

## LONG-TERM ALTERATION OF DAILY MELATONIN, 6-SULFATOXYMELATONIN, CORTISOL, AND TEMPERATURE PROFILES IN BURN PATIENTS: A PRELIMINARY REPORT

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Melatonin, which shows a robust nycthemeral rhythm, plays the role of an endogenous synchronizer, able to stabilize and reinforce circadian rhythms and maintain their mutual phase relationships. Additionally, melatonin is a potent antioxidant and displays immunological properties. Because free radical generation, immune dysfunction, and sleep and metabolic disorders are involved in the short- and long-term pathophysiology of the burn syndrome, we undertook the study of daily urine melatonin, 6-sulfatoxymelatonin (aMT6s, the main hepatic melatonin metabolite), and cortisol variations plus temperature profiles in burn patients using a non-invasive protocol. Eight patients (6 males, 2 females) were studied on three occasions after admission to the intensive care unit (early session: days 1 to 3; intermediate session: day 10; late session: days 20 to 30). Melatonin, aMT6s, and free cortisol levels were determined in urine samples collected at 4 h intervals over a continuous 24 h span. Core temperature was recorded daily. Controls consisted of healthy subjects in the same age range. Cosinor analysis of the data provided an evaluation of mesor, amplitude, and acrophase of circadian rhythms. Also, we calculated day (D), night (N), and 24 h hormone excretions, N/D ratio for melatonin and aMT6s, and D/N ratio for cortisol. These data were analyzed using Kruskal-Wallis test followed by multiple comparisons. Cosinor analysis did not detect a circadian rhythm in melatonin, aMT6s, or cortisol in any of the three sessions. D melatonin excretion displayed a major increase, resulting in a decreased N/D melatonin ratio, and the melatonin mesor (24 h mean) was increased in the early session, compared with controls. For aMT6s, only the early N/D ratio was decreased, and the mesor of the intermediate session increased. These results were not the consequence of hepatic and/or kidney alteration, as the patients' hepatic and renal parameters were in the normal range. The D and N melatonin/aMT6s ratios of controls and patients were similar, and the aMT6s profiles were superimposed on the melatonin ones, mainly during the day. The D, N, and 24 h cortisol values were increased in all sessions, except for the D

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level of the early session. The consistently increased mesors in the three sessions provided confirmation. The core temperature profiles were abnormal in all three sessions, mainly during the night, although there was a tendency toward normalization with time. The individual mesors were consistently increased compared with controls. Globally, the abnormalities we report could participate in the pathophysiology of short- and long-term alterations observed in burn syndrome, especially disturbances of sleep, metabolism, and immune function. (Author correspondence: bruno.claustrat@chu-lyon.fr).

**Keywords** Burn patients; Melatonin; 6-Sulfatoxymelatonin; Free cortisol; Temperature

## INTRODUCTION

In humans, the hormone melatonin is synthesized by the pineal gland and conveys the information of nighttime to the organism. It also plays the role of an endogenous synchronizer, able to stabilize and reinforce circadian rhythms and maintain their mutual phase-relationships (Claustrat et al., 2005). There are few reports of daily hormone and body temperature rhythms in burn patients. Molteni et al. (1979) reported high levels of plasma cortisol, aldosterone, and renin with disappearance of rhythms, whereas Vaughan et al. (1982) observed elevated 24 h plasma cortisol levels in proportion to the extent of body burned, but with the maintenance of a significant circadian rhythm of lower amplitude and normal peak time. Also, the amplitude of the plasma melatonin rhythm was decreased, whereas the mean levels of resting heart rate and body temperature were increased (Vaughan et al., 1985).

Melatonin, cortisol, and temperature rhythms are controlled by a common circadian clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Claustrat et al., 2005). These interplaying rhythms are involved in the regulation of the sleep-wake cycle and internal sleep structure (Gronfier et al., 1999), and more generally in the temporal organization of many physiological functions, including immunity (Guerrero & Reiter, 2002) and antioxidant defenses (Reiter et al., 2000). Free radical generation is associated with thermal injury, and the activation of a proinflammatory cascade after burn injury seems to be important in the development of immune dysfunction, susceptibility to infections, and multiple organ failure (Ward & Till, 1990). Also, a significant proportion of burn patients displays long-term disturbance of sleep and metabolism (Boeve et al., 2002), suggesting the participation of biological rhythm disturbances in chronic insomnia (Lack & Wright, 2007). In view of these data, we longitudinally investigated in an explorative study the 24 h temperature rhythm as well as the daily urine profiles of melatonin, 6-sulfatoxymelatonin (aMT6s, the main hepatic melatonin metabolite; Arendt et al., 1985), and free cortisol in burn patients.

