Research Article

Effect of iron on rat serum melatonin levels under different light/dark cycle patterns

Joaquín Cogo Pagella[1], Marcelo P. Hernando[1,2], Claudio O. Cervino[1*]

[1]Instituto en Ciencias Básicas y Experimentales (ICBE, SeCyT), Universidad de Morón. Machado 914, (1708) Morón, Buenos Aires, Argentina
[2]Departamento de Radiobiología, Centro Atómico Constituyentes (CAC-CNEA). Av. Gral. Paz 1499, (1650) Villa Maipú, Buenos Aires, Argentina
*Correspondence: ccervino@unimoron.edu.ar, Tel: +54 11 6269 0821

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ABSTRACT

Exposure to constant light or darkness for long periods has diverse effects on circadian physiology. Iron (Fe) overloading promotes oxidative stress and causes alterations in cellular structure and function in animals and humans. The aim of this study is to evaluate the interactions among serum melatonin (ML), photoperiod manipulation, and Fe overloading in rats. The results showed that constant darkness exposure for 15 days significantly increased serum ML levels (up to 22%) while the constant light exposure failed to reduce the serum ML level compared to the normal light/dark cycle treated rats. The lost serum ML level usually from the pineal gland under the long term of constant light exposure may be compensated by ML generated by other organs which adapted to the situation. Also, Fe overloading decreased ML production due to this molecule being consumed to scavenge the free radicals induced by the Fe overloading. In addition, we observed interactions among constant light or darkness exposure, Fe overloading and serum ML level. Overall, our results support the hypothesis of ML as scavenging molecule; it may be an effective therapeutic tool in iron-induced oxidative stress.

Key words: Melatonin, iron, photoperiod, light/dark cycle, oxidative stress, circadian rhythm

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1. INTRODUCTION

Melatonin (ML) production of pineal gland is controlled by the light-dark cycle (LDC). The pineal ML is typically synthesized during darkness and suppressed by daylight, resulting in a circadian rhythm (1). Thus, ML is an important component of the circadian system and has been proposed as a link between the presence/absence of light and its effects on animal behavior and physiology (2-5).

ML plays multiple roles in animal physiology. This molecule, produced by the pinealocytes, enters the circulatory system and binds to receptors on a variety of target tissues to exert its physio-biochemical responses (6, 7). The major function of pineal ML is to relay information about changes in photoperiod (3, 8, 9). Additionally, among its physiological functions, this

molecule has sedative, hypnotic, analgesic, and anti-inflammatory properties that make it an attractive alternative for premedication in anesthesia for both animals and humans (6, 10).

ML is not only synthesized in the pineal gland, but other sources of extrapineal ML are present including the Harder gland, retina, skin, intestine and among others (11, 12). ML concentration in some of these tissues appears to be higher than that found in plasma (13). For example, ML levels in the gastrointestinal tract are 10-100 times higher than that in serum (14-16). Also, high level of ML was detected in the thymus of rats which was two orders of magnitude higher than that in serum (17). At the cellular level, ML is positively linked to cell maintenance (18) and mitochondrial activity (19-21).

ML also plays an important role as a free radical scavenger and antioxidant, reducing oxidative stress in organisms (for reviews, see 4, 19, 22). Oxidative stress occurs when the steady-state concentration of reactive oxygen species (ROS) increases in body due to an imbalance between the production of ROS and the availability of antioxidants (23). Brain is the easy target of oxidative stress due to its high iron loading with other factors.

In the current study, we will investigate brain oxidative stress and its relationship with ML using a rat model overloaded with iron (Fe) which is a common model for neuronal oxidative stress (24-27).

