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Abstract

Melatonin regulates the reproductive cycle, energy metabolism and may also act as a potential antioxidant indoleamine. The present study was undertaken to investigate whether long-term melatonin treatment can induce reproductive alterations and if it can protect ovarian tissue against lipid peroxidation during ovulation. Twenty-four adult female Wistar rats, 60 days old (± 250-260 g), were randomly divided into two equal groups. The control group received 0.3 mL 0.9% NaCl + 0.04 mL 95% ethanol as vehicle, and the melatonin-treated group received vehicle + melatonin (100 µg·100 g body weight^{-1}·day^{-1}) both intraperitoneally daily for 60 days. All animals were killed by decapitation during the morning estrus at 4:00 am. Body weight gain and body mass index were reduced by melatonin after 10 days of treatment ($P < 0.05$). Also, a marked loss of appetite was observed with a fall in food intake, energy intake (melatonin 51.41 ± 1.28 *vs* control 57.35 ± 1.34 kcal/day) and glucose levels (melatonin 80.3 ± 4.49 *vs* control 103.5 ± 5.47 mg/dL) towards the end of treatment. Melatonin itself and changes in energy balance promoted reductions in ovarian mass (20.2%) and estrous cycle remained extensive (26.7%), arresting at diestrus. Regarding the oxidative profile, lipid hydroperoxide levels decreased after melatonin treatment (6.9%) and total antioxidant substances were enhanced within the ovaries (23.9%). Additionally, melatonin increased superoxide dismutase (21.3%), catalase (23.6%) and glutathione-reductase (14.8%) activities and the reducing power (10.2% GSH/GSSG ratio). We suggest that melatonin alters ovarian mass and estrous cyclicity and protects the ovaries by increasing superoxide dismutase, catalase and glutathione-reductase activities.

Key words: Melatonin; Ovary mass; Lipid peroxidation; Antioxidant defenses; Superoxide dismutase

Introduction

The rhythm of melatonin (N-acetyl-5-methoxytryptamine) secretion is important for the synchronization of the reproductive response with appropriate environmental conditions in photoperiodic animals (1). Melatonin is known to modulate some physiological functions such as seasonal reproduction, energy metabolism and thermoregulation in mammals (2). Experimental studies have indicated the action of melatonin in nutrition and feed efficiency affecting body mass and adiposity index and both energy intake and expenditure, with melatonin preferentially acting by reducing fat deposits, thus preventing obesity (3,4). However, these controversial effects are not yet fully understood.

It has been shown that melatonin plays key roles in reproductive function due to its stimulation of ovarian activity and the promotion of estrous cyclicity and gonadal atrophy depending on photoperiod length (5). Moreover, melatonin also regulates folliculogenesis and ovulation (6) since acute suppression of luteinizing hormone levels is notable after melatonin treatment (7). Also, it has been emphasized that melatonin affects the axis by directly binding to granulosa cells in the ovary (8).

During ovulation, the mechanisms causing follicular rupture result in exposure of the ovarian surface to deleterious agents such as free radicals (9,10). Thus, repeated

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ovulation and its complex activities are linked to inflammation, reactive oxygen species (ROS) formation, and special cytokine expression, and may also be involved in the etiopathogenesis of ovarian cancer (10,11). Some reports have described the presence of ROS in the female reproductive tract, including the ovaries (12). Under normal conditions, ROS appear to be directly involved in the reproductive physiological functions such as steroidogenesis, oocyte development and fertility (12,13). Interestingly, the major sources of ROS, including ·OH, O_2^- and H_2O_2, are derived from macrophages and neutrophils, as they are present in the ovaries during ovulation (14). The preovulatory follicle has a powerful antioxidant defense, which is depleted by the intense peroxidation. As an example, the activity of glutathione peroxidase (GSH-Px) may also maintain low levels of hydroperoxides inside the ovarian follicles.

