Long-Term Exogenous Melatonin Treatment Modulates Overall Feed Efficiency and Protects Ovarian Tissue Against Injuries Caused by Ethanol-Induced Oxidative Stress in Adult UChB Rats

Luiz Gustavo A. Chuffa, João P. A. Amorim, Giovana R. Teixeira, Leonardo O. Mendes, Beatriz A. Fioruci, Patrícia F. F. Pinheiro, Fábio R. F. Seiva, Ethel L. B. Novelli, Wilson de Mello Júnior, Marcelo Martinez, Camila C. D. Almeida-Francia, and Francisco E. Martinez

Background: Chronic ethanol intake leads to reproductive damage including reactive oxygen species formation, which accelerates the oxidative process. Melatonin is known to regulate the reproductive cycle, food/liquid intake, and it may also act as a potent antioxidant indoleamine. The aim of this study was to verify the effects of alcoholism and melatonin treatment on overall feed efficiency and to analyze its protective role against the oxidative stress in the ovarian tissue of UChB rats (submitted to 10% [v/v] voluntary ethanol consumption).

Methods: Forty adult female rats (n = 10/group) were finally selected for this study: UChB Co: drinking water only; and UChB EtOH: drinking ethanol at 2 to 6 ml/100 g/d + water, both receiving 0.9% NaCl + 95% ethanol 0.04 ml as vehicle. Concomitantly, UChB Co + M and UChB EtOH + M groups were infused with vehicle + melatonin (100 μg/100 g body weight/d) intraperitoneally over 60 days. All animals were euthanized by decapitation during the morning estrus (4 AM).

Results: Body weight gain was reduced with ethanol plus melatonin after 40 days of treatment. In both melatonin-treated groups, it was observed a reduction in food-derived calories and liquid intake toward the end of treatment. The amount of consumed ethanol dropped during the treatment. Estrous cycle was longer in rats that received both ethanol and melatonin, with prolonged diestrus. Following to oxidative status, lipid hydroperoxide levels were higher in the ovaries of ethanol-preferring rats and decreased after melatonin treatment. Additionally, antioxidant activities of superoxide dismutase, glutathione peroxidase activity, and glutathione reductase activity were increased in melatonin-treated groups.

Conclusions: We suggest that melatonin is able to affect feed efficiency and, conversely, it protects the ovaries against the oxidative stress arising from ethanol consumption.

Key Words: Melatonin, Feed Efficiency, Ovary, Ethanol, Lipid Peroxidation.

ACUTE AND CHRONIC ethanol intake is associated with several dysfunctions of female reproduction, including amenorrhea, blockade of ovulation, early menopause, spontaneous miscarriage, and infertility (Carrara et al., 1993; Henderson et al., 2007). Ethanol drinker strains (UChB) are derived from original Wistar rats and have been selectively bred at the University of Chile for almost 60 years (Mardones and Segovia-Riquelme, 1983). These ethanol-preferring rats are considered a special model for the understanding of the basis of alcoholism-linked characteristics such as those found in alcohol-related human diseases.

In mammals, the photoperiodic hormone melatonin (*N*-acetyl-5-methoxytryptamine) is secreted by the pineal gland and it has been documented as an important modulator of reproductive function because of its stimulation of ovarian activity, promotion of estrous cycles, gonadal atrophy (Horton and Yellon, 2001; Ocal-Irez, 1989), and regulation of folliculogenesis and ovulation (Zhao et al., 2000). More directly, ethanol intake has been proposed to induce diurnal melatonin production (Fonzi et al., 1994), and as recently described, chronic ethanol consumption plays a defective role in the synchronization of circadian rhythms (Rosenwasser et al., 2005), resulting in sleep disorders and alcohol-induced mental

From the Structural and Cell Biology Program (LGAC, JPAA, LOM, BAF), Institute of Biology, Universidade Estadual de Campinas—UNICAMP, Campinas; Department of Anatomy (GRT, PFFP, WdMJ, CCDA-F, FEM), Institute of Biosciences, UNESP—Univ Estadual Paulista, Botucatu; Department of Chemistry and Biochemistry (FRFS, ELBN), Institute of Biosciences, UNESP—Univ Estadual Paulista, Botucatu; and Department of Morphology and Pathology (MM), Universidade Federal de São Carlos, São Carlos, Brazil.

Received for publication August 11, 2010; accepted January 4, 2011.

Reprint requests:Francisco Eduardo Martinez, Department of Anatomy, Institute of Bioscience, UNESP—Univ Estadual Paulista, PO Box 510, Rubião Júnior, s/n, 18618-000 Botucatu, SP, Brazil, Tel.: +55-14-3811-6040; Fax: +55-14-3811-6361; E-mail: martinez@ibb.unesp.br

DOI: 10.1111/j.1530-0277.2011.01486.x

dysfunctions (Clark et al., 2007). Melatonin may be useful as a hormonal therapeutic replacement as it minimizes sleep disorders and stress agents arising from chronic alcoholism.

There are some experimental support involving actions of melatonin on nutritional parameters, such as feed efficiency, body mass gain, adiposity index, and both energy intake and expenditure (Korkmaz et al., 2009; Mustonen et al., 2002). These controversial effects are over-linked to specific diet regimen, age, and gender, but none has evaluated the effects associated with chronic ethanol consumption.

It is noteworthy that melatonin presents antioxidant properties both in vivo and in vitro. Melatonin can stimulate the activities and expressions of antioxidant enzymes that contribute to the protection against the damages caused by oxidative stress (Rodriguez et al., 2004). Several studies have demonstrated that melatonin detoxifies a variety of free radicals and reactive oxygen molecules, including hydroxyl radical (OH), superoxide, peroxynitrite, singlet oxygen, and others. Melatonin acting as a free radical scavenger of oxidative products prevents lipid peroxidation in reproductive tissues (Armagan et al., 2006; El-Sokkary et al., 1999; Peyrot and Ducrocq, 2008) and can be an useful tool against the oxidative damage arising from chronic alcoholism (Hu et al., 2009). It is known that the metabolism of ethanol induces reactive oxygen species (ROS) formation, mainly through the P450 CYP2E1 microsomal system evidenced by high rate of oxidized NADPH, where is produced large quantities of superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) both in humans and ethanol-fed rodents (Gouillon et al., 2000; Ronis et al., 2004). Oxidative stress may be a cause of poor oocyte quality, because it produces severe cell damage, including deterioration of membrane lipids, apoptosis, and inhibition of fertilization (Agarwal et al., 2005; Noda et al., 1991). Melatonin also increases the synthesis of enzymes such as superoxide dismutase (SOD), glutathione (GSH), peroxidase (GSH-Px, GSH), reductase (GSH-Rd), and catalase (Armagan et al., 2006; Subramanian et al., 2007; Tan et al., 2007). Furthermore, recent approaches involving chronic ethanol consumption revealed a depletion of mtGSH content (Fernandez-Checa and Kaplowitz, 2005) and a decline in the enzymatic activity of liver Cu–Zn SOD, catalase, and GSH-Px (Polavarapu et al., 1998). The role of melatonin on the female reproductive tract in ethanol-fed rats is still poorly understood.

In view of the above-mentioned findings, this study was designed to investigate the effects of ethanol, associated or not with melatonin treatment, on overall feed efficiency and oxidative stress in the ovaries of UChB ethanol-preferring rats (10% [v/v] ethanol voluntary drinkers).

