Research Article

The effects of endogenous melatonin on brain tissue oxidative stress induced by photoperiodic alterations and iron overloading in rats

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Running Title: Melatonin on brain tissue oxidative stress

Received: March 27, 2024, Accepted: April 23, 2024

ABSTRACT

Melatonin is a potent endogenously occurring antioxidant with the pleiotropic activities to neurodegenerative diseases associated with brain oxidative damage. In this study, we examined the prolonged photoperiodic alterations and iron (Fe) overload on melatonin production and brain oxidative stress in rats. The result showed that the 15 days of constant light (CL) exposure did not low the melatonin production but the 15 days of constant darkness (CD) significantly increased serum melatonin level in rats. The Fe treatment in both CL and CD conditions significantly reduced endogenous melatonin levels and increased brain tissue lipid peroxidation. Fe as a toxic transition metal can induce Fenton reaction to generated hydroxyl radical which can damage the neuronal cell membrane and impair the brain antioxidant system. In the current study, we observed the imbalanced antioxidant defense alterations upon Fe treatment in rat brain including the increased levels of alpha-tocopherol (α-T) and total thiols and the reduced melatonin level and catalase (CAT) activity. We speculated that the reduced melatonin level caused by Fe was due to its consumption since melatonin served as a metal chelator and antioxidant. Understanding these aspects enhances knowledge of brain Fe metabolism and its role in neurodegenerative disorders as well as the potential protective effects of melatonin on this metal.

Key words: Melatonin, iron dextran, photoperiod, oxidative stress, brain, rats

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1. INTRODUCTION

Light plays a pivotal role in animal and human physiology by conveying crucial time-of-day information. The synthesis of the hormone melatonin (ML) in the pineal gland is regulated by the light-dark cycle (LDC) (1). ML, also referred to as N-acetyl-5-methoxytryptamine, is primarily produced by pineal gland of vertebrates and it circulates in the bloodstream as an endocrine hormone binding to its receptors in various target tissues, eliciting specific physiological responses (2). ML is a lipophilic molecule capable of traversing all cellular

membranes, including the blood-brain barrier, and is assumed to be present in all subcellular compartments.

The duration of the ML peak reflects the environmental nighttime since pineal ML is produced during the dark phase and the acute light exposure suppresses its production (3). Thus, plasma ML in mammals exhibits a circadian rhythm with high levels at night and low levels during the day, it peak time occurs between 2:00 and 4:00 a.m. (4). Longer nights result in a longer duration of ML secretion (3). ML is rapidly cleaned from circulation following the cessation of its production as well as its metabolism by the liver. Its secretion profile defines the biological night and it is also referred as chemical expression of dark.

In most mammalian species, circadian rhythms are synchronized by the LDC through entrainment of the suprachiasmatic nucleus (SCN), the master circadian pacemaker (5). Since the SCN is neurologically connected to the pineal gland, the activity of SCN also regulates ML biosynthesis (5, 6). This rhythm appears crucial for, at least, some of actions of ML (7). Increasing evidence suggests that constant light (CL) or constant darkness (CD) can disrupt various aspects of circadian rhythm and have diverse effects on physiology of organisms (8, 9). Cervino *et al.* (10) demonstrated that exposing rats to CL or CD for 15 days affected endogenous ML concentration.

The physiological effects of ML are multiple including bone metabolism, reproductive activity, regulation of the cardiovascular function, immune response and body mass alteration. Additionally, the beneficial effects of ML on brain or gastrointestinal protection, psychiatric disorders, cardiovascular diseases, and oncostatic effects have been reported (11).

ML serves as a primary photoperiodic sensitive molecule (12, 13), but, it also functions as a free radical scavenger and antioxidant (14, 15). Tissues with abundant mitochondria including the heart, brain, and skeletal muscle, rely on efficient oxygen utilization for energy production which also results in the generation of reactive oxygen species (ROS) from mitochondrial electron transport (16). ROS are also produced during metabolism and enzymatic reactions, including hydrogen peroxide (H_2O_2) and superoxide anion (17). Excessive ROS leads to oxidative stress, causing cellular damage (18) including protein damage, lipid peroxidation, and DNA breakage (19). Most of the organisms have equipped antioxidant defense system (19, 20) including enzymatic and non-enzymatic mechanisms (21) to detoxify the ROS. ML is recognized for its antioxidant properties, directly neutralizing ROS and reactive nitrogen species (RNS) (22-24) as well as indirectly enhancing the activities of variety of antioxidative enzymes (25). Metabolites of ML, like AFMK and AMK, also act as efficient free radical scavengers (26), collectively known as the free radical scavenging cascade (27) to maximize antioxidative capacity of ML.

