer, standards, controls, rabbit antimelatonin antiserum, and precipitating reagent.

Data analysis

For the chronobiological study of the plasma and pineal data, 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 were calculated the amplitudes (a 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 hours, 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 analyzed using a non-parametric one-way ANOVA, considering the time as independent variable, followed by a post-hoc Newman-Keuls test for multiple comparisons. A two-way ANOVA with a

Bonferroni test was used for comparisons between groups. We used the significance level 2α= 0.05.

RESULTS

Fig. 1 shows the variations in plasma melatonin levels over a 24-hour period in basal and control conditions, and after 3 days of tryptophan treatment (midway through treatment). In the basal and control group there was a significant rise in the nocturnal values of melatonin with respect to those values during the light period, with the levels of melatonin at all the night hours studied being statistically significant higher) than those obtained at 09:00, 13:00, and 17:00 hours. There was no difference between the values obtained in the basal and control groups. In the animals given tryptophan, the melatonin values were also greater during the dark period than during the light period, with statistically significant differences in the value at 00:00 hours (236.77 pg/ml) with respect to the daytime values.

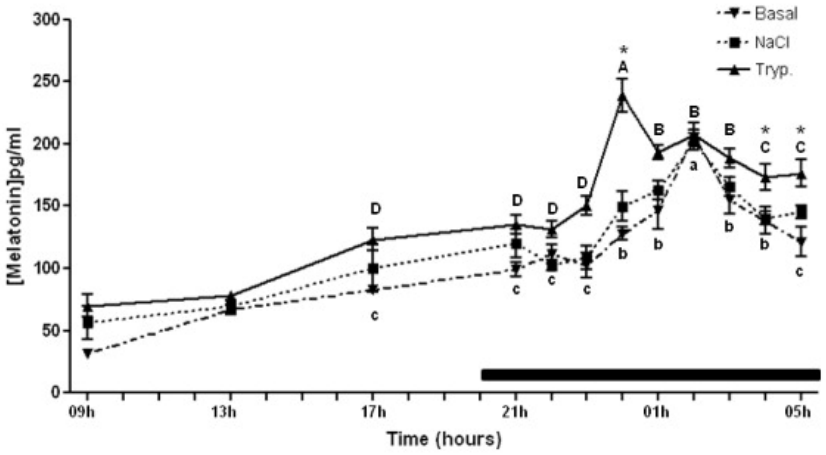

Fig. 1. Plot of the melatonin levels in plasma of Wistar rats over a 24-hour period in basal, control (NaCl), and tryptophan-treated groups after 3 day of treatment (n=360). Each value represents the mean ± SD of ten determinations performed in duplicate. The significance levels for the basal and control groups are the same, because the same statistical data are obtained in both.
Statistically significant with respect the basal and control group. a) Statistically significant with respect at the other hours of the day in basal and control groups. b) Statistically significant with respect at 09, 13, 17, 22, and 23 hours in basal and control groups. c) Statistically significant with respect at 09 and 13 hours in basal and control groups. A) Statistically significant with respect at the other hours of the day in the tryptophan-treated group. B) Statistically significant with respect at 09, 13, 17, 21, 22, and 23 hours in the tryptophan-treated group. C) Statistically significant with respect at 09, 13, 17, 21, and 22 hours in the tryptophan-treated group. D) Statistically significant with respect at 09 and 13 hours in the tryptophan-treated group.

Comparison of the basal, control, and tryptophan-treated values showed a general increase in melatonin levels after the administration of tryptophan at all hours analyzed. This increase was greater during the period of darkness, and statistically significant at 00:00 hours.

Fig. 2 shows the variations in plasma melatonin levels after 7 days of treatment (at the end of treatment). In general, the results are similar to the previous case: the values during the dark period were significantly higher ($p<0.05$) than the daytime values in both untreated groups (basal and control) and in the tryptophan-treated animals. Administration of tryptophan increased the melatonin levels at all times, the differences being statistically significant at 21:00, 22:00, 23:00, 00:00, 01:00, and 05:00 hours, while there were no differences in the basal and control levels.

Fig. 3 shows the circadian variations in the pineal melatonin levels after 7 days of treatment (the end of the treatment). In this case the melatonin levels were measured only at the end of treatment when the rat was sacrificed and the pineal gland removed. As had been observed for the plasma levels, the highest values were reached in the dark period (statistically significant) in all groups.