MATERIALS AND METHODS

Animals and Experimental Design

Forty adult female rats (*Rattus norvegicus albinus*), 60 days old (225 to 240 grams at baseline), were obtained from the Department of Anatomy, Bioscience Institute/Campus of Botucatu (IBB), UNESP—Univ Estadual Paulista. The animals were randomly divided into 4 groups (n = 10/group). UChB EtOH group: rats fed 10% (v/v) ethanol ad libitum (free choice for water or ethanol) drinking from 2.0 to 6.0 ml/100 g body weight (BW)/d and receiving vehicle solution; UChB Co group: Ethanol-naïve rats without access to ethanol, used as a control group, receiving vehicle solution; UChB EtOH + M group: rats fed 10% (v/v) ethanol (free choice for water or ethanol) drinking from 2.0 to 6.0 ml/100 g BW/d and receiving vehicle + melatonin; and UChB Co + M group: without access to ethanol and receiving vehicle + melatonin. When the UChB EtOH rats reached 60 days of age, they were given during 20 days, a choice between 2 bottles containing either water ad libitum (1) or 10% (v/v) ethanol (2). After this period, 10 animals per group displaying ethanol consumption higher than 2.0 ml of ethanol/100 g BW/d were finally selected according to Mardones and Segovia-Riquelme (1983). To predict ethanol intake, blood acetaldehyde levels serve as good marker and they are remarkably high in UChB rats (Tampier et al., 2008). For this study, the preference ratio associated with ethanol-seeking behavior was about 65%. Besides that, to ensure more efficiency and maintenance of constant consumption throughout the experiment, the animals were kept under observation for 10 days when they started with melatonin treatment. Thus, after 90 days old, females received ethanol and melatonin during 60 consecutive days (Fig. 1*A*). After the melatonin treatment, rats in the morning estrus at 4 AM (or Zeitgeber Time 22:00 ZT 22) monitored by vaginal swabs in a dark room using red light were anesthetized and euthanized by decapitation for further analysis. All animals were individually housed in polypropylene cages (43 cm × 30 cm × 15 cm) with laboratory-grade pine shavings as bedding and also maintained under controlled room temperature (23 ± 1°C) and lighting conditions (12L, 12D photoperiod, lights switched on at 6 AM). The rats received a standard rodent chow (3074 SIF; Purina Ltd, Campinas, Brazil) and filtered tap water ad libitum. Experimental protocols followed the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by Ethics Committee on Animal Experimentation (Protocol no. 85/07).

Procedures of Melatonin Administration

For the animals designated to receive exogenous melatonin treatment, successive doses of melatonin (100 μg/100 g BW) (M-5250; Sigma Chemical, St. Louis, MO) were dissolved in 95% ethanol 0.04 ml, using 0.9% saline solution as a vehicle (Chuffa et al., 2011;

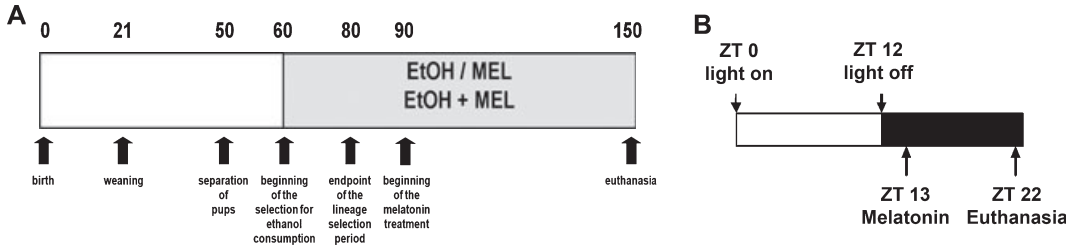

Fig. 1. (**A**) Chronological scheme for overall chronic treatment. (**B**) Schematic protocol used for melatonin (MEL) treatment based on Zeitgeber Time (ZT) corresponding to environmental circadian time. EtOH, ethanol.

Kim and Lee, 2000; Vázquez et al., 2007). The intraperitoneal infusions (only vehicle or vehicle + melatonin) were daily administered between 18:30 and 19:00 PM (ZT 13) (Fig. 1*B*).

Food and Liquid Intake

Feeding content was prepared in lots of 5 days, always at the same time of day (15:00 hours) using a marked test tube and analytical balance (Ohaus Traveler™; Ohaus Corporation, México, D.F, MÉXICO, MX). The profile of liquid ingestion (caloric value of water + ethanol = 7.1 kcal/g ethanol) and food (caloric value of standard chow = 2,930 kcal/kg) were assigned according to the standards of necessary care. Total energy intake (kcal/d) and feed efficiency (weight gain/consumed calories × 100) were evaluated as metabolic parameters. BW was also measured, and melatonin dosages were individually adjusted for each weight. At the end of treatment, the reproductive organs (uterine horn, ovaries, and oviducts) were dissected and weighed. The determination of BW and organ weight was carried out using an analytical balance (OwaLabor, Oschatz, Germany).

Glycemia Measurements

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

Assessment of Estrous Cyclicity

During the second half of the experiment, animals exhibiting estrous cycles were accompanied by colpocytological examination (vaginal swabs). Cells detaching from the vaginal epithelium were removed with a pipette (Lab Mate 0.5 to 10 μl; International Labmate Ltd, St. Albans, UK). The filter tips containing 10 μl of 0.9% saline solution (Marcondes et al., 2002) were discarded after the vaginal secretion had been transferred to clean slides. Colpocytological examination time was fixed at 9 AM. Each slide was analyzed under a Zeiss Axiophot II microscope (Carl Zeiss, Oberköchen, Germany) at 10× and 25× magnification and digitally photographed.

Determination of Lipid Hydroperoxide and Antioxidant Systems

After 60 days of melatonin treatment (100 μg/100 g BW/d), the ovaries were rapidly removed. Each right ovary was weighed, and tissue samples of 40 mg were immediately frozen in liquid nitrogen and stored at −80°C. The ovary samples were homogenized using a motor-driven Teflon Potter Elvehjem tissue grinder (Omni International, Kennesaw, GA) in 1.25 ml of cold 0.1 M phosphate buffer (pH 7.4) with the addition of 1 mM ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 10,000×*g* for 15 minutes. The supernatant fraction was removed for the determination of total proteins (Seiva et al., 2010), lipid hydroperoxide LHP (Jiang et al., 1991), and antioxidant substances (Seiva et al., 2008). LHP was measured by Fe^{2+} to Fe^{3+} oxidation in the presence of xylenol orange at 560 nm. Total antioxidant substances (TAS) were assessed by the inhibition of LHP formation. Spectrophotometric assays were carried out using a spectrophotometer with a temperature-controlled cuvette chamber (UV/visible Ultraspec with Swift II software; Pharmacia Biotech, Cambridge, UK).

The enzymatic antioxidant system was investigated using the extracted supernatant. Afterward, GSH-Px activity (E.C. 1.11.1.9.) was analyzed using GSH oxidation reacted with H_2O_2 and cumene hydroperoxide (Nakamura et al., 1974). GSH-Rd activity was evaluated by monitoring NADPH oxidation (reduced nicotinamide adenine dinucleotide phosphate) at 340 nm (Miller and Blakely, 1992). The reactive mixture included 1 mM Tris buffer, pH 8.0, 5 mM EDTA, 33 mM GSSG, and 2 mM NADPH. The activity of SOD (E.C. 1.15.1.1), observable in a reduction of nitroblue tetrazolium by superoxide radicals, was verified by mixing NADH and phenazine methosulfate at a physiological pH (Ewing and Janero, 1995). Catalase (E.C. 1.11.1.6.) activity was assayed during the decomposition of H_2O_2 to $H_2O + O_2$ (Aebi, 1974). The assays of antioxidant activities were performed at 25°C using a μQuant microplate spectrophotometer (MQX 200 with KCjunior software; Bio-Tek Instruments, Winooski, VT).

Statistical Analysis

Statistical comparisons were performed by 2-way analysis of variance (ANOVA) with post hoc Tukey's test. Nonparametric Kruskal–Wallis test complemented by Dunn were applied according to the chosen parameter. All results are given as means (SEM) or median (min; max) values. Significance was set at $p < 0.01$ and $p < 0.05$. The statistical software used was Sigma Plot version 11.0 (Systat Software, Inc., Chicago, IL) and GraphPad Instat version 4 for graphic design (GraphPad Software, Inc., San Diego, CA).