The use of melatonin has been investigated as a therapeutic strategy to improve oocyte quality in patients failing to get pregnant in earlier *in vitro* fertilization (15). In addition, melatonin has an important role as a ROS scavenger, acting directly and/or indirectly as a hormone preventing attack by free radicals (16). A previous study showed that elevated melatonin levels in preovulatory follicles are able to protect granulosa cells and the oocyte from the free radicals induced by ovulation (17). Melatonin also positively increases both antioxidant enzyme activity and gene expression of superoxide dismutase (SOD), catalase and GSH-Px, which are fundamental to detoxify most of the ROS produced (18).

Although melatonin treatment has been widely used as an effective and useful adjuvant for several therapies, including to maintain oocyte quality (15), its adverse time-dependent effects are still obscure and require further study. The study of long-term exposure to melatonin may provide new insights into the understanding of the physiological and cellular mechanisms underlying metabolic changes, appetite signaling and protective factors in female reproduction. Additionally, it should be remembered that possible changes in nutritional status could improve or compromise the reproductive viability. Melatonin can contribute to decreasing fat pad storages (4), providing a better lifespan, and may reduce lipid oxidation, which would be of benefit for reproduction, in addition to having a known detoxifying role. However, the role of melatonin in such events remains unclear and conflicting results have been observed.

The present study was undertaken to determine if long-term melatonin administration is able to induce reproductive changes and how melatonin exerts its protective action related to lipid oxidation and antioxidant activities in the rat ovary during ovulation.

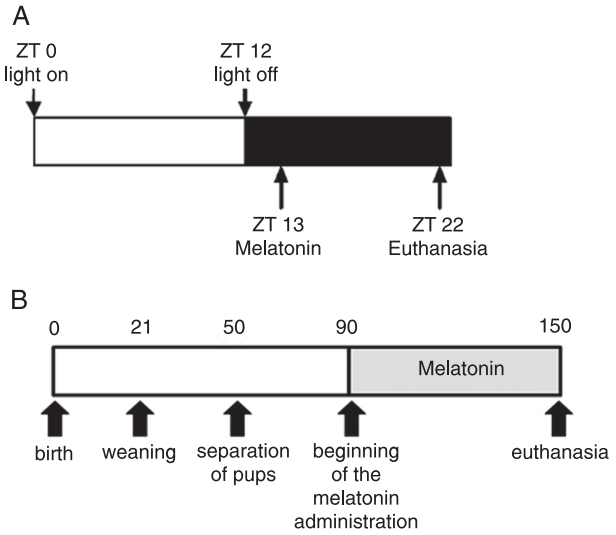

Figure 1. *A*, Protocol for melatonin treatment based on Zeitgeber time (ZT) corresponding to the environmental circadian time. *B*, Rats received 100 µg melatonin·100 g body weight^{-1}·day^{-1}, *ip*, for 60 days.

Material and Methods

Animals and experimental design

Twenty-four adult female rats (*Rattus norvegicus albinus*), 60 days old (± 250-260 g), were obtained from the Department of Anatomy, Bioscience Institute, Botucatu Campus (IBB, UNESP). The rats were divided into two groups of 12 animals each: control, rats receiving standard chow and tap water *ad libitum* and 0.04 mL 95% ethanol + 0.3 mL 0.9% NaCl (1:7, v/v) as vehicle; melatonin-treated group, rats receiving standard chow and tap water *ad libitum* and vehicle + melatonin. All animals were housed in polypropylene cages (43 cm x 30 cm x 15 cm) with laboratory-grade pine shavings as bedding under conditions of controlled room temperature (23 ± 1°C) and lighting (12-h light/12-h dark photoperiod, lights switched on at 6:00 am). At 90 days of age, females received melatonin daily for 60 consecutive days (Figure 1A and B). After the period of melatonin treatment, rats cycling in the morning of estrus (period of ovulation) at 4:00 am (or Zeitgeber time 22:00, ZT 22, corresponding to the environmental circadian time set) and monitored by vaginal swabs in a dark room using a red light, were anesthetized and killed by decapitation for further analysis. The experimental protocols were approved by the Ethics Committee of the Institute of Bioscience, UNESP, Botucatu, SP, Brazil (protocol #85/07).