The brain is one of the most vulnerable organs to free radical attack due to its high oxygen consumption. Oxidative damage is a common factor in the neuropathology of various neurodegenerative disorders, such as Alzheimer's disease (28-30), Parkinson's disease, and others (31). Numerous studies have shown that the decreased ML levels in serum and cerebrospinal fluid in patients with neuropathological diseases (23, 32). To increase ML production with enhanced free radical scavenging activity makes a crucial strategy for treatment and management of these diseases (33).

Exposure to toxic transition metals like iron (Fe) can result in multiple organ damage in humans by oxidative stress. For example, elevated blood level of free ferrous iron, caused by excessive ingestion, reacts with peroxides to generate highly reactive free radicals capable of damaging DNA, proteins, lipids, and other cellular components. Due to its ability to undergo one-electron reactions, Fe^{2+} catalyzes, via the Fenton reaction, the conversion of H_2O_2 into the hydroxyl radical (●OH), one of the most toxic ROS (34). Once generated, it immediately reacts with cellular lipids, proteins, and DNA to destroy their normal structures (35). Fe-mediated oxidative damage is implicated in several neurological disorders (36-38). While Fe appears

essential for normal brain function, but, its excessive accumulation induces generation of ROS (39). In patients with Parkinson's disease, brain Fe content and lipid peroxidation are reportedly higher than that in the healthy controls (40). Studies in patients with Alzheimer's disease have also shown significantly elevated Fe levels in several brain regions (36, 41).

Therefore, in the current study, we will explore the potential protective effects of ML on Fe-induced oxidative stress in rat brains, particularly in animals exposed to different photoperiods. This inquiry may provide insights into potential therapeutic or preventive strategies for mitigating or averting the detrimental effects of Fe on the brain in forthcoming research.

2. MATERIALS AND METHODS

2.1. Chemicals.

ML, Fe-dextran and other chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Animals.

Fifty-five male Sprague Dawley rats, weighing between 220 and 245 g (8-10 weeks of age), underwent a 2-week acclimation period at a temperature of 22±2 ºC. During this period, they had unrestricted access to water and food, and were subjected to a 12:12 h LDC (light ON: 8:00 a.m.) before treatments. The rats were then randomly divided into 11 groups as follows:

1. Control (CG): Animals were only injected with vehicle under normal LDC.
2. Iron (FeG): Rats were injected with Fe-D (iron-dextran) under normal LDC.
3. Iron+Melatonin (FeMG): Rats were injected with Fe-D plus ML under normal LDC.
4. Constant light (CL): Rats exposed to continuous light (LL) for 15 days.
5. CL/LDC: Rats exposed to LL for 15 days and then returned to the 12:12 LDC.
6. FeG+CL: Rats were injected Fe-D and exposed to LL for 15 days.
7. FeG+CL/LDC: Rats exposed to Fe-D and LL for 15 days and then returned to 12:12 LDC.
8. Constant dark (CD): rats exposed to continuous dark (DD) for 15 days.
9. CD/LDC: Rats exposed to DD for 15 days and then returned to 12:12 LDC.
10. FeG+CD: Rats were injected Fe-D and exposed to DD for 15 day.
11. FeG+CD/LDC: Rats exposed to Fe-D and DD for 15 days and then returned to the 12:12 LDC.

All groups are also listed in the Table 1.

ML or Fe-D were given intraperitoneal (i.p.) injections 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 used 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 in accordance with the guidelines set forth by the Canadian Council on Animal Care (https://www.ccac.ca/en/standards/guidelines/) and adhered to the ARRIVE guidelines (https://arriveguidelines.org/).

Table 1. Groups and time of treatments.

Groups[b]	Day	D1			D15			D30		
	Hour	8 am	12 pm	16 pm	8 am	12 pm	16 pm	8 am	12 pm	16 pm
	ZT	0	4	8	0	4	8	0	4	8
1. CG		--	cv	bs	--	--	--	--	--	--
2.FeG		Fe-D	cv	bs	--	--	--	--	--	--
3.FeMG		Fe-D	ML	bs	--	--	--	--	--	--
4. CL		--	--	--	ss	--	bs	--	--	--
5.CL/LDC		--	--	--	--	--	--	ss	--	bs
6.FeG+CL		--	--	--	Fe-D	--	bs	--	--	--
7.FeG+CL/LDC		--	--	--	--	--	--	Fe-D	--	bs
8.CD		--	--	--	ss	--	bs	--	--	--
9.CD/LDC		--	--	--	--	--	--	ss	--	bs
10.FeG+CD		--	--	--	Fe-D	--	bs	--	--	--
11.FeG+CD/LDC		--	--	--	--	--	--	Fe-D	--	bs

cv: control vehicle (ip), ss: saline solution (ip), ML: melatonin 50 mg/kg (ip), Fe-D: Fe-dextran 500 mg/kg (ip), bs: blood sample collection, CG: control group, FeG: iron group, FeMG: Fe-D+melatonin group, CL: constant light, CL/LDC: CL and returned to light/dark cycle of 12:12h (LDC), FeG+CL: FeG + constant light, FeG+CL/LDC: FeG + constant light and returned to LDC, CD: constant dark, CD/LDC: constant dark and returned to LDC, FeG+CD: FeG + constant dark, FeG+CD/LDC, FeG + constant dark and returned to LDC, ZT: zeitgeber time.