RESULTS

After 60 days of treatment, there were significant differences on BW among the groups. The interaction between ethanol and melatonin promoted a constant decline in BW gain after the second half of treatment, when compared with animals receiving only ethanol (Fig. 2*A*). In the final days of treatment, the UChB Co + M group had higher BW gain compared with animals that were given both melatonin and ethanol (Fig. 2*A*). Initially, based on the time points examined, UChB EtOH and UChB EtOH + M rats exhibited an identical drop in food consumption, differently from the controls (Fig. 2*B*). Only the UChB Co + M rats had increased food intake at several time points, indicating the effects of melatonin, especially during the second half of treatment. After 40 days of treatment, the interaction between melatonin and ethanol caused a reduction in food consumption average than those receiving only ethanol (Fig. 2*B*). Furthermore, after 50 days of treatment, animals receiving ethanol in combination with melatonin showed a marked loss of appetite with a fall in food intake. Feed efficiency was reduced in UChB EtOH rats and became even more pronounced when melatonin was given. The group that received ethanol and melatonin had the lowest amount of stored calories, half of that found in UChB Co + M rats (Fig. 3*A*). Glucose levels were reduced after melatonin treatment, except in the animals receiving ethanol, despite these animals already demonstrated a considerable reduction in glucose levels (Fig. 3*B*). There was a negative correlation between ethanol intake and exogenous melatonin administration throughout the 60 days of treatment ($r = -0.79$; $p < 0.001$), evidenced by a mild reduction in total ethanol consumption observed prior to starting and during the experiment (Fig. 3*C*).

In melatonin-treated rats, energy and food intake declined toward the end of treatment. As shown in Table 1, during overall 60-day treatment, calories derived from ethanol and food, in the presence of melatonin, were poorly taken by the

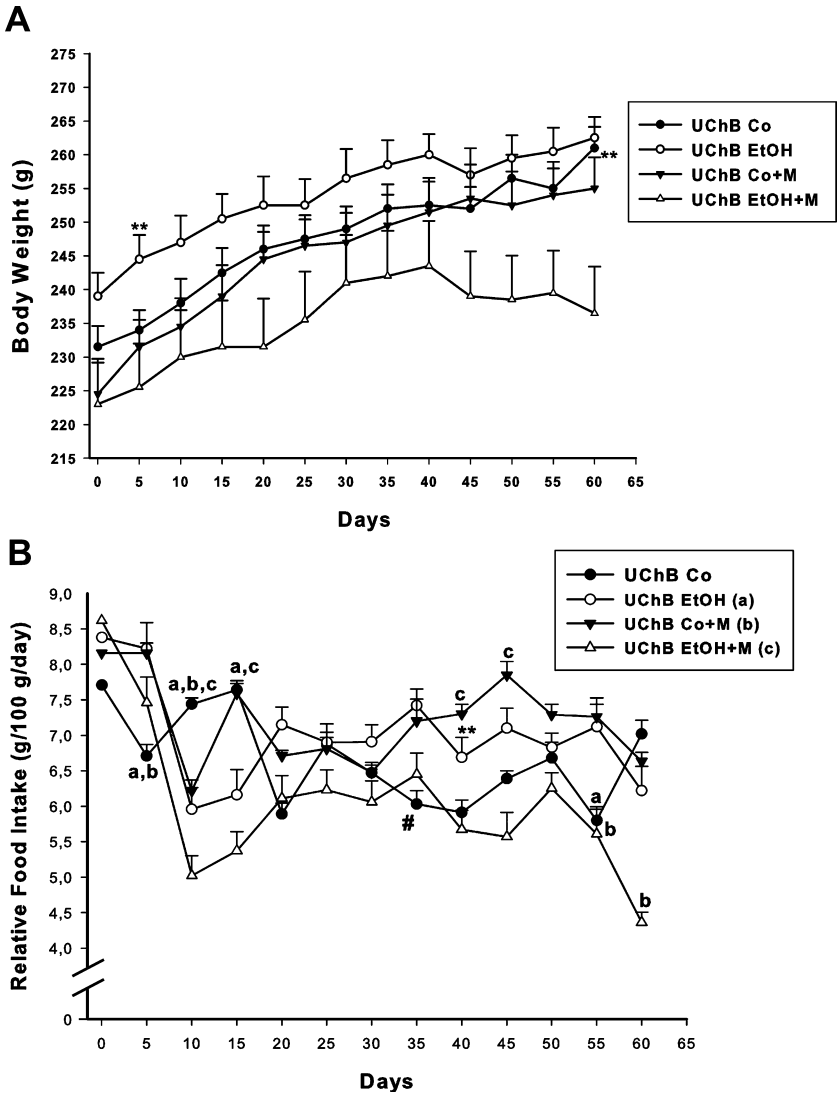

Fig. 2. (**A**) Ethanol (EtOH) and melatonin (M) effects (100 μg/100 g/d) on body weight gain in all experimental groups during 60 days of treatment. $^{**}p < 0.01$ versus UChB EtOH + M group at 5, 20, 45 to 60 days. At the end of treatment, UChB EtOH + M showed significant difference from UChB Co + M group. (**B**) Influence of melatonin and ethanol administration on relative food intake over 60 days in rats receiving standard chow. $^{**}p < 0.01$ versus UChB EtOH + M group on days 40 to 60. $^{\#}p < 0.01$ versus UChB Co + M group on days 35 to 55. $^{a-c}$Significant difference from UChB EtOH, UChB Co + M and UChB EtOH + M groups, respectively. Values are expressed as mean ± SEM (n = 10/group). Two-way ANOVA with Tukey's post hoc test. Day 0: food consumption before beginning of the treatment.

UChB EtOH rats. Following the experiment, the interaction between ethanol and melatonin contributed to the decrease in food and total energy intake, but not the energy provided by ethanol itself. In ethanol-preferring rats, the relative daily water intake was significantly decreased over the experiment, regardless of melatonin. It was also proved that after melatonin administration, total ethanol intake was reduced by 14.4% whereas untreated animals increased their consumption by about 11.3% (Table 1).

During second half of daily melatonin treatment, from the 4th until the 8th week of intervention, the vaginal smears revealed estrous cycle irregularities, namely, more extensive cycles in UChB EtOH + M rats than in other groups, with an elevated frequency of prolonged diestrous phase when compared with those receiving only ethanol. After 4 weeks of cytological examination, the increase in metaestrous stage was even more evident in UChB Co + M group (Table 2). Despite these irregularities, there were no anovulatory cycles.

The relative and total ovarian weights were significantly reduced in animals receiving the ethanol plus melatonin in relation to those receiving either ethanol or melatonin (Table 3). Also, the combination of ethanol and melatonin negatively affected the uterine horns weights. On the other hand, melatonin had no significant effect on oviduct weight (Table 3).