Melatonin administration

For the animals designated to receive exogenous melatonin treatment, successive doses of melatonin (100 µg/100 g body weight; M-5250, purchased from Sigma Chemical, USA) were dissolved in 0.04 mL 95% ethanol, using 0.3 mL 0.9% NaCl (1:7, v/v) as vehicle (19). The injections (only vehicle or vehicle + melatonin) were administered daily *ip* between 6:30 and 7:00 pm (ZT 13; Figure 1A).

Food and liquid intake

The diet of the animals was prepared in lots of 5 days, always at the same time of day (3:00 pm) using a marked

test tube and an analytical scale (Ohaus Traveler™, Analytica S.A, Colombia). Liquid and food consumption (caloric value of standard chow = 2930 kcal/kg) was determined. Total energy intake (kcal/day) and feed efficiency (weight gain/consumed calories x 100) were evaluated as metabolic parameters. Body weight was also measured. At the end of treatment, the reproductive organs (uterine horn, ovaries and oviducts) were dissected and weighed. Body weight and organ weight were measured using an analytical balance (OwaLabor, Germany).

Glycemia measurements

The glycemic index was measured with a blood glucose sensor (One Touch Ultra System Kit, Lifescan, Italy) using blood samples from the caudal vein of the animals. To avoid variation, all samples were assessed in an equal volume collected from each animal after 12 h of fasting.

Assessment of estrous cyclicity

During the second part of the experiment, animals exhibiting estrous cycles were monitored by colpocytological examination (vaginal smears) (20). Cells detaching from the vaginal epithelium were removed with a pipette (Lab Mate 0.5-10 µL, UK). The filter tips containing 10 µL 0.9% saline were discarded after the vaginal secretion had been transferred to clean slides. Colpocytological examination time was at 9:00 am. Each slide was analyzed under a Zeiss Axiophot II microscope (Carl Zeiss, Germany) at 10X and 25X magnification and digitally photographed.

Determination of lipid hydroperoxide and antioxidant systems

After 60 days of melatonin treatment (100 µg·100 g body weight^{-1}·day^{-1}), the ovaries were removed rapidly. Each ovary was weighed and tissue samples of 40 mg were frozen immediately in liquid nitrogen and stored at -80°C. The ovary fragments were homogenized using a motor-driven Teflon Potter Elvehjem (Scientific Ltd., England) tissue homogenizer in 1.25 mL cold 0.1 M phosphate buffer, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 10,000 *g* for 15 min. The supernatant fraction was removed for the determination of total proteins, lipids, hydroperoxide, serum antioxidant capacity, and total antioxidant substances (test Kit Randox Laboratories Ltd., Crumlin, Co., UK).

Lipid hydroperoxide (LHP) was measured by Fe^{2+} to Fe^{3+} oxidation, using a 100-mL sample and a 900-mL reaction mixture containing 250 mM $FeSO_4$, 25 mM H_2SO_4, 100 mM xylenol orange, and 4 mM butyl hydroxytoluene in 90% (v/v) methanol. Absorbance was measured at 560 nm (21). Total antioxidant substances (TAS) were measured by the inhibition of LHP formation and measured at 500 nm (22). Spectrophotometric assays were carried out using a spectrophotometer with a temperature-controlled cuvette chamber (Ultraspec with Swift II software, Pharmacia Biotech, UK).

The enzymatic antioxidant system, reduced glutathione (GSH) and oxidized glutathione (GSSG), was investigated using the extracted supernatant (22). GSH-Px activity (E.C. 1.11.1.9) was then analyzed using glutathione oxidation reacted with hydrogen peroxide and cumene hydroperoxide. GSH reductase (GSH-Rd) activity was determined by monitoring NADPH oxidation at 340 nm. The mixture contained 1 mM Tris buffer, pH 8.0, 5 mM EDTA, 33 mM GSSG and 2 mM NADPH. SOD activity (E.C. 1.15.1.1) was determined with NADH and phenazine methosulfate (PMS) and reduction of nitroblue tetrazolium (NBT) by superoxides at physiological pH. The complete reaction system consisted of 50 mM phosphate buffer, pH 7.4, 0.1 mM EDTA, 50 mM NBT, 78 mM NADH, and 3.3 mM PMS. Catalase (E.C. 1.11.1.6) activity was assayed during the decomposition of H_2O_2 to H_2O + O_2. The assays of antioxidant activities were performed at 25°C using a µQuant microplate spectrophotometer (MQX 200 with KCjunior software, Bio-Tek Instruments, USA). All chemicals were purchased from Sigma.