2.3. Melatonin treatment.

ML was dissolved in absolute ethanol (Sintorgan®) and, then diluted with 0.9% saline to a final concentration of 9.6 mg/mL. The ethanol concentration in the final solution was less than 3.3%. ML solution was administered to the FeMG group (Group 3) at a single dose of 50 mg/kg body weight at 12:00 pm (ZT = 4), following dosing protocols established in our previous studies (42, 43) and consistent with literature reports (44-46). At this time, the endogenous ML levels are minimal (47), regardless of Fe presence. Serum ML concentrations were evaluated 4 hours post intraperitoneal injection.

The control group (CG) received vehicle injections with the same volume as ML injections at 12:00 am (ZT = 4).

Samples from rats that were injected with exogenous ML under the photoperiod conditions designed in this experiment were reserved for the analysis of other antioxidant molecules not shown in this study.

2.4. Iron treatment.

Each rat in FeG, FeMG, FeG+CL, and FeG+CD was received a single dose of 500 mg/kg body weight of Fe-dextran by ip at 8 am (ZT= 0).

Four hours later (ZT= 4), the ML was given (Table I). This time interval was selected due to the maximum Fe concentration occurred 6 hours after its injection (48).

Blood samples were collected 8 hours after acute Fe-D overload to assess its impact on ML levels, given that Fe levels remained high in plasma at this time (48). This approach aimed to ensure elevated ML values in the presence of Fe-D to evaluate its antioxidant function. Notably, the serum ML concentration in the CG served as a reference for ML content in blood without treatments, and its value was significantly lower than that observed in the FeG group.

2.5. Schedules of light exposure.

Four groups of rats were exposed to constant light illumination for 15 days with three fluorescent tubes (Philips TL-D 36W 840 Super 80; light color: 4000K - cold white; luminous flux: 3350 Lm). The light intensity at the cage level was around 41 μW/cm^2 (ILT2400 Hand-Held Light Meter). On day 15, saline solution and Fe-D were administered to CL and FeG+CL, as described above. Meanwhile, CL/LDC and FeG+CL/LDC were returned to the acclimation photoperiod (12L:12D, light ON: 8:00 a.m.) for an additional 15 days. At the end of this period, saline solution and Fe-D were injected, as indicated in Table I.

Cage cleaning, feeding, and animal handling were conducted under red light (incandescent lamp, 25W) positioned 3 meters away from the animals.

Another four groups of rats were kept in constant darkness for 15 days. On day 15, saline solution and Fe-D were administered to CD and FeG+CD, as previously described. Meanwhile, CD/LDC and FeG+CD/LDC were returned to the acclimation photoperiod (12L:12D, light ON: 8:00 a.m.) for an additional 15 days. At the end of this period, saline solution and Fe-D were injected, following the protocol outlined in Table I.

2.6. Brain and blood sample preparations.

Brain and blood sample preparation procedures were standardized across all experimental groups to account for the influence of the photoperiod on circadian rhythms. Blood collection was conducted during the lighting phase of the LDC at 4 p.m. (ZT= 8), aiming to compare plasma ML levels during CL or CD cycles with those of the control groups, rather than focusing solely on absolute levels.

Blood samples were collected from anesthetized animals in a CO_2 chamber, and brains were removed at specific times following Fe treatment, as previously described. All samples were obtained between 4:00 and 4:30 p.m., immediately after completion of the experiment. In Groups 1 to 3, samples were collected eight hours after Fe inoculation, while in Groups 4, 6, 8, and 10, sampling occurred after 15 days, and in Groups 5, 7, 9, and 11, after 30 days (see Table I).

Five milliliters of blood were drawn from the heart, transferred to tubes, and left at room temperature for 10–20 minutes to allow clotting. Following centrifugation at 3000 rpm for 10 min., the serum samples were transferred to Eppendorf tubes and stored at -20°C until analysis.

Brain tissue was promptly excised and immediately frozen at -80°C for subsequent analysis.

2.7. Melatonin measurement.

Serum ML levels were assessed using the ELISA assay (Melatonin ELISA Kit, ab213978, Abcam) following the manufacturer's instructions. This colorimetric method measures the level of color produced, which is directly proportional to the level of the primary antibody bound to the proteins at the bottom of the wells.