Fe is an essential bioactive element required for cellular and body's normal physiology. Clinical and epidemiologic observations indicate that increased Fe storage status is a risk factor in several diseases (28, 29). Fe is an important mediator of cell oxidative damage, especially, under the condition of its overloading to catalyze an increase in the steady state concentration of ROS (28). Free radical-mediated oxidative stress in cells is one of the main causes of alterations in cellular structure and function due to Fe overloading (30, 31). The toxicity of superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) arises from their Fe-dependent conversion into the extremely reactive hydroxyl radical (•OH) via Haber–Weiss reaction to generate severe damage to membranes, proteins, and deoxyribonucleic acid (DNA) (32). Naturally, organisms have antioxidative mechanisms including proteins that preserve Fe homeostasis and keep most of this metal sequestered, preventing free Fe from catalyzing free radical reactions (33). Ferroptosis (34) is a mechanism for non-apoptotic, iron-dependent, oxidative cell death. Ferroptosis is crucially involved in neurological diseases, including neurodegeneration, stroke and neurotrauma (for review, 35).

Several studies have shown the suppressive effect of ML against iron-induced oxidative stress (27, 36, 37).

The administration of different compounds and quantities of Fe, either as a dietary supplement or intraperitoneal injection, leads to an increase of Fe in several tissues and plasma (38). Among the various formulations used for iron supplementation, Fe-dextran (Fe-D) injection seems to be a suitable model for the study of iron toxicity since the pathological and clinical consequences of its administration resemble those of acute Fe overloading in humans (29).

In a Fe-D intraperitoneal injection model of rats, Galleano and Puntarulo (39) reported a 12-fold increase of Fe concentration in plasma 20 h after injection compared to control values. Similarly, in a previous study of the same model, we found a 5-fold increase of Fe in the brain 6 h after injection compared to control values (25). Since the studies related to the relationship between circulating ML level and iron are scarce (40-42), the aim of this work is to study the effects of Fe overloading on serum ML levels under the conditions of photoperiod 12L:12D or under the constant LDC.

2. MATERIAL AND METHODS

2.1. Experimental designs.

Forty male Sprague-Dawley rats weighing between 215 to 245 g were acclimated for 2 weeks at a room temperature of 22±2º C and under a normal light-dark cycle (LDC) of 12L:12D (lights on at 8:00 am). Then, the rats were randomly divided into 8 groups, with 5 animals per group, and were housed in cages with *ad libitum* access to food and water.

The experimental groups were as follows (Table I): (1) control group (CG), in which the animals were only injected with vehicle under normal LDC; (2) ML, in the rats were only injected ML dissolved in the vehicle; (3) LL, in which rats exposed to constant light; (4) DD, in which rats were exposed to constant dark; (5) Fe group (IG), in which the rats were injected with Fe-D under normal LDC; (6) IG+ML, in which the rats were injected with Fe plus ML under normal LDC; (7) IG+LL, in which the rats were injected Fe and exposed to constant light, and (8) IG+DD, in which rats were injected Fe and exposed to constant dark (See the details of each treatment below).

In all groups, to prevent viscera penetration, intraperitoneal (i.p.) injections of ML or Fe-D were performed in the lower left abdominal quadrant of each rat using a 25 G x 5/8 needle, with a dosing volume of 1.2 mL/rat. The zeitgeber time (ZT, where ZT0 = lights on) was considered for the time of injections (ZT= 0 for Fe-D and ZT= 4 for ML).

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Morón (Acta Nº 1, 3/21/2022). Experimental animals were treated according to the Canadian Council on Animal Care (https://ccac.ca/en/guidelines-and-policies/the-guidelines/) as well as according to ARRIVE guidelines (https://arriveguidelines.org/).

Table I. Experimental design.

Groups	Day	D1			D15		
	Hour	8 am	12 pm	16 pm	8 am	12 pm	16 pm
	ZT	0	4	8	0	4	8
CG		--	cv	bs	--	--	--
ML		--	ML	bs	--	--	--
LL		--	--	--	--	cv	bs
DD		--	--	--	--	cv	bs
IG		Fe-D	cv	bs	--	--	--
IG+ML		Fe-D	ML	bs	--	--	--
IG+LL		--	--	--	Fe-D	cv	bs
IG+DD		--	--	--	Fe-D	cv	bs

Groups: CG, control; ML, melatonin; LL, constant light exposure; DD, constant dark exposure; IG, iron; IG+ML, iron+melatonin; IG+LL, IG in constant light exposure; IG+DD, IG in constant dark exposure. ZT: zeitgeber time. Procedure: cv, control vehicle (ip); ML, melatonin 50 mg/kg (ip); Fe-D, Fe-dextran 500 mg/kg (ip); bs, blood sample collection.