With respect to ovarian tissues, total protein concentration was high in the UChB EtOH and UChB Co + M groups. The interaction between ethanol and melatonin showed a significant decrease in TAS concentrations when compared with

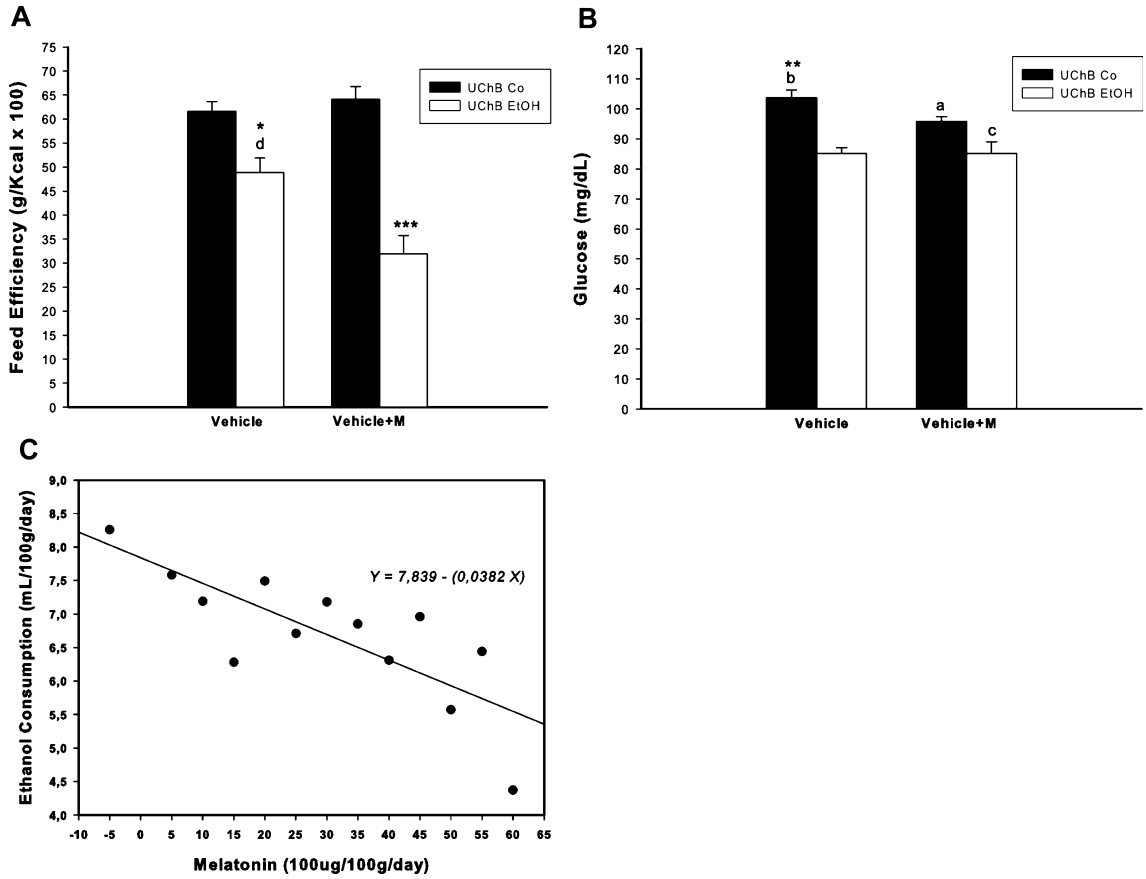

Fig. 3. (**A**) Feed efficiency (%) over the 60 days of melatonin administration (100 μg/100 g/d) and ethanol (EtOH) intake. $^{d}p < 0.05$ versus UChB EtOH + M group; $^{*}p < 0.05$, $^{***}p < 0.001$ versus UChB Co + M group. (**B**) Effects of melatonin administration alone or in combination with ethanol intake on plasma glucose levels of nonfasted rats. $^{b}p < 0.01$ versus UChB EtOH group; $^{a,c}p < 0.05$ versus UChB Co and UChB EtOH + M groups, respectively; $^{**}p < 0.01$. Values are expressed as mean ± SEM ($n = 10$/group). Two-way ANOVA complemented by Tukey's test. (**C**) Scattergram showing the correlations between melatonin administration and ethanol consumption throughout the 60 days ($r < -0.79$; $p < 0.001$). Day 0: beginning of the melatonin treatment.

Table 1. Status of Food Consumption (g/d), Total Energy Intake (kcal/d), Relative Water/Ethanol Intakes (ml/100 g/d), and Ethanol-Derived Calories (kcal/d) in Rats Receiving Only Vehicle and/or Melatonin.

	Vehicle		Melatonin	
Parameters	UChB Co	UChB EtOH	UChB Co	UChB EtOH
Food consumption	16.32 ± 0.40	15.90 ± 0.38	17.03 ± 0.51	14.49 ± 0.57[a]
Energy intake (food + ethanol)	47.83 ± 1.17	49.59 ± 1.26	49.90 ± 1.50	46.36 ± 1.90[a,b]
Relative water intake	13.47 ± 0.15	9.89 ± 0.30[c]	12.58 ± 0.44	7.48 ± 0.58[a]
Ethanol intake (PT)	0.00	4.86 ± 0.67	0.00	6.58 ± 0.62
Ethanol intake (AT)	0.00	5.48 ± 0.57	0.00	5.63 ± 0.57*
Ethanol calories	0.00	2.99 ± 0.14	0.00	3.41 ± 0.21

Values are expressed as mean ± SEM. $N = 10$/group.
Means followed by lowercase letters indicate statistical differences among the groups ($p < 0.05$). Two-way ANOVA with post hoc Tukey's test.
PT, prior treatment; AT, after treatment; EtOH, ethanol.
[a,b] $p < 0.05$ versus UChB EtOH and UChB Co + M groups, respectively.
[c] $p < 0.01$ versus UChB Co group.
* $p < 0.05$ versus UChB EtOH + M (PT).

animals receiving only melatonin (Table 4). The LHP/TAS ratio was enhanced after ethanol intake, because LHP levels increase with chronic alcoholism; however, no changes were found after melatonin treatment (Table 4 and Fig. 4).

LHP levels were significantly reduced after melatonin treatment. In UChB EtOH group, the levels of LHP were higher than those found in control group in which the ethanol-treated rats evidenced the highest levels of LHP formation (note that melatonin improved LHP levels at 18.5% in UChB EtOH group) (Fig. 4). SOD and GSH-Px activities were increased with the interaction of ethanol on melatonin, compared with both groups receiving either

Table 2. Effects of Ethanol and Melatonin on Estrous Cyclicity (Days) and Frequency (%) of Lengthiness in Each Phase Among the Experimental Groups (N = 10/Group).

Groups	Cycle duration (days)	Estrus persistent/cycle (%)	Metaestrus persistent/cycle (%)	Diestrus persistent/cycle (%)
UChB Co	7.45 ± 0.50	6.67 (0; 26.67)	13.33 (0; 40.00)[a]	6.66 (0; 10.00)
UChB Co + M	6.55 ± 0.34	13.33 (0; 30.00)	18.33 (6.67; 40.00)	15.00 (6.67; 26.67)
UChB EtOH	6.45 ± 0.44	16.67 (0; 26.67)	16.67 (0; 43.33)	10.00 (3.33; 23.33)
UChB EtOH + M	10.35 ± 1.10**	6.67 (0; 30.00)	16.66 (0; 36.67)	21.66 (3.33; 46.67)[b]

Values are expressed as mean ± SEM and median (minimum–maximum).
EtOH, ethanol.
[a]$p < 0.05$ versus UChB Co + M group.
[b]$p < 0.05$ versus UChB EtOH group. Kruskal–Wallis test complemented by Dunn.
**$p < 0.01$ versus UChB Co + M and UChB EtOH groups, respectively. Two-way ANOVA with post hoc Tukey's test.

Table 3. Data of Total (grams) and Relative (g/100 g Body Weight) Reproductive Organs Weights in Female Rats Receiving Vehicle or Melatonin at the End of the Treatment.