Thus, in the basal and control animals the peaks were obtained at 01:00 hours, whereas in the tryptophan-treated group the peak was obtained at 23:00 hours. Tryptophan administration produced an advancing of the peak of synthesis as well as an increase in melatonin levels at all the times analyzed, the increase being significant ($p<0.05$) at 21:00, 22:00 23:00, and 00:00 hours. Table 1 lists the cosinor parameters (amplitude, MESOR, and acrophase) for the melatonin levels of the basal, control, and tryptophan-treated rats. In general, there were significant rises ($p<0.05$) in the tryptophan-treated plasma MESORs on days 3 and 7. In particular, the MESORs for the tryptophan-treated group were 45% and 21% higher than the basal and control values halfway through the treatment (day 3), and 45% and 52% higher, respectively, at the end of the treatment. In the pineal gland, after 7 days of treatment, the MESOR was 46% and 47% higher ($p<0.05$) than in the basal and control groups, respectively. There were no significant between-group differences in the amplitudes of any of the three parameters analyzed. The acrophase values in the plasma on day 3 were reached at 02:14, 01:52, and 01:23 hours, and on day 7 at 02:14, 01:53, and 01:16 hours in the basal, control, and tryptophan-treated groups, respectively. In the pineal gland, the respective acrophases were reached at 03:01, 02:43, and 00:44 hours. The administration of tryptophan hence produced an advance in the acrophase in all three parameters analyzed (plasma on days 3 and 7, and pineal on day 7).

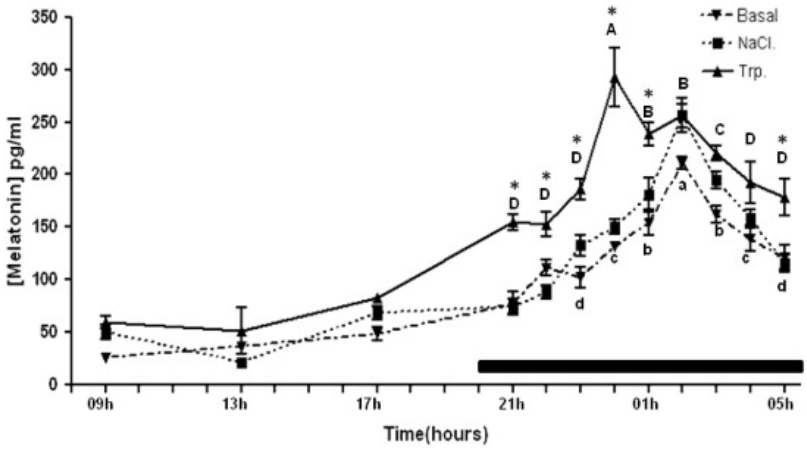

Fig. 2. **Levels of melatonin in the plasma of basal, control, and tryptophan-treated rats over a 24-hour period after 7 days of treatment. Each value represents the mean ± SD of ten determinations performed in duplicate (n=360). The significance levels for the basal and control groups are the same, because the same statistical data are obtained in both.**

Statistically significant with respect to the basal and control group. a) Statistically significant with respect at the other hours of the day in basal and control groups. b) statistically significant with respect at 09, 13, 17, 21, 22, and 05 hours in basal and control groups. c) statistically significant with respect at 09, 13, 17, and 21 hours in basal and control groups. d) Statistically significant with respect at 09, 13, and 17 hours in basal and control groups. A) Statistically significant with respect d at 09, 13, 17, 21, 22, 23, 01, 03, 04, and 05 hours in tryptophan-treated group. B) Statistically significant with respect at 09, 13, 17, 21, 22, 23, 04, and 05 hours in tryptophan-treated group. C) Statistically significant with respect at 09, 13, 17, 21, 22, and 23 hours in tryptophan-treated group. D) Statistically significant with respect at 09, 13, and 17 hours in tryptophan-treated group.