2.2. Melatonin treatment.

The ML group (ML) and Fe+melatonin group (IG+ML) received a single dose of 50 mg/kg body weight of ML (Sigma-Aldrich, St. Louis, MO, USA), which was administered at 12:00

pm (ZT= 4). The ML dose was established according to our previous work (10) and reported in the literature (43-45).

ML solution was prepared with the milligrams of ML to be used in five rats according to their weights. ML was first dissolved in 0.2 mL of absolute ethanol (Sintorgan®) and then diluted with 0.9% saline till to a final volume of 6.0 mL. The final ethanol concentration was less than 3.3%, and a final ML concentration was 9.6 mg/mL.

Both in the condition with or without Fe, ML was injected during the circadian time ZT= 4, when endogenous ML levels are minimal (46). The serum ML concentration in blood was evaluated after 4 h of its intraperitoneal injection.

The vehicle (CG) was also injected with the volume as ML rats at 12:00 pm (ZT= 4).

2.3. Iron treatment.

To evaluate the effect of Fe on ML levels, blood samples were obtained 8 hours after acute Fe-D overloading and at this period the serum Fe level was high (25).

In the Fe groups (IG; IG+ML; IG+LL, and IG+DD), each animal received a single dose of 500 mg/kg body weight of (Sigma-Aldrich, St. Louis, MO, USA) at 8 am (ZT= 0).

Four hours later (ZT= 4) after Fe-D injection, ML at the dose of 50 mg/kg body weight was injected in the IG+ML group (Table I). The delay in ML administration was due to the fact that maximum Fe concentration was determined 6 h after its injection (25). This procedure ensures high ML level to match the high Fe-D concentration, therefore, to better evaluate its antioxidant capacity. In fact, the serum ML concentration in CG is the reference of the normal serum ML content and can be used to compared the melatonin in the IG group (Table II).

2.4. Treatment with constant light exposure.

Two groups of rats were kept in an environment illuminated with two fluorescent tubes (Philips TL-D 36W 840 Super 80; light color: 4000K - cool white; luminous flux: 3350 Lm). Rats were treated with constant light exposure (LL) for 15 days. On day 15, the ML or Fe-D were injected in CG+LL and IG+LL, respectively, as described above.

2.5. Treatment with constant dark exposure.

Two groups were kept in an environment with constant dark (DD) for 15 days. On day 15, the ML or Fe-D were injected in CG+DD and IG+DD groups, respectively, as described above. The cage cleaning, feeding, and handling of the animals were carried out with red light (incandescent lamp, 25W) kept 3 m away from the animals.

2.6. Preparation of blood samples.

To avoid the influence of the photoperiod on circadian rhythms, blood collection in all experimental groups was at 4 pm (ZT= 8).

Blood samples were taken from the heart of anesthetized animals in a CO_2 chamber. Samples were taken four hours after vehicle or ML injection in CG and ML groups, and eight hours after Fe-D injection in IG and IG+ML groups. Similarly, the process of blood collection was repeated on day 15 for CG+LL, IG+LL, CG+DD and IG+DD groups. All samples were collected from 4 to 4:30 pm immediately after being brought out from the experiment. Five-milliliter blood was collected and stored at room temperature for 10-20 min to clot. After centrifugation at 3000 rpm for 10 min, the serum was collected and stored at -20º C for future analysis. All the procedures described were performed under dim artificial light.

2.7. Melatonin measurement.

Serum ML levels were measured using the ELISA assay (Melatonin ELISA Kit (ab213978), Abcam), according to the instructions provided by the manufacturer.

2.8. Statistical analyses.

Two-Way ANOVA and *post-hoc* All Pairwise Multiple Comparison Procedures (Tukey Test) were used for statistical analysis of the data (SigmaStat 3.5, Systat Software, Inc). All data were expressed as mean and standard deviation (SD) with a significance level of $p < 0.05$.