	Vehicle		Melatonin	
Parameters	UChB Co	UChB EtOH	UChB Co	UChB EtOH
Ovary weight	0.098 ± 0.01	0.098 ± 0.01	0.098 ± 0.02	0.069 ± 0.02[a,b]
Ovary relative weight	0.037 ± 0.00	0.037 ± 0.00	0.038 ± 0.01	0.029 ± 0.01[a,b]
Oviduct weight	0.034 ± 0.01	0.034 ± 0.00	0.033 ± 0.01	0.030 ± 0.01
Oviduct relative weight	0.013 ± 0.01	0.013 ± 0.00	0.013 ± 0.00	0.013 ± 0.01
Uterine horn weight	0.52 ± 0.13	0.51 ± 0.13	0.50 ± 0.06	0.40 ± 0.11[a]
Uterine horn relative weight	0.19 ± 0.04	0.19 ± 0.06	0.20 ± 0.02	0.17 ± 0.04

Values are expressed as mean ± SEM. N = 10/group.
Two-way ANOVA complemented by the Tukey's test.
EtOH, ethanol.
[a]$p < 0.01$ versus UChB EtOH group.
[b]$p < 0.001$ versus UChB Co + M group.

Table 4. Determinations of Total Protein (%) mg Protein/mg Tissue, Total Antioxidant Substances (% TAS) and Lipid Hydroperoxide (LHP)/TAS Ratio (g/Tissue) in Ovarian Tissue of 10% (v/v) Ethanol (EtOH)-Preferring Rats Receiving Melatonin at Doses of 100 μg/100 g BW/d for 60 Days.

	Vehicle		Melatonin	
Parameters	UChB Co	UChB EtOH	UChB Co	UChB EtOH
Protein	38.80 ± 3.33[a,b]	50.10 ± 6.26	48.60 ± 4.66	42.89 ± 5.53
TAS	64.84 ± 1.67	63.22 ± 2.25	67.02 ± 1.49	61.70 ± 1.98[b]
LHP/TAS	9.54 ± 0.27[a]	13.22 ± 0.23	9.84 ± 0.15	11.69 ± 0.32

Values are expressed as mean ± SEM. N = 7 animals/group.
Letters indicate statistical differences among the groups ($p < 0.05$). Two-way ANOVA complemented by Tukey's test.
[a]$p < 0.001$ versus UChB EtOH.
[b]$p < 0.05$ versus UChB Co + M.

ethanol or melatonin (Fig. 5*A* and 5*B*). Catalase activity did not differ between ethanol-treated rats, and conversely, when melatonin alone was given, the catalase activity was completely restored (Fig. 5*C*). All melatonin-treated animals showed an increase in GSH-Rd activity compared with rats that received or not ethanol (Fig. 5*D*).

DISCUSSION

Appetite regulation and energy intake are fundamental for the maintenance of caloric balance and BW. Ethanol and melatonin are thought to interact directly on the maintenance of BW, mainly during the second half of treatment. Previous studies involving several doses and routes of melatonin administration in rats found an associated BW reduction in those that had been castrated (Puchalski et al., 2003), in adult rats (Bojková et al., 2008; Rasmussen et al., 2001) and in those consuming a high fat diet (Prunet-Marcassus et al., 2003). On the other hand, when melatonin is used in nocturnal progressive doses ranging from 3,000 to 15,000 pg/ml, it promotes food intake and BW gain in both male and female rats (Angers et al., 2003). Ethanol per se produces weak satiating signals and in humans, depending on metabolism and nutritional status, appears to increase food consumption and appetite (Yeomans, 2004; Yeomans et al., 1999). These differences may rely on factors such as age, gender, or body composition of the experimental animals. Taken together, melatonin seems to be related with the periodicity of food

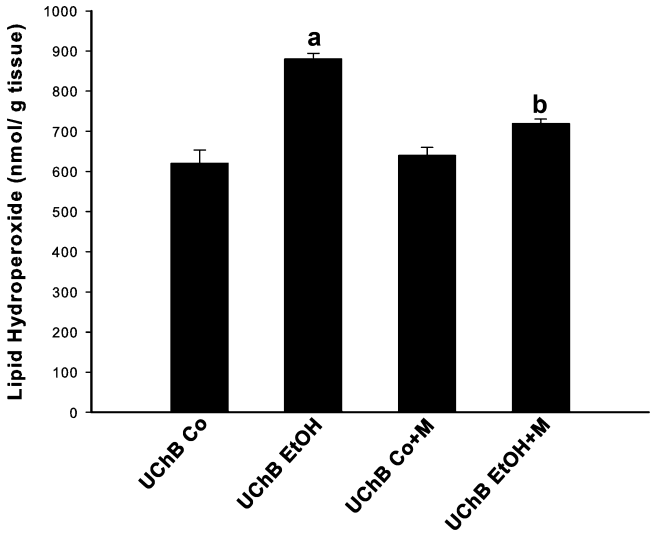

Fig. 4. Lipid hydroperoxide concentration (nmol/g tissue) in ovarian tissue of control and ethanol (EtOH)-treated rats, receiving or not melatonin (M) (100 μg/100 g/d) after 60 days of treatment. [a]$p < 0.05$ versus UChB Co group; [b]$p < 0.001$ versus UChB EtOH group. Values are means ± SEM ($n = 10$/group). Two-way ANOVA complemented by Tukey's test.

intake by influencing the gastrointestinal tract (Bubenik, 2002). Taking into account that NADH, produced by ethanol-acetaldehyde conversion, plays key roles in cellular bioenergetics and can modulate fatty acid synthesis as well as suppress β-oxidation (Lieber, 2004), these mechanisms could explain, in part, the differences in BW associated with food consumption by the animals receiving either ethanol or melatonin and both ethanol and melatonin. In addition, the caloric value of ethanol (empty calories) did not contribute to BW gain, and furthermore, lipid synthesis is blocked by the inhibition of glucose-6-phosphate dehydrogenase (Ayene et al., 2002), which is a key enzyme in the pentose shunt for generating NADPH and essential for lipogenesis. Similar approaches have been obtained with rats submitted to various diet regimens and exposed to chronic ethanol intake for 20 weeks (Smith et al., 2008) and 8 consecutive weeks (Monteiro et al., 2009).

Scalera and colleagues (2008) also reported reduction in food consumption and increased leptin levels in melatonin-treated rats. In rabbits, treatment with melatonin was followed by 4 weeks of reduced food consumption, BW, and glucose levels (Hussein et al., 2007). Also supporting our findings, Strbák and colleagues (1998) demonstrated that when ethanol consumption rises, food consumption is often reduced, depending on age and particular lineage. In female rats, ethanol preference has also been linked to a reduction in water consumption (Bell et al., 2004). Considering our rat model (UChB), the low water intake occurred because of increased ethanol/ethanol + water ratio. As observed in the UChB Co + M group, ethanol deprivation may lead to withdrawal-induced food intake increases (Kampov-Polevoy et al., 2004; Krahn et al., 2006). In this condition, the melatonin would interact synergistically.

Feed efficiency was reduced concurrently with ethanol consumption and became more pronounced when combined with melatonin, resulting in a low calorie-derived BW gain. This association suggests changes in energetic metabolism and in the ability to store energy as fat mass. Additionally, glucose blood levels were lower in the presence of ethanol or melatonin. In fact, it is well documented that ethanol inhibits gluconeogenesis and melatonin plays a role in carbohydrate metabolism related to insulin sensitivity (Muhlbauer et al., 2009; She et al., 2009). As expected, the effects of ethanol in combination with melatonin promoted a greater reduction in glucose levels. Similar studies have demonstrated that both melatonin (Prunet-Marcassus et al., 2003) and chronic ethanol consumption (Choi et al., 2006) induced low feed efficiency in rats beyond the effects that ethanol alone has on malnutrition (DiCecco and Francisco-Ziller, 2006).

Ethanol consumption decreased after the beginning of melatonin treatment. Accordingly, the ethanol intake dropped when melatonin (25 μg/animal/d) was administered over 11 weeks (Rudden and Symmes, 1981). Noticeably, it has been reported the prominent actions of melatonin on the opioidergic system, producing the analgesic effect in a dose-dependent manner that is similar to naloxone (opioid receptor antagonist) (Yu et al., 2000). Furthermore, whether melatonin is able to stabilize the reward system (Tahsili-Fahadan et al., 2005), it could be avoiding the ethanol reinforcement. Nevertheless, the effects of melatonin on ethanol dependence syndrome remain to be investigated (Arnedt et al., 2007; Fonzi et al., 1994).