2.8. TBARS content measurement.

TBARS content was assessed using a modified fluorescence method (49). An aliquot (50 mg) of brain samples was homogenized with 1 ml of 40 mM potassium phosphate buffer and then, 1ml of120 mM KCl (pH 7.4), 0.1 ml of (4% w/v) BHT and 0.25 ml (3% v/v) SDS were added. After thorough mixing, 1 ml of 0.1N HCl, 0.15 ml of (10% w/v) phosphotungstic acid, and 0.5 ml of (0.7% w/v) 2-thiobarbituric acid were added. The mixture was heated for 45 min. in boiling water, and TBARS were then extracted into 1 ml of n-butanol. Following a brief

centrifugation, the fluorescence of the butanol layer was measured at λ_{ex}= 515 nm and λ_{em}= 555 nm. The results were expressed as nmol TBARS (malondialdehyde equivalents) per milligram of protein. Protein content was determined in each experiment as previously described by Lowry *et al*. (50), utilizing a 4 ml/ml dilution of the homogenate and employing bovine serum albumin as a standard fresh protein solution. Malondialdehyde standards were prepared from 1,1,3,3-tetramethoxypropane.

2.9. α-tocopherol (α-T) measurement.

The α-T content in the brain homogenates was quantified by filtering 5 ml samples through reverse-phase HPLC with electrochemical detection using a Bioanalytical Systems LC-4C amperometric detector equipped with a glassy carbon working electrode at an applied oxidation potential of +0.6 V (51). Extraction from the samples was carried out using 1 ml of methanol and 4 ml of hexane. After centrifugation at 600 g for 10 minutes, the hexane phase was separated and evaporated under N_2. Extracts were reconstituted in methanol:ethanol (1:1 v/v) and injected for HPLC analysis. The HPLC conditions included isocratic reversed phase; column: Supelcosil LC-8; dimensions: 3.3 cm × 4.6 cm × 3 μm; mobile phase: 20 mM lithium perchlorate in methanol/water 99/1 (v/v); flow rate: 1 ml/min; retention time: αT = 0.8 min and βC = 1.6 min. Synthetic D,L-α-T from phytol (Sigma-Aldrich, St. Louis, MO, USA) was used as a standard reference.

2.10. Total thiols content measurement.

Total thiols were determined spectrophotometrically in whole brain tissue in the presence of 20 mM EDTA at a concentration of 75 mg/ml described by Sedlak and Lindsay (52). The 5,5-dithiobis(2-nitrobenzoic acid) was used to measure absorbance at λ= 412 nm using glutathione (GSH) as the reference to make the standard curve.

To determine the GSH content in the brain, reverse-phase HPLC with electrochemical detection was employed. A Supelcosil LC-18-DB column was used, which was equilibrated with a mobile phase of 20 mM NaH_2PO_4 at pH 2.7. The brain samples were homogenized (200 mg/ml) in $HClO_4$ 1 M-EDTA 2 mM, followed by centrifugation at 29,000 g for 20 min. at 4 ºC. Then, 20 ml of the filtered supernatants were diluted 1/20 (v/v) in the mobile phase and injected into the HPLC system.

2.11. Catalase activity measurement.

The activity of the antioxidant enzyme CAT was determined following the spectrophotometric method described by Aebi (53). The brain tissues were homogenized in a potassium phosphate buffer (30 mM with KCl 120 mM, pH 7.4) at a 1:9 ratio (P/V). The homogenate was centrifuged at 600g for 10 minutes, and the supernatant was collected for the measurements. Measurements were conducted in a potassium phosphate buffer (50 mM, pH 7), in the presence of 10 mM H_2O_2. The rate of H_2O_2 decomposition was monitored at 240 nm and 25 °C to determine enzyme activity. The units of CAT represent the amount of enzyme catalysing the elimination of 1 μM H_2O_2 per min.

2.12. Statistics analyses.

The data were presented as mean ± SE and analyzed by ANOVA following post-hoc Tukey's test (SigmaStat 3.5, Systat Software, Inc). The statistically significant difference was set up $p < 0.05$. The separate ANOVA was performed for groups with and without Fe. The

one-way ANOVA was used to compare subgroups as Control (without Fe) in three main groups (basal CG, CL, and CL/LDC) to assess the effect of light on serum ML; Fe-dextran in three main groups (basal FeG, FeG+CL, and FeG+CL/LDC) to evaluate the effect of light plus Fe on serum ML; Control (without Fe) in three main groups (basal CG, CD, and CD/LDC) to explore the impact of darkness on serum ML; Fe-dextran in three main groups (basal FeG, FeG+CD, and FeG+CD/LDC) to examine the effect of darkness plus Fe on serum ML.