## MATERIALS AND METHODS

### Subjects

This prospective study, which involved non-invasive biological sampling, was approved by the institutional Ethics Committee of Hospices Civils de Lyon and met the ethical standards of this journal (Portaluppi et al., 2008). Eight burn patients (six men/two women whose ovarian cycle provisionally ceased after burn injury), aged from 19 to 33 yrs (mean  $\pm$  SD:  $25 \pm 6$  yrs), were admitted in the intensive care unit (ICU) within 24 h after burn injury. The burned skin surface was  $>30\%$  (mean  $\pm$  SD:  $53 \pm 16\%$ , range 37–87%). The abbreviated burn severity index (ABSI; Tobiasen et al., 1982) was between 6 and 12 (median 8). During the first 72 h in the ICU, patients received inotropic support ( $n = 4$ ) with norepinephrine, antibiotic therapy ( $n = 4$ ) for systemic infection, sporadic paracetamol injection ( $n = 4$ ) when fever rose  $>39^\circ\text{C}$ , and furosemide ( $n = 1$ ). All patients were sedated with midazolam, sufentanyl, and ketamine the first week of hospitalization and then received morphine as analgesic treatment. Plasma cortisol response to corticotropin stimulation was normal, as were the biochemical indexes of hepatic and renal functions. None of the patients received corticoid treatment during the first three weeks of hospitalization. The mean duration of hospitalization in the ICU was 46 days (range 22–85 days). Environmental conditions were those of ICU wards, including artificial lighting from cardiopulmonary monitoring and during hourly nursing rounds. The luminance in the room was 180–200 lx during the day and  $<5$  lx during the night, except during nursing rounds (150–200 lx for  $\sim 10$  min/h). During this time span, patients were not submitted to direct lightening and usually their eyes were closed, especially during the early session. Controls consisted of 14 healthy subjects (12 men/2 women investigated during their follicular phase) of the same age range (18–30 yrs) as the burn patients who collected their urines at home. They did not display marked morningness or eveningness, as assessed by the Horne and Östberg (1976) test.

### Hormone and Temperature Studies

Daily urine excretions of melatonin, aMT6s, and free cortisol plus core temperature were studied on three occasions after admission to the ICU (early session: days 1 to 3; intermediate session: day 10; late session: days 20 to 30). A previous report had shown that both urinary melatonin and aMT6s assays correlate significantly with daily plasma melatonin profiles and can be used as non-invasive methods to study melatonin secretion (Paakkonen et al., 2006). Urine samples were collected at 4 h intervals for 24 h, beginning at 08:00 h. Urine samples were kept at  $4^\circ\text{C}$