Following chronic ethanol consumption, the main time-dependent effects over 60-day treatment have already been confirmed by our previous study in which ovarian tissue was structurally compromised (Chuffa et al., 2009). The ethanol-induced alterations include higher incidence of degenerating granulosa cells and follicular atresia, fluctuations in follicular fluid composition, ultrastructural changes such as autophagy, excessive lipid droplets formation, as well as disruption of follicular basement membrane. In this context, melatonin administration may act as a protective factor by preventing ethanol-induced ovarian failures.

In this study, rats receiving only ethanol or melatonin presented persistent estrus, while the positive interaction between ethanol and melatonin promoted the longest estrous cycles, which featured an extended diestrous phase. It is known that melatonin dissolved in drinking water can increase the frequencies of diestrous or estrous phases in rodents (Kachi et al., 2006) by modulation of GnRH hormones. Furthermore, rats receiving melatonin (200 μg/100 g BW/d) showed longer estrous periods, reduction in polycystic ovary syndrome, and ovary weight (Prata Lima et al., 2004). As seen before, there may also be a positive interaction between ethanol and melatonin for decreasing ovarian weights, because they act on reproduction-linked hormones. It was clearly demonstrated that high melatonin levels is directly related to functional hypogonadotropic hypogonadism (Bergiannaki et al., 1995; de Roux

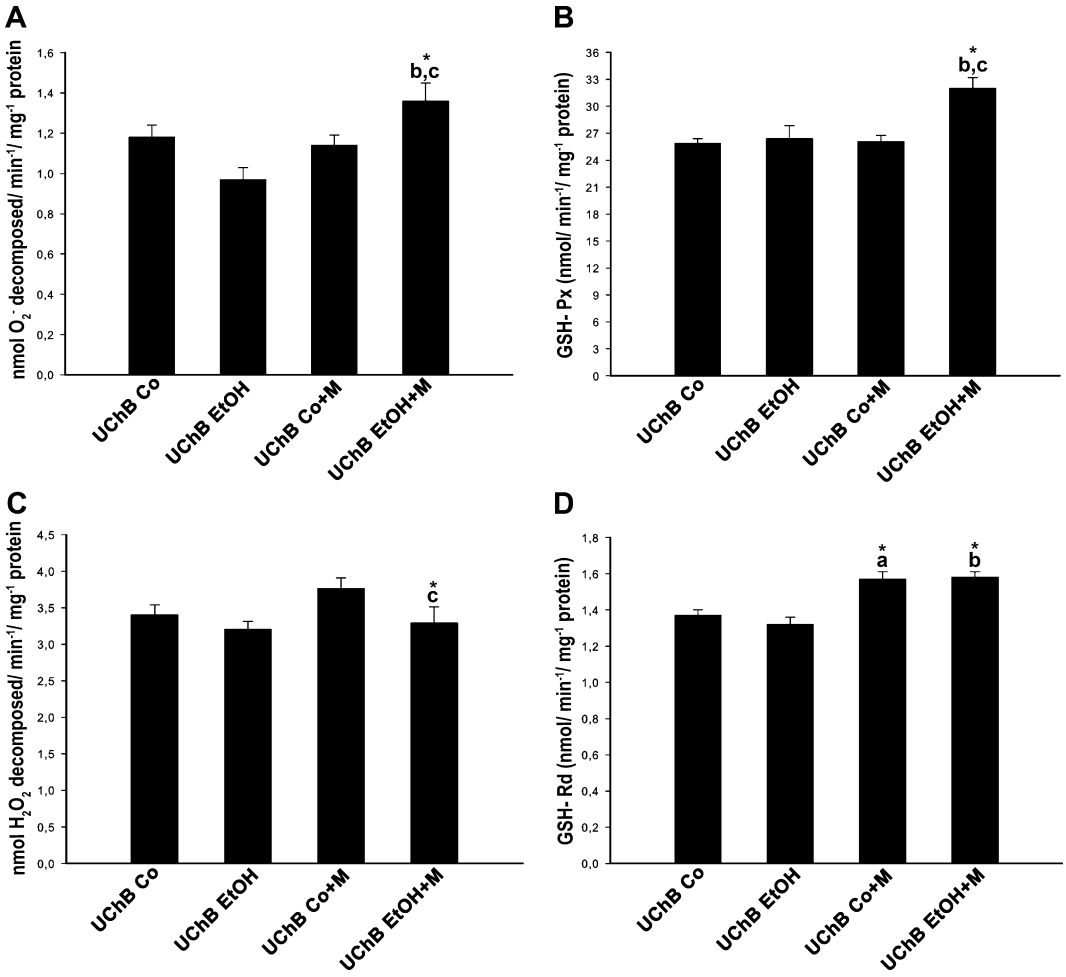

Fig. 5. Antioxidant activity (nmol/mg protein) in ovarian tissue of UChB rats receiving or not melatonin (M) (100 μg/100 g/d) after 60 days of treatment. (**A**) Activity of superoxide dismutase. (**B**) Activity of glutathione peroxidase (GSH-Px). (**C**) Activity of catalase. (**D**) Activity of glutathione reductase (GSH-Rd). $^{a-c}$Significant differences from UChB Co, UChB EtOH, UChB Co + M groups, respectively; $^*p < 0.05$. Values are expressed as means ± SEM (n = 10/group). Two-way ANOVA complemented by Tukey's test. EtOH, ethanol.

et al., 2003). Moreover, administration of exogenous melatonin induces a decrease in luteinizing hormone (LH) secretion, which in turn blocks the ovulation, leading to an extensive luteal phase (Voordouw et al., 1992), thus providing endocrine disruption and gonadal atrophy as it was also noted. Consistently, our previous study has found a reduction in relative ovary weight and an extensive estrous phase in UChB strain and it was associated with fall in LH levels (Chuffa et al., 2009). In this context, the addition of melatonin may suppress further release of GnRH, resulting in prolonged diestrous. On the other hand, Dardes and colleagues (2000) pointed out that low serum levels of melatonin tend to increase the duration of estrus, while treatment with melatonin was effective in regulating the estrous cycle.

The total protein concentration in ovaries of UChB EtOH and UChB Co + M rats was enhanced, suggesting increased enzymatic activity because of cellular metabolism. Particularly, in UChB EtOH rats, melatonin did not influence TAS concentration. However, the LHP/TAS ratio was increased when only ethanol was given. Indeed, to validate these findings, it is necessary to analyze the individual enzymatic and nonenzymatic antioxidant status.

There seems to be a little doubt that ethanol-induced oxidative stress is linked to the metabolism of ethanol. As a result of ethanol and subsequent acetaldehyde oxidation, there is a significant increase in the hepatic NADH/NAD^+ redox ratio (Das et al., 2005). Under a variety of pathophysiological conditions, including acute and chronic ethanol consumption, the increased levels of NADPH-oxidase generates O_2^- and H_2O_2, which in the presence of Fe^{3+}, produces powerful oxidants such as the OH (Kessova and Cederbaum, 2003), enhancing the lipid peroxidation (Le Lan et al., 2004). Ethanol is well reported to deplete GSH levels via its own pro-oxidative character or by inhibiting the mitochondrial GSH transporter (Kannan et al., 2004; Wheeler et al., 2003). Interestingly, the LHP levels were higher in animals consuming ethanol, similar to those described by other authors (Ashakumary and Vijayammal, 1996), and underwent a remarkable reduction after the administration of melatonin, confirming a positive interaction.