Statistical analysis

The Student *t*-test for independent samples was used. Data are reported as means ± SEM and statistical significance was set at $P < 0.05$. The statistical software used was Sigma Plot version 11.0 and GraphPad Instat version 4 for graphic design.

Results

After 60 days of melatonin treatment, there were significant differences in body weight. Melatonin-treated rats exhibited a body weight reduction after 10 days of treatment, with a remarkable maintenance of body weight gain between days 35-60 (Figure 2). As expected, melatonin

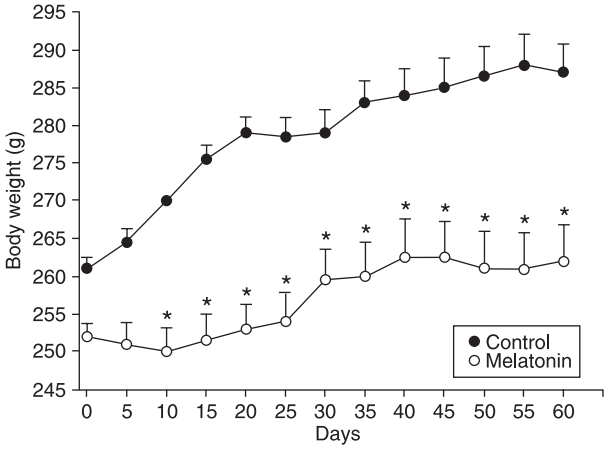

Figure 2. Effects of melatonin (100 µg·100 g body weight^{-1}·day^{-1}) on body weight gain during 60 days of treatment. Data are reported as means ± SEM for 10 animals per group. *$P < 0.01$ *vs* control group on days 10 to 60 of treatment (Student *t*-test).

treatment also reduced total weight gain and body mass index by decreasing fat mass (Table 1). Although overall food efficiency and liquid consumption were not affected by melatonin, there was a reduction in total and relative food consumption, as well as a reduced energy intake and glucose levels throughout 60 days of melatonin administration (Table 1, Figure 3).

During the second half of the daily melatonin treatment, when changes in total energy balance were detected, alterations in the reproductive cycles were observed, such as estrous irregularities, namely, more extensive cycles in melatonin-treated rats than controls, with a high frequency of continuous metaestrus and diestrus (Table 2). During this period, melatonin had no influence on estrous stage. Total ovarian mass was significantly reduced in melatonin-treated rats; however, the relative ovarian mass remained unchanged because of the reduced final body weight of the animals (Table 2).

Table 3 shows that total protein concentration remained unchanged in ovarian tissues after melatonin treatment. LHP levels increased during spontaneous ovulation and decreased when melatonin was given. Moreover, TAS were higher in melatonin-treated rats than controls, although the LHP/TAS ratio was unchanged because of the high LHP formation in control animals (Table 3). With respect to antioxidant activities, melatonin increased SOD, GSH-Rd and catalase activities in ovarian tissues during ovulation. Only GSH-Px activity was unchanged after melatonin treatment. Additionally, the reducing power (GSH/GSSG ratio) was higher in melatonin-treated rats than in controls, thus

Table 1. Nutritional parameters of female rats after 60 days of melatonin treatment.

Parameters	Control (N = 12)	Melatonin (N = 12)
Body weight gain (g)	36.5 ± 10.6	22.5 ± 11.6*
Body mass index (g/cm^2)	0.78 ± 0.05	0.66 ± 0.08*
Total food consumption (g/day)	19.57 ± 0.46	17.55 ± 0.43*
Liquid consumption (mL/day)	12.42 ± 0.23	11.06 ± 0.23
Energy intake (kcal/day)	57.35 ± 1.34	51.41 ± 1.28*
Feed efficiency (g/kcal)	63.0 ± 4.56	61.5 ± 4.76
Blood glucose (mg/dL)	103.5 ± 5.47	80.3 ± 4.49*

Data are reported as means ± SEM. *P < 0.05 *vs* control group (Student *t*-test).