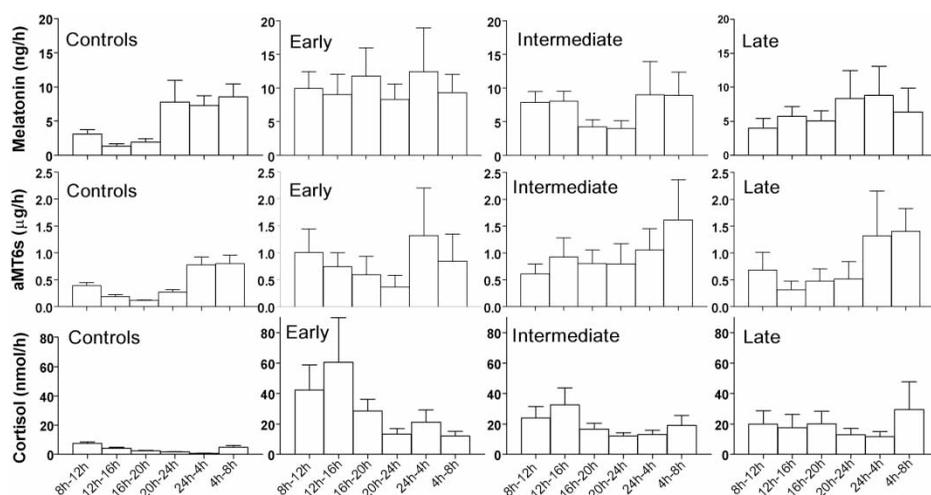
until the end of collection, and then aliquoted and stored at  $-20^{\circ}\text{C}$  until assayed. Urinary melatonin, aMT6s, and free cortisol levels were determined by radioimmunoassay or radiocompetition as previously reported (Brun et al., 1987; Harthé et al., 1991; Murphy, 1968). Quantity rates (quantities/time span) were calculated for each parameter. This resulting expression takes into account a possible dilution of urine related to the hydration of patients. Core temperature was recorded hourly through a bladder temperature sensor connected to an indwelling catheter in the early and intermediate sessions and a clinical thermometer in the late session. Due to dressing replacement (every day or more) and/or surgical operation when patients experienced hypothermia, non-relevant temperature values were excluded from the profiles. Reference values had previously been determined in healthy controls of the same age-range (Brun et al., 1998).

### Statistics

Results of the analyses of the hormone and temperature profiles are expressed as mean  $\pm$  SEM in figures. Rhythms of hormone excretion were studied by mean cosinor analysis on the 4 h urine blocks (Faure et al., 1990). The Chronos-Fit program (Zuther & Lemmer, 2005) provided the mesor (the 24 h mean of the data points) and an estimation of the amplitude (A, one-half the total peak-to-trough difference) and acrophase ( $\phi$ , peak time), with 95% confidence intervals, of the best fitting cosine function. In each case,  $F$  statistic was calculated to examine the appropriateness of the adjusted function and of the time-dependence of data (zero-amplitude test). The sinusoidal test was met when the calculated  $F$  was inferior to the value read in an  $F$ -table. Hormone concentrations were time-dependent when the null hypothesis  $H_0$  was rejected by the zero amplitude test (calculated as  $F > F_{0.95}$ ). Also, the urinary data were divided into 12 h (day, D, 08:00–20:00 h and night, N, 20:00–08:00 h) and 24 h blocks; then, the N/D melatonin and aMT6s ratios and the D/N cortisol ratio were calculated. The data analyzed using Graphpad Prism 4 program were submitted to the non-parametric Kruskal-Wallis rank sum test followed by multiple post-hoc comparisons using Dunn's test. Individual temperature profiles were analyzed by the Chronofit program (Zuther & Lemmer, 2005).

### RESULTS

Patients displayed good compliance with the non-invasive protocol. Daily variations and findings of the mean cosinor of hormone excretion are presented in Figure 1 and Table 1, respectively. No significant rhythm was detected in patients by cosinor analysis for any of the



**FIGURE 1** Mean daily profiles of urinary melatonin, aMT6s, and free cortisol excretions in patients (early session,  $n = 8$ ; intermediate session,  $n = 6$ ; late session,  $n = 4$ ) and controls.

hormones (calculated  $F < F_{0.95}$ ), so that only mesors could be compared between patients and controls.

## Melatonin

The Kruskal-Wallis test showed heterogeneity of the D melatonin levels and N/D ratios between groups (see Figures 2a [left] and 2c). Compared with controls, the D melatonin levels of the patients were significantly increased in all three sessions. Due to this increase, the N/D ratios were decreased in all three sessions (see Figure 2c). The 24 h urinary melatonin levels did not display such heterogeneity, although there was a tendency for an early increase followed by a progressive normalization with time in patients (see Figure 2b). The early session increase of mesor confirmed this tendency (see Table 1).