Undoubtedly, melatonin has an important role in attenuating ROS deleterious effects by promoting mRNA synthesis for antioxidant enzymes (Rodriguez et al., 2004). Considering these functions, the protective effect observed in the ovaries is believed to be attributed to both properties of melatonin as ROS scavenger and increasing the antioxidant status (Chuffa et al., 2011). Similar results elucidating the protective effect of melatonin and other antioxidants have been found in the testis of ethanol-treated rats (Oner-Iyidogan et al., 2001). According to Amanvermez and colleagues (2005), the chronic ethanol consumption leads to lipid and protein oxidation in rat ovaries and also reduces the GSH content in testes. Replacement therapy using melatonin and 17β-oestradiol in ovariectomized rats caused reduction in lipid peroxidation and increased GSH content and SOD activity (Feng and Zhang, 2005).

As previously described, ROS such as OH, peroxyl radicals (ROO^{-}), and H_2O_2 are known to be detrimental to the oocyte (Tamura et al., 2008), and in this study, doses of melatonin increased SOD and GSH-Px activities in ethanol-preferring rats. Similar evidence has been documented after treatment with vitamin E in adult rat ovaries (Rao et al., 2009). Moreover, supporting these findings, daily injection of melatonin (10 mg/kg) in rat tissues increased SOD activity after 7 days (Ozturk-Urek et al., 2001), while Liu and Ng (2000) found higher SOD activity after a single injection of melatonin (5 mg/kg). In accordance with our results, Tomas-Zapico and Coto-Montes (2005) and Rodriguez and colleagues (2004) emphasized that administration of melatonin promotes increased activity of SOD, GSH-Px, and GSH-Rd. Melatonin did not affect catalase activity in the ovaries. It is likely that this enzyme is being used for peroxisome metabolism during chronic ethanol consumption.

In conclusion, we demonstrated that there are interactions between melatonin and ethanol on feed efficiency, with melatonin acting as a hormone regulating weight gain, stored calories, and ethanol intake. Moreover, the administration of melatonin, although has caused estrous cycle disruption, was able to protect the ovaries of UChB rats against oxidative stress, preventing attack by free radicals, and promoting antioxidant defenses.

ACKNOWLEDGMENTS

We thank Mr. Wanderley Thiago da Silva for animal care and Mr. Gelson Rodrigues for technical support.

FINANCIAL SUPPORT

FAPESP—São Paulo Research Foundation (2007/59967-7 and 2008/56229-8) and CAPES.

REFERENCES

Aebi H (1974) Catalase, in *Methods of Enzymatic Analysis* (Bergmeyer HU ed.), pp 673–677. Academic Press, New York.

Agarwal A, Gupta S, Sharma RK (2005) Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 3:28.

Amanvermez R, Demir S, Tunçel OK, Alvur M, Agar E (2005) Alcohol-induced oxidative stress and reduction in oxidation by ascorbate/L-cys /L-met in the testis, ovary, kidney, and lung of rat. Adv Ther 22:548–558.

Angers K, Haddad N, Selmaoui B, Thibault L (2003) Effect of melatonin on total food intake and macronutrient choice in rats. Physiol Behav 80:9–18.

Armagan A, Uz E, Yilmaz HR, Soyupek S, Oksay T, Ozcelik N (2006) Effects of melatonin on lipid peroxidation and antioxidant enzymes in streptozotocin-induced diabetic rat testis. Asian J Androl 8:595–600.

Arnedt JT, Conroy DA, Brower KJ (2007) Treatment options for sleep disturbances during alcohol recovery. J Addict Dis 26:41–54.

Ashakumary L, Vijayammal PL (1996) Additive effect of alcohol and nicotine on lipid peroxidation and antioxidant defence mechanism in rats. J Appl Toxicol 16:305–308.

Ayene IS, Stamato TD, Mauldin SK, Biaglow JE, Tuttle SW, Jenkins SF, Koch CJ (2002) Mutation in the glucose-6-phosphate dehydrogenase gene leads to inactivation of ku DNA end binding during oxidative stress. J Biol Chem 277:9929–9935.

Bell RL, Rood ZA, Hsu CC, Lumeng L, Li TK, Murphy JM, McBride WJ (2004) Effects of concurrent access to a single concentration or multiple concentrations of ethanol on ethanol intake by periadolescent high-alcohol-drinking rats. Alcohol 33:107–115.

Bergiannaki JD, Paparrigopoulos TJ, Syrengela M, Stefanis CN (1995) Low and high melatonin excretors among healthy individuals. J Pineal Res 18:159–164.

Bojková B, Orendás P, Friedmanová L, Kassayová M, Datelinka I, Ahlersová E, Ahlers I (2008) Prolonged melatonin administration in 6-month-old Sprague-Dawley rats: metabolic alterations. Acta Physiol Hung 95:65–76.

Bubenik GA (2002) Gastrointestinal melatonin: localization, function and clinical relevance. Dig Dis Sci 47:2336–2348.

Carrara O, Oger-Jeannin V, Desechalliers JP (1993) Disorders of the hypothalamo–hypophyseal–ovarian axis in chronic alcoholic women. Rev Med Interne 14:9–13.

Choi MJ, Kim MJ, Chang KJ (2006) The effect of dietary taurine supplementation on plasma and liver lipid concentrations and mineral metabolism in rats fed alcohol. Adv Exp Med Biol 583:243–250.

Chuffa LG, Amorim JP, Teixeira GR, Mendes LO, Fioruci BA, Pinheiro PF, Seiva FR, Novelli EL, Mello Júnior W, Martinez M, Martinez FE (2011) Long-term melatonin treatment reduces ovarian mass and enhances tissue antioxidant defenses during ovulation in the rat. Braz J Med Biol Res 44:217–223.

Chuffa LGA, Padovani CR, Martinez FE (2009) Ovarian structure and hormonal status of the UChA and UChB adult rats in response to ethanol. Maturitas 62:21–29.

Clark JW, Fixaris MC, Belanger GV, Rosenwasser AM (2007) Repeated light–dark phase shifts modulate voluntary ethanol intake in male and female high alcohol-drinking (HAD1) rats. Alcohol Clin Exp Res 31:1699–1706.

Dardes RC, Baracat EC, Simões MJ (2000) Modulation of estrous cycle and LH, FSH and melatonin levels by pinealectomy and sham-pinealectomy in female rats. Prog Neuropsychopharmacol Biol Psychiatry 24:441–453.

Das SK, Nayak P, Vasudevan DM (2005) Consequences of ethanol consumption. J Ind Soc Toxicol 1:1–10.

de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E (2003) Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci USA 100:10972–10976.

DiCecco SR, Francisco-Ziller N (2006) Nutrition in alcoholic liver disease. Nutr Clin Pract 21:245–254.

El-Sokkary GH, Reiter RJ, Tan DX, Kim SJ, Cabrera J (1999) Inhibitory effect of melatonin on products of lipid peroxidation resulting from chronic ethanol administration. Alcohol Alcohol 34:842–850.

Ewing JF, Janero DR (1995) Microplate superoxide dismutase assay employing a nonenzymatic superoxide generation. Anal Chem 232:243–248.

Feng Z, Zhang J (2005) Long-term melatonin or 17β-estradiol supplementation alleviates oxidative stress in ovariectomized adult rats. Free Radic Biol Med 39:195–204.

Fernandez-Checa JC, Kaplowitz N (2005) Hepatic mitochondrial glutathione: transport and role in disease and toxicity. Toxicol Appl Pharmacol 204:263–273.

Fonzi S, Solinas GP, Costelli P, Parodi C, Murialdo G, Bo P, Albergati A, Montalbetti L, Savoldi F, Polleri A (1994) Melatonin and cortisol circadian secretion during ethanol withdrawal in chronic alcoholics. Chronobiologia 21:109–112.