3. RESULTS

The results are presented in Table II. A significant difference was observed in serum ML levels among groups for both conditions (with/out Fe, $p < 0.001$) also for different treatment (control, melatonin, LL and DD, $p < 0.001$) and their interactions ($p < 0.001$) (Figure 1).

Table II. Serum levels of melatonin (pg/mL) in different groups.

Groups	Mean	SD	Median	Minimum	Maximum	*p*
CG	289.4	32.65	289.0	249.7	340.1	---
ML	504.4	91.43	503.0	425.0	653.6	< 0.001*vs* CG
LL	317.6	36.68	317.6	281.9	374.6	0.854 *vs* CG
DD	353.1	16.92	353.1	336.2	371.0	0.044 *vs* CG
IG	199.9	61.50	229.1	115.9	267.1	0.016 *vs* CG
IG+ML	455.1	95.18	454.1	347.5	604.8	< 0.001*vs* IG 0.172 *vs* ML
IG+LL	33.9	25.26	22.4	7.5	62.4	< 0.001*vs* IG < 0.001 *vs* LL
IG+DD	32.9	20.18	19.8	17.4	57.3	< 0.001*vs* IG < 0.001 *vs* DD

SD: standard deviation.

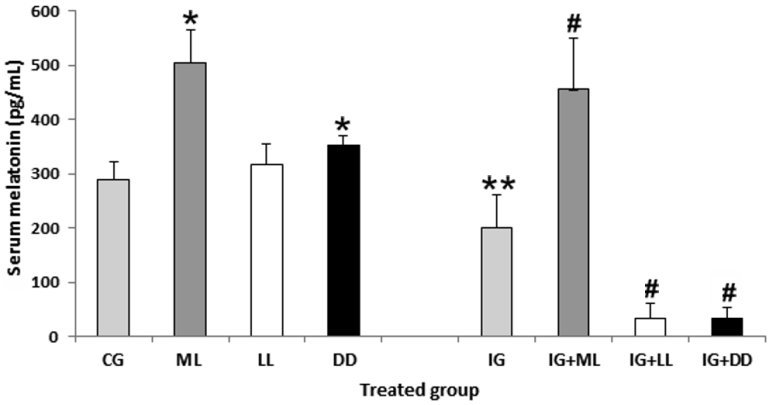

Fig. 1. Serum levels of melatonin (pg/mL) in different experimental groups

Data were expressed as *mean ± SD. *: p < 0.05 vs CG, #: p < 0.05 vs IG, **p < 0.05 vs CG. CG, control group; ML, melatonin group; LL, continuous light exposure; DD, continuous dark exposure; IG, iron group; IG+ML, iron+melatonin group; IG+LL, IG in continuous light exposure; IG+DD, IG in continuous dark exposure.*

Injection of ML (50 mg/kg) resulted in ML increase (74%) in serum compared to the CG, showing significant differences even 4 hours after ML administration in the illuminated phase of the LDC. Injection of Fe-D (500 mg/kg) caused a significant decrease (31%) of serum ML compared to CG. Administration of Fe-D and ML (IG+ML) resulted in a significant increase of serum ML level compared to IG, but slightly lower than the ML group ($p > 0.05$).

While a constant light exposure of 15 days produced a slight but not significant increase in endogenous ML compared to the control ($p > 0.05$). In the presence of ion, an abruptly reduced serum ML up to 17% was observed compared to the CG group ($p < 0.001$) and to 11% in the CG+LL group compared to the IG group ($p < 0.001$) (Table II and Figure 1).

The rats with constant dark exposure of 15 days (DD) had a significant higher ML level (22% higher) than that in the CG ($p < 0.05$); however, in the presence of Fe, this increase was dropped to 16% of its original level (IG+DD vs IG) ($p < 0.001$) and to 9% compared to the CG+DD ($p < 0.001$) (Table II and Figure. 1).