## aMT6s

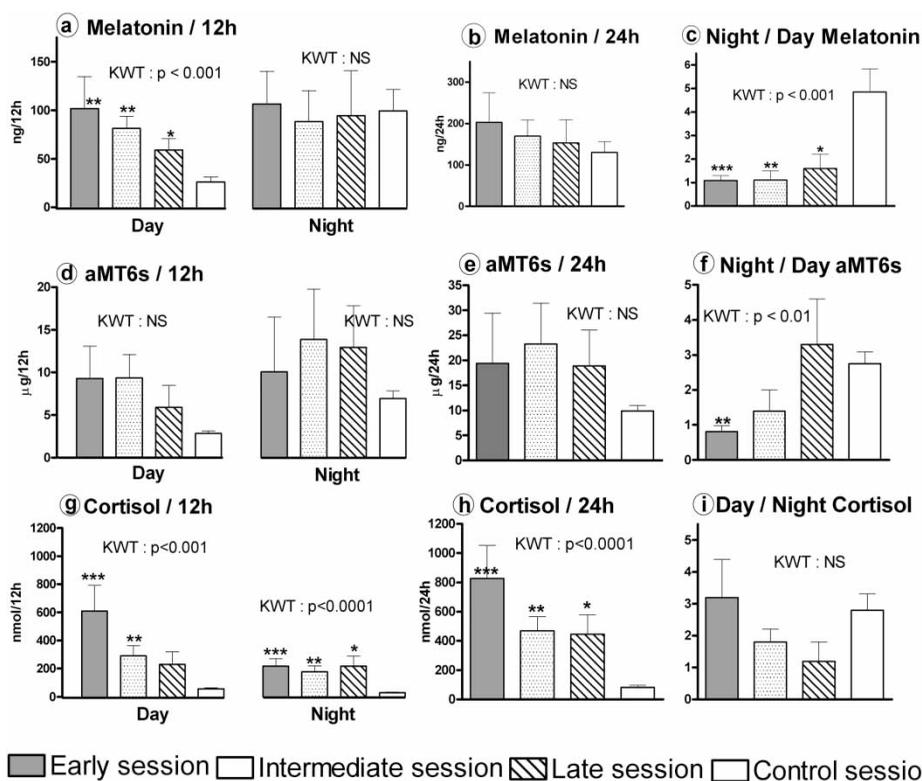
The D, N, and 24 h aMT6s levels did not display heterogeneity by the Kruskal-Wallis test (see Figures 2d and 2e). Compared with controls, there was a tendency, however, of an increase in aMT6s in the early and intermediate sessions in patients, and this was confirmed by increased mesors in the same sessions (see Table 1). Also, visual inspection of the data showed an evolution of the D aMT6s levels like that of the D melatonin ones. In addition, the melatonin/aMT6s ratio (an index of melatonin metabolism) did not differ between controls and patients (D and N ratios in controls:  $0.009 \pm 0.001$  and  $0.017 \pm 0.012$ ; D and N ratios in the early,

**TABLE 1** Cosinor parameters (95% confidence levels) of urinary melatonin, aMT6S, and free cortisol profiles

Variables	Cosinor parameters	Controls	Patients (early session)	Patients (intermediate session)	Patients (late session)
Melatonin	Mesor (ng/h)	2.8–5.4	6.8–12.8 <sup>†</sup>	4.9–9.3	4.2–8.6
	Amplitude (ng/h)	0.9–4.6	—	—	—
	Acrophase (h:min)	22:52–04:00	—	—	—
	Sinusoidality test	$F_{78}^3 = 1.1$ (2.73)	$F_{42}^3 = 0.35$ (2.83)	$F_{24}^3 = 0.34$ (3.01)	$F_{12}^3 = 0.14$ (3.49)
	Amplitude 0 test	$F_{81}^2 = 4.4$ (3.1)*	$F_{45}^2 = 0.09$ (3.23)	$F_{27}^2 = 1.43$ (3.35)	$F_{15}^2 = 0.96$ (3.68)
aMT6S	Mesor (ng/h)	272–417	419–1197 <sup>†</sup>	625–1311 <sup>†</sup>	308–931
	Amplitude (ng/h)	141–346	—	—	—
	Acrophase (h:min)	03:07–06:20	—	—	—
	Sinusoidality test	$F_{78}^3 = 0.13$ (2.73)	$F_{42}^3 = 0.42$ (2.83)	$F_{24}^3 = 0.70$ (3.01)	$F_{12}^3 = 0.42$ (3.49)
	Amplitude 0 test	$F_{81}^2 = 11.3$ (3.1)*	$F_{45}^2 = 0.55$ (3.23)	$F_{27}^2 = 0.64$ (3.35)	$F_{15}^2 = 1.71$ (3.68)
Cortisol	Mesor (ng/h)	2.6–3.7	20.2–49 <sup>†</sup>	14.4–24.7	11.1–26.2
	Amplitude (ng/h)	A = 1.9–3.5	—	—	—
	Acrophase (h:min)	$\phi = 10:04$ –12:22	—	—	—
	Sinusoidality test	$F_{78}^1 = 0.51$ (3.95)	$F_{42}^3 = 0.75$ (2.83)	$F_{24}^3 = 0.39$ (3.01)	$F_{12}^3 = 0.44$ (3.49)
	Amplitude 0 test	$F_{79}^4 = 26.8$ (2.5)*	$F_{45}^2 = 2.95$ (3.23)	$F_{27}^2 = 3.09$ (3.35)	$F_{15}^2 = 0.42$ (3.68)