3. RESULTS

3.1. The effects of photoperiods and Fe on serum melatonin level.

The results showed that 15 days of CL exposure or CL returned to the normal photoperiod had no significant effect on serum ML levels compared to the normal photoperiod control (LDC) ($p > 0.05$). However, CD significantly increased the serum ML levels compared to the LDC and when the CD rats returned to LDC, their serum ML level significantly lower than that in the normal photoperiod. Fe treatment significantly reduced serum ML levels in all groups but especially in both CL and CD rats, their melatonin levels from 199.7±100.80 to 33.9±26.43 and 32.9±20.18 pg/ml, respectively ($p < 0.01$) (Figure 1). When CL-FeG group returned to the normal photoperiod (CL/LDC), the serum ML level still maintained at low (55.3±17.37pg/ml); however, when the CD-FeG rats returned to normal photoperiod (CD/LDC), their serum ML level increased to 150.8±77.89 pg/ml which was comparable to the Fe-treated LDC group (Figure 1).

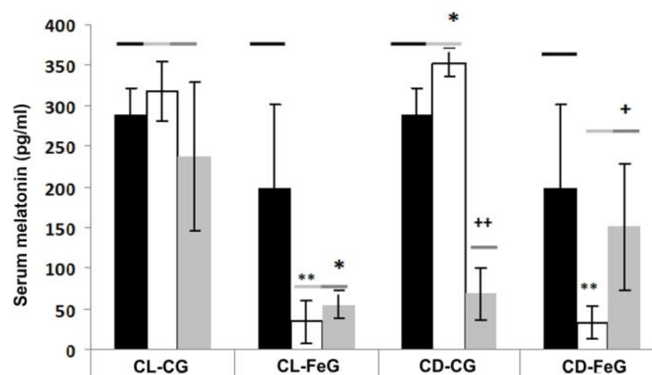

Fig. 1. Effects of photoperiod alteration and Fe treatments on serum melatonin of rats.
*LDC: 12L:12D light-dark, CL: constant light, CD: constant dark. Black bars represent basal LDC, white bars represent CL or CD, and grey bars represent returned LDC. CG: control group; FeG: iron group. Data are presented as mean ± SE (n = 5). Horizontal lines at the same height indicate no significant differences (Tukey Test) between treatments for each condition for CL or CD. *: p < 0.05 and **: p < 0.001 comparing basal LDC vs CL or CD conditions or +: p < 0.05 and ++: p < 0.001 comparing CL or CD vs returned LDC conditions.*

3.2. The effects of photoperiods and Fe treatments on TBARS content.

The prolonged period of CL and CD had no significant effects on the brain TBARS contents compared to their respective controls ($p > 0.05$). Unexpectedly, when the CL returned to LDC, the brain TBARS content was significantly increased compared to control; however, this alteration was not observed in CD rats. Fe treatment exhibited the same results in CL rats as mentioned above. Fe treatment in CD group not only significantly increased the brain TBARS content compared to its control but, this value was further significantly elevated when they returned to LDC (Figure 2).

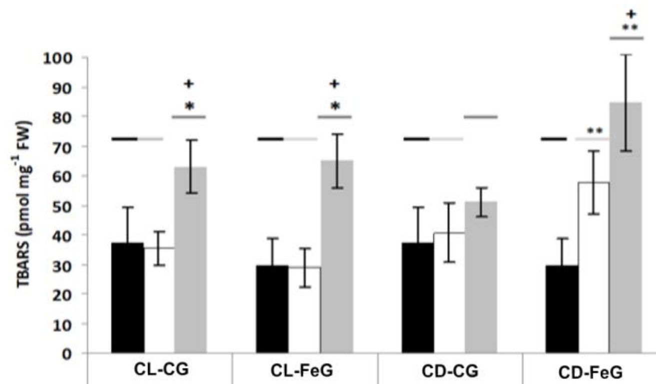

Fig. 2. The effects of photoperiods and Fe treatments on TBARS content.

*LDC: 12L:12D light-dark, CL: constant light, CD: constant dark. Black bars represent basal LDC, white bars represent CL or CD, and grey bars represent returned to LDC. CG, control group; FeG, iron group. Data are presented as mean ± SE (n = 5). Horizontal lines at the same height indicate no significant differences (Tukey Test) between treatments for each condition for CL or CD. *: p < 0.05 and **: p < 0.001 comparing basal LDC vs CL or CD conditions or +: p < 0.05, ++: p < 0.001 comparing CL or CD vs returned LDC conditions.*

3.3. The effects of photoperiods and Fe treatments on α-T content of rat brain.

The results showed that CL, CL-FeG both significantly increased the α-T content of rat brain compared to their respective controls and these values were further significantly elevated when they were returned to the LDC. The CD and CD-FeG also significantly increased the α-T content of rat brain, when they were returned to LDC these increased values were reduced but still significantly higher than those of their respective controls ($p < 0.05$) (Figure 3).