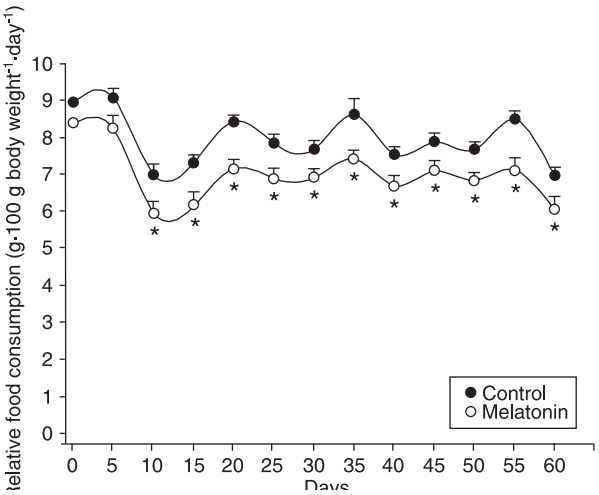

Figure 3. Influence of melatonin administration (100 µg·100 g body weight^{-1}·day^{-1}) on relative food intake for 60 days of rats receiving standard chow. Data are reported as means ± SEM for 10 animals per group. *P < 0.01 *vs* control group on days 10 to 60 of treatment (Student *t*-test). Day 0: Food consumption before the beginning of melatonin administration.

Table 2. Total and relative ovarian mass and duration of estrous cycles in female rats throughout 60 days of melatonin treatment.

Parameters	Control (N = 12)	Melatonin (N = 12)
Ovarian mass (g)	0.094 ± 0.01	0.075 ± 0.02*
Ovarian relative mass (g/100 g body weight)	0.035 ± 0.01	0.031 ± 0.01
Estrous cycle duration (days)	5.06 ± 0.20	6.90 ± 0.71*
Estrus (h/cycle)	23.92 ± 0.61	21.60 ± 0.92
Metaestrus (h/cycle)	6.72 ± 0.39	25.92 ± 0.39*
Diestrus (h/cycle)	34.56 ± 1.55	91.14 ± 1.48*

Data are reported as means ± SEM. *P < 0.05 *vs* control group (Student *t*-test).

Table 3. Oxidative and antioxidant status of rat ovaries after 60 days of melatonin administration.

Parameters	Control (N = 12)	Melatonin (N = 12)
Ovary protein (mg/100 mg tissue)	26.25 ± 2.52	35.74 ± 5.95
LHP (nmol/g tissue)	450.7 ± 15.3	419.5 ± 14.2*
TAS (%)	52.50 ± 1.47	65.06 ± 4.03*
LHP/TAS (g/tissue)	8.27 ± 0.59	7.47 ± 0.41
SOD (nmol/mg protein)	1.48 ± 0.12	1.88 ± 0.05*
GSH-Px (nmol/mg protein)	32.3 ± 1.22	30.6 ± 1.72
GSH-Rd (nmol/mg protein)	1.32 ± 0.01	1.55 ± 0.01*
CAT (nmol/mg protein)	2.85 ± 0.25	3.73 ± 0.16*
GSH/GSSG	10.5 ± 0.7	11.7 ± 0.6*

Data are reported as means ± SEM. LHP = lipid hydroperoxide; TAS = total antioxidant substances; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; GSH-Rd = glutathione reductase; CAT = catalase; GSH = reduced glutathione; GSSG = oxidized glutathione. *P < 0.05 *vs* control group (Student *t*-test).

emphasizing the reduced glutathione formation associated with increasing GSH-Rd activities during exhaustive antioxidant renovation.