\*Indicates significant 24 h rhythm.

<sup>†</sup>Indicates results significantly different by comparison with the 95% confidence interval of controls.



**FIGURE 2** Melatonin, aMT6s, and free cortisol excretions in patients (early, intermediate, and late sessions) and controls. Day (D) and night (N) excretions (left); 24 h excretion (middle); N/D or D/N ratio (right). Kruskal Wallis test (KWT): N.S., non-significant. Post-hoc comparisons between patient and control sessions: \* $p < 0.05$ ; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

intermediate, and late patient sessions, respectively:  $0.031 \pm 0.011$  and  $0.050 \pm 0.022$ ,  $0.011 \pm 0.003$  and  $0.027 \pm 0.015$ , and  $0.029 \pm 0.022$  and  $0.0077 \pm 0.0033$ ; Kruskal-Wallis test non-significant). Finally, the N/D aMT6s ratios displayed heterogeneity and an early decrease in patients compared with controls (see Figure 2f), with normalization of the ratio with time.

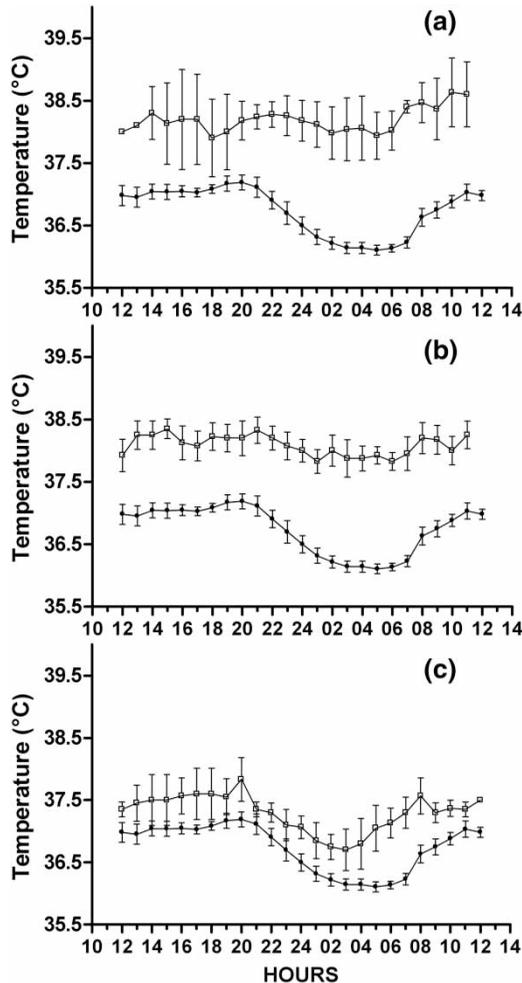
## Cortisol

The Kruskal-Wallis test showed heterogeneity of the D, N, and 24 h urinary free cortisol levels between groups (see Figures 2g and 2h). The D and 24 h excretions were significantly increased in all sessions, except for the D excretion of the early session, and they displayed a progressive decline with time. However, the N excretion remained increased compared with controls; consistent with this observation, the mesors were consistently increased (see Table 1). Circadian rhythm detection approached near-statistical significance in the early and intermediate

sessions (see Table 1). Due to both increases of D and N values, the D/N ratio was not significantly altered over time (see Figure 2i).