4. DISCUSSION

In the present study we demonstrated that high levels of Fe in blood were associated with a decreased ML production. In addition, the potential associations between serum ML and the different photoperiodic exposure including the constant light (LL) or darkness (DD) exposure, and the high level of Fe in blood were uncovered.

The serum ML levels and their variations in our study were consistent with those observed in the previous studies (46-49). Comparing to the baseline of animals who were exposed to a normal 12L:12D photoperiod with ML injection had a significantly high serum ML concentrations. This result was different from those observed by Farhadi *et al*. (49) in which the oral ML administration did not change serum ML levels in rats exposed to 12L:12D light/dark cycle for 10 days. This difference may be due to the ML dose differences since the dose in our study was five times higher than that they used and the delivery methods were also different.

In addition to pineal gland, the retina and CNS, together with their interconnections, constitute the main circadian axis (1, 24, 50). The LDC mediates a series of non-visual responses through this circadian axis that involves, among others, the phase change of the internal circadian clock and the rhythm and ML production levels, both daily and seasonally.

In vertebrates, including humans, constant light exposure alters many aspects of their circadian rhythm and has negative effects on their physiology. The negative physiological effects of constant light exposure include alterations in growth (51), the accelerated aging (52), the increased depression and anxiety (53), an increased risk of cancer (54), and generally, a negative impact on immune function (55, 56). The absence of a circadian signal is likely to be a strong driver that underlies these effects. However, similar effects are evidenced in response to an extension (in the number of hours) of exposure to light or dim light during the period of natural darkness (57, 58). All together, these studies suggest that the light in natural darkness could be the reason for behavioral and physiological alterations (59, 60).

There are no conclusive results from the literature on the effects of a constant or prolonged period of illumination on serum ML levels and their circadian rhythms. Our results showed that after exposure to constant light for 15 days (LL), serum ML concentrations of the rats did not exhibit significant changes compared to the rats with normal LDC. The results were consistent with Farhadi *et al.* (49) in which, they also demonstrated that 10 days of constant light exposure failed to induce serum ML changes in rats. However, in several other studies, it was reported that animals kept under constant light for one or two weeks, were enough to inhibit their ML production of the pineal glands in chickens (40), hamsters (61) and rats (62, 63).

In the present study, the lack of change in serum ML after constant light exposure could be explained by (a) the function of the pineal gland in the long term of constant light exposure is not affected (64, 65), and (b) the decreased pineal ML secretion with long term of illumination was compensated by other ML secreting organs, such as the Harder gland, restoring the serum ML level (3, 49).

On other hand, behavioral and neural consequences of exposure to constant darkness have been reported in laboratory animals (66, 67). Rats under constant darkness for several weeks exhibited distinct behavioral and anatomical features that are similar to characteristics of depressed patients in humans (68). In humans, it has been demonstrated that the circadian system is disturbed during the polar winter, largely due to insufficient bright light (69).

In this study, the constant dark exposure for 15 days in rats (DD) significantly increased their serum ML levels (up to 22%). The results were consistent with the observations of the other studies (49, 63, 70, 71). Reiter *et al*. (1) have documented that ML production in the pineal gland starts at the beginning of the dark stage of the day, and that the maximum production occurs in the middle of the night. Cardinali and Pévet (72) demonstrated that longer nights result in a longer duration of ML secretion. Then, in relation to our results, it is possible that during constant dark exposure, the function of ML production system is increased.

In addition to light, other factors including Fe can also influence the ML production. Fe is essential to life, but, an excess has toxic effects due to its ability to catalyze free radical-producing reactions (73). Iron-mediated oxidative stress has been classically linked to cell death by apoptosis and more recently to ferroptosis, which represents a form of non-apoptotic cell death dependent on iron. The Fe levels in different tissues are dependent on the administration protocol, in oral, or in parenteral form (25, 74, 75). For example, parenteral Fe administration sharply increases serum Fe, exceeding the physiological capacity to bind circulating Fe and leading to a situation of oxidative stress in plasma.

In this study, we have observed that the acute administration of Fe-D significantly decreases the blood ML levels (31%), at 8 h after injection. When ML was injected to the rats with Fe overloading, a non-significant decrease in serum ML was observed compared to ML group.