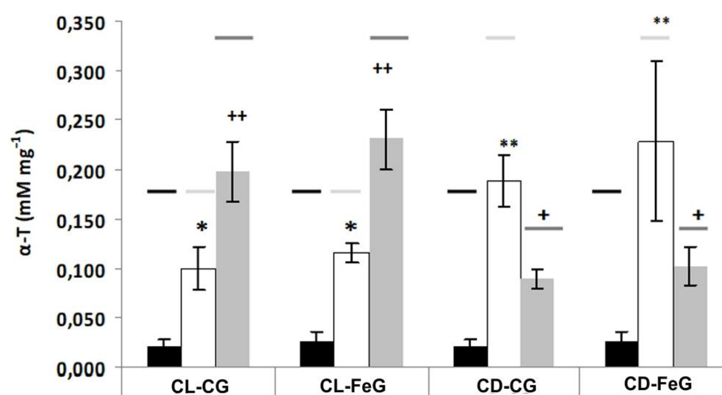

Fig. 3. The effects of photoperiods and Fe treatments on α-T content of rat brain.

*LDC: 12L:12D light-dark, CL: constant light, CD: constant dark, Black bars represent basal LDC, white bars represent CL or CD, and grey bars represent returned LDC s. CG, control group; FeG, iron group. Data are presented as mean ± SE (n = 5). Horizontal lines at the same height indicate no significant differences (Tukey Test) between treatments for each condition for CL or CD. *: p < 0.05, **: p < 0.001 comparing basal LDC vs CL or CD conditions or +: p < 0.05, ++: p < 0.001 comparing CL or CD vs returned LDC conditions.*

3.4. The effects of photoperiods and Fe treatments on total thiol content of rat brain.

The results showed that CL and CD significantly increased the total thiol content of rat brain and Fe-treatment did not further increase these values in both photoperiodic conditions

compared to their respective controls ($p < 0.01$), when they were returned to the LDC, these increased values turned back to normal ranges of their control, except the CD-FeG, in which, the total thiol content of rat brain still higher than that of its control ($p < 0.05$) (Figure 4).

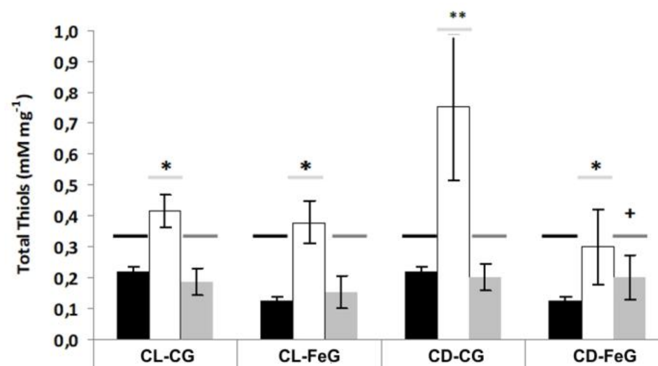

Fig. 4. The effects of photoperiods and Fe treatments on total thiol content of rat brain.
*LDC: 12L:12D light-dark, CL: constant light, CD: constant dark. Black bars represent basal LDC, white bars represent CL or CD, and grey bars represent returned LDC. CG, control group; FeG, iron group. Data are presented as mean ± SE (n = 5). Horizontal lines at the same height indicate no significant differences (Tukey Test) between treatments for each condition for CL or CD. *: p < 0.05, **: p < 0.001 comparing basal LDC vs CL or CD conditions or +: p < 0.05, ++: p < 0.001 comparing CL or CD vs returned LDC conditions.*

3.5. The effects of photoperiods and Fe treatments on CAT activity of rat brain.

The results showed that CL and CL-FeG did not significantly impact the CAT activity of the brain, however, when the CL rats were returned to the LDC, the CAT activity was significantly reduced compared to the control ($p < 0.05$). CD and CD-FeG significantly reduced CAT activity compared to their respective controls ($p < 0.01$); however, when the CD-FeG rates were returned to LDC, their CAT activity recovered to the control level (Figure 5).