### Core Temperature

The mean daily profiles of core body temperature for each session are presented in Figure 3. Five, four, and three individual temperature profiles were available in the early, intermediate, and late sessions, respectively. Individual mesors were above the upper limit of the 95% confidence interval of controls whatever the session (see Table 2). In the early session, four of the five studied patients displayed a significant circadian rhythm, but its amplitude was low. In the intermediate session, two



**FIGURE 3** Mean daily core body temperature profiles in patients (●: a, early; b, intermediate; c, late session) and controls (□).

**TABLE 2** Cosinor parameters of temperature profiles of patients and controls

Patients	Early session			Intermediate session			Late session		
	M (°C)	A (°C)	φ (h:min)	M (°C)	A (°C)	φ (h:min)	M (°C)	A (°C)	φ (h:min)
1	38.12	0.46	10:21	38.29	—	—	37.59	0.36	17:13
2	37.71	0.33	22:37	37.49	0.19	16:02	36.93	0.31	20:14
3	38.34	—	—	—	—	—	37.6	—	—
4	38.11	0.24	19:35	38.25	—	—	—	—	—
5	38.46	0.36	15:59	38.22	0.52	18:23	—	—	—
Controls	36.70–36.76°C*	0.47–0.55°C*	15:47–16:27*						

When the circadian rhythm is not significant, only the mesor (M) is given. A is amplitude and φ is acrophase (referenced to local 00:00 h).

\*95% confidence intervals (see Kocher et al., 2006).

patients displayed a significant rhythm with low or normal amplitude. Finally, a significant rhythm was observed in two of the three patients studied in the late session, but the amplitude was low. Globally, all patients displayed an increased temperature mainly during the night and an abnormal acrophase, except patient 5 (early session) and patient 2 (intermediate session).

## DISCUSSION

Using a non-invasive protocol, we observed major alterations of the daily profiles of urinary melatonin, aMT6s, and cortisol plus the 24 h core body temperature rhythm in burn patients, which persisted for several weeks after injury. Although the sample of patients was small, the results are clear. We did not investigate another patient group as a hospital control that would have received similar treatment or displayed the same recovery kinetics, and this constitutes a shortcoming of this study. We do not believe the observed alteration of melatonin secretion is related to the ICU environment. For example, the episodic lighting during the night did not evoke a decrease in nocturnal melatonin excretion and was not sufficient to shift the secretion pattern from the night to the day. In this regard, Bojkowski et al. (1987) showed that a 300 lx light intensity administered for 30 min is necessary to obtain slight melatonin suppression in healthy subjects instructed to continuously look directly at the light. Due to the dramatic increase of the D melatonin excretion, the N/D melatonin variation was abolished in the three study sessions. This result does not parallel that of Vaughan et al. (1985), who reported normal (low) plasma melatonin levels during the day and decreased nighttime values on the post-burn day. Also, the urinary melatonin excretion rates were more disturbed than the aMT6s ones. This was not related to the medical hydration of the patients and/or abnormal melatonin metabolism, as the patients displayed kidney and hepatic parameters in the normal range. In addition, the D and N melatonin/aMT6s ratios did not differ between patients and controls in any session, and the D and N aMT6s levels were of the same order of magnitude (around 1 $\mu$ g/h) as those reported in critically ill patients with severe sepsis (Mundigler et al., 2002). A further study including combined plasma and urine sampling could clarify the discrepancy between both profiles, although it is difficult to carry out for ethical reasons. Finally, one could ask whether destruction of the skin, a site of melatonin biosynthesis, may contribute to abnormal circulating melatonin levels (Slominski et al., 2005). This hypothesis is weak but, at the very least, the cutaneous melatonergic system, which is involved in preserving the integrity of skin from oxidative stress (Fischer et al., 2008), is probably impaired in burn patients.