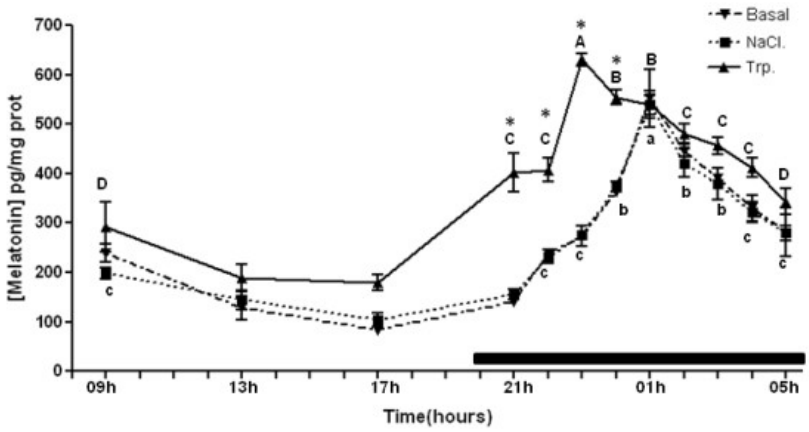

Fig. 3. Levels of melatonin in pineal gland in the basal, control, and tryptophan-treated groups over a 24-hour period after 7 days of treatment. Each value represents the mean ± SD of ten determinations performed in duplicate (n=360). The significance levels for the basal and control groups are the same, because the same statistical data are obtained in both. Statistically significant with respect to the basal and control group. a) Statistically significant with respect at the other hours of the day in the basal and control groups. b) Statistically significant with respect to the values obtained at 09, 13, 17, 21, 22, 23, and 05 hours in the basal and control groups. c) Statistically significant with respect at 13, 17, and 21 hours in the basal and control groups. A) Statistically significant with respect at the other hours of the day in the tryptophan-treated group. B) Statistically significant with respect at 09, 13, 17, 21, 22, 02, 03, 04, and 05 hours in the tryptophan-treated group. C) Statistically significant with respect at 09, 13, 17, and 05 hours in the tryptophan-treated group. D) Statistically significant with respect at 13 and 17 hours in the tryptophan-treated group.

DISCUSSION

The amino acid tryptophan is the precursor in the synthesis of the hormone melatonin. This hormone is considered to be the biological clock responsible for synchronizing biological rhythms (Berger et al. 1983). This clock, located in the suprachiasmatic nucleus (SCN), is in turn synchronized by light and non-light signals (Sánchez et al. 2008b). These rhythms must be maintained for the proper functioning of the organism and the maintenance of homeostasis (Berger et al. 1988, Skwarlo-Santa 1996, Rodriguez et al. 1999, Barriga et al. 2001).

All mammals studied up to now present a similar circadian rhythm of melatonin, with the higher levels during darkness due to increased activity of N-acetyltransferase, and lower levels during the light period. This has been corroborated in earlier studies by our research group (Sánchez et al. 2004, Paredes et al. 2005) not only in mammals (rats) but also in birds (Paredes et al. 2007b). Since melatonin is synthesized from the amino acid tryptophan, the amount of this amino acid available in the blood and brain will affect the circulating levels of melatonin (Esteban et al. 2004). Thus, the use of diets rich in tryptophan will produce an increase in the amount of tryptophan available in the blood and brain, and consequently a greater synthesis of melatonin.

For these reasons, the present work was aimed at assessing the effect of the administration of 125 mg/kg of tryptophan for 7 days on the levels of melatonin over a 24-hour period in the plasma and pineal gland of rats. Up to now, studies performed on this topic have only considered a specific time, not a 24-hour period. However, we consider that it is important to know how a variable changes over a period of 24 hours, since experiments based on obtaining data only at specific hours are of doubtful validity. This is especially so with respect to their clinical application using a chronotherapeutic pattern of administration, when it is important to choose the optimal time of administration and thus achieve the greatest effectiveness.

The results showed the basal and control groups to have similar patterns of circadian variation in plasma melatonin levels, with higher values in the dark period and lower in the light period, and a

Table 1. **Cosinor parameters for the melatonin levels in plasma and pineal gland of rats**

	Amplitude (pg./ml or pg/mg prot.)			MESOR (pg/ml or pg./mg prot.)			Acrophase (time hh:mm)		
	Basal	Control	Tryptophan	Basal	Control	Tryptophan	Basal	Control	Tryptophan
Plasma (day 3)	57.10 ± 42.39	49.66 ± 34.75	62.67 ± 37.78	88.85 ± 25.61	106.32 ± 21.06	129.10 ± 22.93*	02:14 ± 3:12	01:52 ± 3:01	01:23 ± 2:34
Plasma (day 7)	57.10 ± 42.39	88.81 ± 55.56	102.90 ± 42.28*	88.85 ± 25.61	84.43 ± 33.66	128.73 ± 25.67 *	02:14 ± 3:12	01:53 ± 2:79	01:16 ± 1:42
Pineal (day 7)	160.29 ± 105.8	157.38 ± 100.48	180.81 ± 88.87*	226.96 ± 63.36	225.35 ± 60.37	330.80 ± 53.17*	03:01 ± 2:33	02:43 ± 2:78	00:44 ± 2:03