Discussion

Appetite regulation and energy sources are fundamental for the maintenance of caloric balance and body weight gain. Melatonin reduced body weight gain, body mass index, total and relative food consumption as well as energy intake and glucose levels in the present study. There are several possible explanations regarding the action of melatonin as a metabolic hormone disruptor. Melatonin seems to be directly related to the periodicity of food intake (23). Molecular evidence suggests a stronger interaction between melatonin-induced clock genes and cell metabolism, including the control of glucose homeostasis and adipogenesis (24-26), thereby reducing the lipid content. Moreover, melatonin influences the satiety process and reduces blood glucose levels (27,28). Taken together, our findings concerning the nutritional balance could be attributed to one or more of these factors. Similarly, a previous study on the effects of melatonin detected body weight reduction in animals (29). It has also been demonstrated that pinealectomized rats do not show a greater body weight and, conversely, when they are exposed to a short-day photoperiod, a marked reduction of body weight is seen (30). Furthermore, these differences may depend on factors such as gender, age, or body composition of the animals receiving appropriate treatment.

In the present study, rats receiving melatonin showed a reduction in ovarian weight and, additionally, longer estrous cycles, which featured an extended metaestrous and diestrous phases. It is known that melatonin can increase the diestrous frequencies or estrous phases in rodents (31) by modulating GnRH hormones, but the exact mechanisms involved are poorly understood. Thus, it seems true that nutritional improvements were not effectively responsible for maintaining regular cycles. Moreover, rats receiving melatonin (200 µg·100 g body weight^{-1}·day^{-1}) and exposed to continuous light showed longer estrous periods and reduction of ovarian weight (32). It is clear that melatonin does not act directly on hypothalamic GnRH neurons and its responsiveness does not change with the photoperiod (33). Disturbances involving neuroendocrine regulation of reproduction appear to be associated with hypogonadotropic hypogonadism (34) where expression of *Kiss 1* is down-regulated by melatonin (35). Thus, our findings could be partially explained if melatonin suppressed GnRH hormones.

It is noteworthy that spontaneous ovulation is a source of ROS generation due to the intensive ovarian activity (36). Conversely, there is a body of evidence suggesting that melatonin plays key roles as a ROS scavenger (15,37). Under a variety of physiological conditions, including ovulation or follicular rupture, the proteolytic cascade and vascular changes lead to ROS production (38). It seems clear that increased levels of NADPH-oxidase generate superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2), which in the presence of Fe^{3+} produces powerful oxidants such as hydroxyl radicals, enhancing lipid peroxidation (39). In melatonin-treated rats, LHP levels were reduced and TAS concentration was increased, although the LHP/TAS ratio was not modified by melatonin. Interestingly, lipid hydroperoxide levels were higher during spontaneous ovulation and underwent a remarkable reduction after the administration of melatonin, which quenched hydroxyl radicals within ovarian cells, confirming the protective effect of the hormone as a ROS scavenger preserving fatty acid integrity against aldehyde formation and finally improving the lipid profile (40). Also, another sub-product formed as a result of melatonin scavenging hydrogen peroxide, i.e., N(1)-acetyl-N(2)-formyl-5-methoxykynuramine, is also a potent scavenger.

Melatonin had an important effect on the ovaries, inducing higher SOD, catalase and GSH-Rd activity compared to control. It is well known that melatonin has an important role in transcriptional mRNA synthesis (i.e., gamma-glutamyl cysteine synthetase and others) for antioxidant enzymes (18), suggesting that melatonin regulation is receptor-mediated, thereby most likely implicating the MT1/MT2 receptors via second messengers such as cAMP, phospholipase C or intracellular calcium concentration, and thus, it might have improved the antioxidant profile if they are activated. The antioxidant defense system is an integrated array of enzyme and non-enzyme antioxidants. In control rats, higher ovarian LHP levels indicated that the oxidation of GSH to GSSG, reducing the GSH/GSSG ratio, led to lipoperoxidation and oxidative stress. During the reduction of hydrogen peroxide, GSH is oxidized to GSSG and GSH-Rd is activated to convert oxidized GSSG to reduced GSH. Curiously, besides increasing the GSH/GSSG ratio, melatonin also enhanced GSH-Rd activity. Our findings seem to agree with these mechanisms.

This study demonstrated that long-term melatonin treatment modulates body weight gain, calorie storage and food intake. Although melatonin reduced ovarian mass and caused estrous cycle disturbances, it was able to protect the ovaries against lipid peroxidation by reducing LHP levels during ovulation. These protective effects could be due to the properties of melatonin both as a ROS scavenger and as an agent increasing antioxidant activities.

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