We feel that the type of medications administered to the burn patients had little influence on the results. Opioids show large interspecies differences in their effects on melatonin secretion. For example, in the domestic pig, morphine does not alter plasma melatonin concentration when administered in the morning or evening of the diurnal light-dark cycle (Lewczuk et al., 1999), whereas it displays a stimulatory effect, at least in vitro, on bovine pinealocytes (Chuchuen et al., 2004) and rat pineal gland (Chetsawang & Govitrapong, 2005). No such data are available for humans. Also, the abnormal N/D melatonin variation was not a consequence of benzodiazepine treatment. Indeed, adinazolam was not associated with any change in aMT6s output during a six-week treatment in depressed patients (Kennedy et al., 1992). In addition, ketamine, which was episodically administered during the day for dressing replacement and/or surgical operation, blocks the effects of the excitatory neurotransmitter glutamic acid at the N-methyl-D-aspartate (NMDA) receptors (Gunduz-Bruce, 2009). The acute administration of NMDA antagonists at the beginning of the dark period induces an inhibition of melatonin production in hamsters (Vuillez et al., 1998). These conditions of administration are quite different from ours. Further, the impaired melatonin secretion could not be related to pineal  $\beta$ -receptor response to elevated norepinephrine levels due to major stress or treatment, as the human pineal gland displays poor responsiveness to circulating catecholamines and adrenergic agonists (Berlin et al., 1995; David et al., 1987). Finally, corticosteroids could modify the density and sensitivity of pineal  $\beta$ -receptors, as demonstrated for lymphocytes (Davies et al., 1980). However, the chronic situation of hypercortisolism should have decreased melatonin secretion as a result (Claustrat et al., 1984).

In agreement with a previous study involving the determination of free plasma cortisol (Garrel, 1996), urinary free cortisol displayed an initial increase, mainly during the night, that was maintained over the complete study. Hypercortisolism could have reinforced clock alteration, as glucocorticoids suppress gene expression of vasopressin, a main neurotransmitter in the SCN (Liu et al., 2006). Also, this sustained hypercortisolism could participate in osteoporosis, which is a major after-effect of burn injury (Pandit et al., 1993).

Although there was a tendency toward recovery, abnormal temperature profiles, which were observed mainly during the night, persisted one month after injury. This is in agreement with experimental data that showed both an increase in body temperature and a decrease of its circadian variation following burn injury in the rat (Caldwell et al., 1999).

Sleep disorders are a frequent complication of burn injury for a variety of reasons, including those associated with trauma and treatment (i.e., ICU environment, pain, itching, and medication; see Boeve et al., 2002; Jaffe & Patterson, 2004; Raymond et al., 2004). Both long-term

cortisol hypersecretion and abnormal decrease of evening temperature could be supplementary major factors (Krauchi, 2007; Vgontzas & Chrousos, 2002). The inappropriate secretion of melatonin could have played a role in the alteration of the temperature profile. In physiological conditions, melatonin reinforces the nocturnal decrease of core body temperature (Strassmann et al., 1991), a phenomenon that facilitates sleep propensity. This is the consequence of peripheral vasodilation related to stimulation of melatonin receptors present in the peripheral vasculature (Krauchi et al., 2000). In burn patients, vascular receptors might be desensitized due to the dramatic increase of daytime melatonin secretion.

Other physiological functions that display a circadian organization and that are influenced by the melatonin signal, especially immune and antioxidative defenses, are affected in burn patients. Burn patients display severe alterations of the immune system, mainly in IL-2, IL-6, and the soluble forms of IL-2 and CD25 receptors (Jobin et al., 2000; Peteiro-Cartelle et al., 1999). Interactions between the pineal gland and the immune system are bidirectional, as interleukins and cytokines affect melatonin synthesis and release (Withyachumnarnkul et al., 1990).

Oxidative stress plays an important role during sepsis and burn trauma and may be involved in the development of organ injury (Horton, 2003). Melatonin is a potent free radical scavenger and displays antioxidative properties (Tan et al., 2002). Moreover, it is a major skin protectant with a wide spectrum of effects upon the maintenance of skin homeostasis against different stress-inducing events, including burns (Fischer et al., 2008). Exogenous melatonin displays protective effects on burn injury in rats (Tunali et al., 2005). At the present time, experimental evidence indicates that endogenously produced melatonin is relevant as a physiological antioxidant (Reiter et al., 2005). Also, beneficial pleiotropic actions of melatonin have been observed in septic newborns (Gitto et al., 2001). Considering the synchronizing role of melatonin as well as its immunomodulatory and antioxidative activity during the natural immune response, reinforcement of the night/day variation by the administration of a supraphysiological melatonin dose could be beneficial after burn injury (Maldonado et al., 2007). We suggest undertaking such an investigation in controlled conditions.

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