Each value represents the mean ± SD of 10 determinations (*) 2α=0,05 with respect to the values obtained in the basal and control group. Amplitude and MESOR are expressed in pg/ml in plasma and pg/mg protein in pineal.

peak at 02:00 hours both halfway through and at the end of the treatment. The same circadian variations were obtained for the levels of melatonin in the pineal gland, with the peak being observed at 01:00 hours. The concentrations measured in the pineal were far greater than those in plasma because the pineal is where the synthesis of the hormone principally takes place. This rhythm in melatonin levels was maintained after tryptophan administration, with the peak concentrations occurring at 00:00 and 23:00 hours in plasma and pineal, respectively.

This administration of tryptophan in a daily single dose of 125 mg/kg at 19:00 hours, one hour before lights-off, produced an increase in the plasma levels of melatonin throughout the 24 hours, both halfway through and at the end of the treatment, although this increase was much higher during the period of darkness. Esteban et al. (2004) also found increased levels of circulating melatonin at night in the plasma of rats when tryptophan was administered at night (20:00 hours), although in this case the melatonin levels were analyzed only at two specific times. Similar results were obtained by Cubero et al. (2006) in ringdove, who observed a greater increase in serum melatonin levels when 125 mg/kg of tryptophan was administered than when the dose was 300 mg/kg. This gave the impression that an excessive concentration of tryptophan could saturate the enzyme responsible for its transformation into melatonin. However, a study by Paredes et al. (2007b) in the same species did not confirm this idea, because they observed the greater increase in melatonin with the 300 mg/kg dose. Although they administered the tryptophan in the morning (09:00 hours), the greatest increases in melatonin levels corresponded to hours of darkness. They found practically no differences in melatonin levels between the measurements made halfway through and those made at the end of the treatment.

In the case of the pineal gland, the peak appeared during the dark period, at 01:00 hours in the basal and control groups, and at 23:00 hours in the tryptophan-treated group. The administration of tryptophan led to increased melatonin levels at all the times analyzed, this increase being greater in the dark period than in the light period. Studies performed by Young and Anderson (1982) found an increase in pineal melatonin levels following an intraperitoneal injection of tryptophan to rats, and this increase was greater when the administration was at night than when it was during the day. Similar results were reported by Brzozowski et al. (1997) who found increased plasma melatonin levels following the intragastric administration of tryptophan (25–200 mg/kg) to Wistar rats.

In addition, the administration of tryptophan produced a rise in the MESOR of the plasma and pineal (of around 45% and 46% with respect to the basal group, and 52% and 47% with respect to the control group, respectively), as well as an advance in the acrophase of melatonin with respect to the basal and control groups. This leads us to hypothesize that the 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 indole occurred two to three hours following the administration of the amino acid, a time lag 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 hour afterwards, although after 3 hours 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).

In sum, the administration of 125 mg/kg of tryptophan for 7 days one hour before the light turn off, increased the levels of melatonin over the 24-hour period, in both plasma and pineal. This suggests that diets enriched with tryptophan can achieve the same physiological and therapeutic effects as supplemental melatonin, because they will increase the levels of the hormone. Also, knowing the time of day when melatonin reaches its peak levels, and seeing the effects of tryptophan administration on these levels, can help determine at what time it is best to administer tryptophan for it to have the greatest effect on melatonin, and thus increase the effectiveness of treatments involving melatonin.

ACKNOWLEDGEMENTS

The authors would like to express their thanks to Ms Elena Circujano Vadillo for her technical assistance and to Manuel Molina and Manuel Mota of the Department of Mathematics (Section of

Statistics and Operations Research) of the Faculty of Science for their advice and collaboration in the statistical analyses of the data. S.D. Paredes was the beneficiary of a grant from the Consejería de Economía, Comercio e Innovación – Fondo Social Europeo (Junta de Extremadura, POS07012). Soledad Sánchez Mateos held a grant awarded by the Fundación Fernando Valhondo Calaff.

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