Several studies have reported that ML has suppressive effect against iron-induced oxidative stress and injury (42) and in several tissues, including the brain and liver. The mechanisms may be involved in the antioxidative capacity of melatonin including: (i) as a free radical scavenger (76-80), (ii) as an antioxidative enzyme activator (81, 82), (iii) as a Fe chelator (36), (iv) regulating Fe metabolism (83).

Hernando *et al.* (27) have suggested that the increased ROS as a result of the presence of Fe in rat brains can be scavenged by ML and thus decrease its level as we have observed in the study. On the other hand, when ML functions as an iron-chelator, a decreased ML concentration in blood is expected when Fe level is high after acutely administered. Unfortunately, with the ELISA kit used in this study it was not be possible to detect the complex of melatonin-iron.

Othman *et al*. (83) demonstrated that ML ameliorated oxidative stress caused by adriamycin via regulating Fe levels. In the study, where ML (15 mg/kg) was used before and concurrently with adriamycin in rats, it significantly decreased plasma Fe levels compared with rats treated only with adriamycin.

Iron injections in rats could also resulted in the ML accumulation in liver and kidney of these rats (84, 85). This increased ML level could be used to protect these organs against the ROS. Therefore, the increase in hepatic and renal ML may be due to an increase in the biosynthesis of extrapineal ML for each organ (86, 87) or ML mobilization from the plasma to these organs, with the consequence of the reduced serum ML.

The influence of the LDC on ROS levels in the rats CNS has been studied. Both constant light (LL) and constant darkness (DD) alter the oxidative state of brain structures, with changes

in the levels of various antioxidant enzymes (88). Also, constant light influences circadian oscillations of circulatory lipid peroxidation, antioxidants, and some biochemical variables (89) and was associated with increased oxidative stress and adriamycin-induced nephropathy (90) in rats. On the other hand, ML acts as a direct scavenger of toxic radicals and stimulates the activity of the antioxidant enzyme GSH-Px to detoxify the hydroxyl radical produced by constant light exposure (82). Túnez *et al.* (90) reported that all pathological changes induced by both adriamycin and constant light were reversed to normal by ML administration. ML treatment decreased lipid peroxides, favored the recovery of reduced glutathione, antioxidant enzyme activity, and parameters of renal function.

In the LL group, no differences in the endogenous ML concentration were determined in the rats without Fe injection compared to the control group. However, in the DD group without Fe injection an increase in serum ML concentration (up to 22%) was observed due to the result of changes in photoperiod.

The fact that serum ML decreased 17% in the presence of Fe regardless of light exposure compared to its initial values (IG) supported the hypotheses of ML as a radical scavenger or iron-chelator. In presence of Fe, an increase in the ROS level (data not shown) under the LL or DD for 15 days caused physiological responses in relation to the ML concentration.

Overall, our results support the hypothesis of ML as scavenging molecule, stimulated by the presence of ROS generated by increased Fe level and exposure to LL and DD. Thus, it is expected that ML supplementation will restore the redox balance through the suppression of ROS (22).

In conclusion, the results confirmed that prolonged exposure to constant light or darkness, especially to darkness, had profound effects on the circadian axis. We demonstrated that constant darkness exposure in rats leads to an increase in serum ML, while constant light has no influence on ML level, possibly due to compensation of ML productions by other organs. Both exogenous ML and photoperiodic manipulation, if administered properly, can gradually modify the circadian system and modulate endogenous ML. Additionally, the increased Fe significantly lowered the ML level due to the mechanism that Fe increased ROS formation and ML was consumed as a free radical scavenger. The results support the idea that this molecule may be an effective therapeutic tool in iron-induced oxidative stress.

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AUTHORSHIP

COC contributed to the conception of this study and wrote the original draft of the article. JCP, MPH, and COC contributed with the experimental design and data acquisition and analysis. All authors contributed to data interpretation and critically revised the manuscript and approved the final version of the paper.

CONFLICT INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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