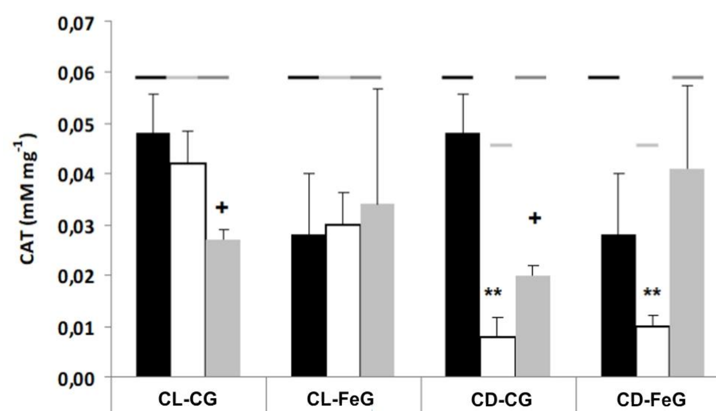

Fig. 5. The effects of photoperiods and Fe treatments on CAT activity of rat brain.
*LDC: 12L:12D light-dark exposure, CL: constant light, CD: constant dark. Black bars represent basal LDC, white bars represent CL or CD, and grey bars represent returned LDC. CG, control group; FeG, iron group. Data are presented as mean ± SE (n = 5). Horizontal lines at the same height indicate no significant differences (Tukey Test) between treatments for each condition for CL or CD. *: p < 0.05, **: p < 0.001 comparing basal LDC vs CL or CD conditions or +: p < 0.05, ++: p < 0.001 comparing CL or CD vs returned LDC conditions.*

4. DISCUSSION

It has been well known that the acute exposure of light at night suppresses ML production in vertebrates. In the current study, we further explored the effects of the prolonged light exposure on ML production in rats, i.e., these rats were exposed to CL for 15 days, as comparison, some rats were also kept at the CD for 15 days. The results showed that prolonged CL did not low the serum ML production compared to the control group and this could be explained by the adaptation of the animals to the environmental changes and/or the photoreceptors of retinal cells had lost sensitivity upon to the long-lasting illumination. This result was consistent with previous report (10). In contrast, the pronged CD significantly increase serum ML level compared to the normal LDC rats. When these CD rats were returned to normal LDC, their serum ML levels dropped below normal. However, Fe treatments in all photoperiodic conditions including CL and CD caused dramatically serum ML reduction. Coincidently, this ML decline caused by Fe overloading in some degree associated with brain oxidative stress such as the lipid peroxidation. These results could be explained by the reduced protection with altered ML production as well as enzymatic or non-enzymatic antioxidants.

Additionally, in CD animals with Fe-treatment, there were the increases in α-T and total thiols while the decreases in CAT activity and ML level in the brain, which support the notion of the increased ROS generation, as measured by elevated lipid damage. The increased syntheses of thiols and α-T represent an adaptive response of the brain to oxidative stress (48, 54).

We previously demonstrated that after Fe-D injection, the maximum Fe concentration in the brain of rats occurred at 6 hours and accompanied the increased lipid damage in different brain areas, especially in the cortex (48). Lockman *et al.* (55) showed varying susceptibility to Fe overloading in neuronal cells and astrocytes in rats. Triggs and Willmore (56) demonstrated that intracerebral injection of Fe^{2+}, particularly into the hippocampus, significantly increases lipid peroxidation levels and induces neuronal damage (57).

Lipid peroxidation, a fundamental cellular deteriorative process, is one of the primary effects induced by oxidative stress and occurs in tissues due to the rich in polyunsaturated fatty acids (58). The ROS can serve as the signal molecule of cells but, when ROS formation exceeds the antioxidant defense capability or disrupts redox signaling it will cause oxidative stress and affects cell functionality (59). Fe overloading can disrupt the balance of the antioxidant system, leading to a severe loss of total antioxidant status. Neuronal damage caused by ROS overproduction is directly linked to the pathophysiology of major neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease (60).

Antioxidant enzymes, such as CAT, acts in coordination with non-enzymatic molecules like GSH and α-T to counteract the oxidative stress in tissues (61). Our results suggest that endogenous ML may prevent brain oxidative damage by activating antioxidative defense enzymes, including the upregulation of superoxide dismutase mRNA levels (62) and the enhancement of GSH peroxidase activity (63).

One of the actions of ML involves directly scavenging free radicals (64, 65). Through this action, ML can halt the initiation and propagation of lipid peroxidation. Moreover, several studies have shown that ML can bind to various metals, including Fe (III) (66, 67) to attenuate the Fenton's reaction that leads to oxidative damage. All these actions of melatonin may be suppressed by Fe overloading, therefore, resulting in brain oxidative injury, as demonstrated in the present study. Previous studies have shown that Fe treatment significantly reduced the administrated ML levels in brains of rats (43). As a result, ML pretreatment could potentially ameliorate the altered brain histopathologies resulting from increased ROS levels induced by Fe overloading. Additionally, several metabolites of ML are capable of detoxifying free radicals and their derivatives (68). ML can rapidly cross the blood-brain barrier and accumulate

in brain cells with high levels (69). Consequently, it is accessible to all tissues and cells, where it scavenges free radicals (70, 71). Upon returning rats to LDC conditions, there was a decrease in ML concentrations in both CL and CL/FeG groups (CL/FeG, $p < 0.05$). These decreases were with the increased lipid peroxidation observed in both experimental groups. A similar trend was observed in Fe-treated rats CD group returned to LDC. The lower than normal ML values suggest that after long-term exposure to CD conditions, the ML synthetic machinery of the pineal gland and the circadian axis became sensitive to light exposure upon returning to the normal LDC (10).

In addition, the CAT activity is significantly increased in the cortex (2.7-fold) after acute Fe overloading, with no changes in the hippocampus and striatum areas when compared to control rats (54). In the present study, there was a decreased activity of CAT in the brain of rats that were exposed to CD, not only in the control group (GC) but also in Fe-treated rats, as well as in both control groups of LDC treatments. The activity of CAT, like that of other antioxidant enzymes, is sometimes diminished under conditions of intensive oxidative stress. This decline in CAT activity under such conditions is attenuated when ML is present (72). This effect may be attributed to the general protective effect of ML on macromolecules, including proteins. Coincidentally, we observed the increased lipid damage in both CL and CD-FeG brain with decreased ML values.

Low molecular weight thiols, such as glutathione (GSH), are essential cellular components with diverse metabolic and homeostatic roles. GSH, the most abundant non-protein thiol, functions in detoxification, cell growth regulation, and immune maintenance (73). Redox-sensitive transcription factors respond to varying ROS levels, coordinating biological responses accordingly. Excessive ROS triggers glutathiolation of proteins (74), facilitated by sulfenic acid or nitrosation formation (75-77). Under oxidative stress, cysteines oxidize to reactive sulfenic acid, reacting with GSH. Proteins like aldose reductase (76, 78), branched-chain aminotransferase (79), hemoglobin (80), and actin (81) undergo glutathiolation via sulfenic acid intermediates. Although glutathione was not measured in this study, increased total thiol values in CL and CD conditions suggest elevated GSH levels due to ROS from light exposure or Fe-D.

The α-Tocopherol is a lipid-soluble antioxidant crucial for safeguarding against lipid peroxidation (17). By scavenging ROS and integrating into cell membranes, it shields them from oxidative damage, with each α-T molecule protecting approximately 100 membrane phospholipids (82). The *in vitro* studies on cholestanetriol-treated porcine ovarian granulosa cells revealed that α-T administration significantly improved cell viability and normalized the activities of superoxide dismutase (SOD) and CAT, highlighting its therapeutic antioxidant potential (83). α-T is primarily stored in the liver, adipose tissue, and muscles (48), with its hepatic regulation ensuring homeostasis. The tocopherol transfer protein facilitating its secretion into circulating lipoproteins for delivery to target tissues (84, 85). Under both CL and CD conditions, α-T levels increased not only in the granule cell but also in the fascia dentata granule cell layer, effectively preventing lipid peroxidation. However, this protective effect seemed insufficient in rats treated with Fe under CD. Piloni *et al*. (54) also demonstrated α-T consumption in the cortex area of the rat brain. Despite increased α-T content observed in both the GC and FeG under CL conditions, it failed to prevent lipid damage in either case.

In summary, in the current study we have observed: 1. To expose rats to prolonged CD for 15 days led to lipid peroxidation, ML depletion and reduced total thiols, elevated α-tocopherol (α-T), and decreased CAT activity which indicated the inadequate antioxidant responses. 2. Fe treatment in both CL and CD significantly reduced ML production and increased brain tissue oxidative stress. 3. When the rats in CL and CD with/out Fe treatments were returned to normal LDC they had a further decreased ML and increased lipid peroxidation in brain tissue. This novel observation requires further investigation. 4. In the same conditions,

α-T levels increased after CL and Fe exposure but decreased after CD. Moreover, total thiols were depleted after CL exposure, regardless of Fe presence or not. CAT activity increased upon returning to LDC conditions compared to CD, irrespective of Fe presence.

These findings underscore the brain's vulnerability to oxidative stress induced by Fe overload and light conditions, affecting antioxidant consumption (such as total thiols, α-T or ML) and enhancing the activity of others (like CAT). A deeper understanding of these dynamics will significantly advance our comprehension of brain Fe metabolism and its implications in neurodegenerative disorders.

ACKNOWLEDGMENTS

The research leading to these results received funding from University of Morón Foundation under Grant Agreement No 15-004/16, 2016-2018. We would like to acknowledge and thank Dr. Omar Iodice (CONICET) and Dra. María Cruz Miraglia for his technical assistance. We especially thanks to both anonymous reviewers and the editor for comments and suggestions.

AUTHORSHIP

COC and MPH contributed to the study conception and design. Material preparation, data collection and analysis were performed by the three authors. The first draft of the manuscript was written by MPH and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

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

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Please cite this paper as:
Hernando, M., Cogo Pagella, J. and Cervino, C. 2024. The effects of endogenous melatonin on brain tissue oxidative stress induced by photoperiodic alterations and iron overloading in rats. Melatonin Research. 7, 1 (Apr. 2024), 103-119. DOI:https://doi.org/https://doi.org/10.32794/mr112500170.