Gouillon Z, Lucas D, Li J, Hagbjork AL, French BA, Fu P, Fang C, Ingelman-Sundberg M, Donohue TM Jr, French SW (2000) Inhibition of ethanol-induced liver disease in the intragastric feeding rat model by chlormethiazole. Proc Soc Exp Biol Med 224:302–308.

Henderson J, Gray R, Brocklehurst P (2007) Systematic review of effects of low-moderate prenatal alcohol exposure on pregnancy outcome. BJOG 114:243–252.

Horton TH, Yellon SM (2001) Aging, reproduction, and the melatonin rhythm in the Siberian hamster. J Biol Rhythms 16:243–253.

Hu S, Yin S, Jiang X (2009) Melatonin protects against alcoholic liver injury by attenuating oxidative stress, inflammatory response, and apoptosis. Eur J Pharmacol 616:287–292.

Hussein MR, Ahmed OG, Hassan AF, Ahmed MA (2007) Intake of melatonin is associated with amelioration of physiological changes, both metabolic and morphological pathologies associated with obesity: an animal model. Int J Exp Pathol 88:19–29.

Jiang ZY, Woollard ACS, Wolf SP (1991) Lipid hydroperoxide measurements by oxidation of Fe^{2+} in the presence of xylenol orange. Comparison with the TBA assay and on iodometric method. Lipids 26:853–856.

Kachi T, Tanaka D, Watanabe S, Suzuki R, Tonosaki Y, Fujieda H (2006) Physiological pineal effects on female reproductive function of laboratory rats: prenatal development of pups, litter size and estrous cycle in middle age. Chronobiol Int 23:289–300.

Kampov-Polevoy AB, Eick C, Boland G, Khalitov E, Crews FT (2004) Sweet liking, novelty seeking, and gender predict alcoholic status. Alcohol Clin Exp Res 28:1291–1298.

Kannan M, Wang L, Kang YJ (2004) Myocardial oxidative stress and toxicity induced by acute ethanol exposure in mice. Exp Biol Med 229:553–559.

Kessova I, Cederbaum AI (2003) CYP2E1: biochemistry, toxicology, regulation and function in ethanol-induced liver injury. Curr Mol Med 3:509–518.

Kim JK, Lee CJ (2000) Effect of exogenous melatonin on the ovarian follicles in γ-irradiated mouse. Mutat Res 449:33–39.

Korkmaz A, Topal T, Tan DX, Reiter RJ (2009) Role of melatonin in metabolic regulation. Rev Endocr Metab Disord 10:261–270.

Krahn D, Grossman J, Henk H, Mussey M, Crosby R, Gosnell B (2006) Sweet intake, sweet-liking, urges to eat, and weight change: relationship to alcohol dependence and abstinence. Addict Behav 31:622–631.

Le Lan C, Ropert M, Lainé F, Medevielle M, Jard C, Pouchard M, Le Treut A, Moirand R, Loréal O, Brissot P (2004) Serum ceruloplasmin and ferroxidase activity are not decreased in hepatic failure related to alcoholic cirrhosis: clinical and pathophysiological implications. Alcohol Clin Exp Res 28:775–779.

Lieber CS (2004) Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. Alcohol 34:9–19.

Liu F, Ng TB (2000) Effect of pineal indoles on activities of the antioxidant defense enzymes superoxide dismutase, catalase, and glutathione reductase, and levels of reduced and oxidized glutathione in rat tissues. Biochem Cell Biol 78:447–453.

Marcondes FK, Bianchi FJ, Tanno AP (2002) Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol 62:609–614.

Mardones J, Segovia-Riquelme N (1983) Thirty-two years of selection of rats by ethanol preference: UChA and UChB strains. Neurobehav Toxicol Teratol 5:171–178.

Miller AC, Blakely WF (1992) Inhibition of glutathione reductase activity by a carbamoylating nitrosourea: effect on cellular radiosensitivity. Free Radic Biol Med 12:53–62.

Monteiro R, Soares R, Guerreiro S, Pestana D, Calhau C, Azevedo I (2009) Red wine increases adipose tissue aromatase expression and regulates body weight and adipocyte size. Nutrition 25:699–705.

Muhlbauer E, Gross E, Labucay K, Wolgast S, Peschke E (2009) Loss of melatonin signaling and its impact on circadian rhythms in mouse organs regulating blood glucose. Eur J Pharmacol 606:61–71.

Mustonen AM, Nieminen P, Hyvärinen H (2002) Effects of continuous light and melatonin treatment on energy metabolism of the rat. J Endocrinol Invest 25:716–723.

Nakamura W, Hojoda S, Hayashi K (1974) Purification and properties of rat liver glutathione peroxidase. Biochim Biophys Acta 358:251–261.

Noda Y, Matsumoto H, Umaoka Y, Tatsumi K, Kishi J, Mori T (1991) Involvement of superoxide radicals in the mouse two-cell block. Mol Reprod Dev 28:356–360.

Ocal-Irez TG (1989) Effect of pineal indoleamine, 5-metoxyindole-3acetic acid, on the estrous cycle and reproductive organs of female wistar albino rats. Brain Res 493:1–7.

Oner-Iyidogan Y, Gurdol F, Oner P (2001) The effects of acute melatonin and ethanol treatment on antioxidant enzyme activities in rat testes. Pharmacol Res 44:89–93.

Ozturk-Urek R, Bozkaya LA, Tarhan L (2001) The effects of some antioxidant vitamin- and trace element-supplemented diets on activities of SOD, CAT, GSH-Px and LPO levels in chicken tissues. Cell Biochem Funct 19:125–132.

Peyrot F, Ducrocq C (2008) Potential role of tryptophan derivatives in stress responses characterized by the generation of reactive oxygen and nitrogen species. J Pineal Res 45:235–246.

Polavarapu R, Spitz DR, Sim JE, Follansbee MH, Oberley LW, Rahemtulla A, Nanji AA (1998) Increased lipid peroxidation and impaired antioxidant enzyme function is associated with pathological liver injury in experimental alcoholic liver disease in rats fed diets high in corn oil and fish oil. Hepatology 27:1317–1323.

Prata Lima MF, Baracat EC, Simoes MJ (2004) Effects of melatonin on the ovarian response to pinealectomy or continuous light in female rats: similarity with polycystic ovary syndrome. Braz J Med Biol Res 37:987–995.

Prunet-Marcassus B, Desbazeille M, Bros A, Louche K, Delagrange P, Renard P, Casteilla L, Pénicaud L (2003) Melatonin reduces body weight gain in Sprague Dawley rats with diet-induced obesity. Endocrinology 144:5347–5352.

Puchalski SS, Green JN, Rasmussen DD (2003) Melatonin effects on metabolism independent of gonad function. Endocrine 21:169–173.

Rao MV, Chawla SL, Sharma SR (2009) Protective role of vitamin E on nickel and/or chromium induced oxidative stress in the mouse ovary. Food Chem Toxicol 47:1368–1371.

Rasmussen DD, Mitton DR, Larsen SA, Yellon SM (2001) Aging-dependent changes in the effect of daily melatonin supplementation on rat metabolic and behavioral responses. J Pineal Res 31:89–94.

Rodriguez C, Mayo JC, Sainz RM, Antolín I, Herrera F, Martín V, Reiter RJ (2004) Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res 36:1–9.

Ronis MJ, Hakkak R, Korourian S, Albano E, Yoon S, Ingelman-Sundberg M, Lindros KO, Badger TM (2004) Alcoholic liver disease in rats fed ethanol as part of oral or intragastric low-carbohydrate diets. Exp Biol Med 229:351–360.

Rosenwasser AM, Fecteau ME, Logan RW (2005) Effects of ethanol intake and ethanol withdrawal on free-running circadian activity rhythms in rats. Physiol Behav 84:537–542.

Rudden PK, Symmes SK (1981) Pineal gland and melatonin influence on chronic alcohol consumption by hamsters. Pharmacol Biochem Behav 14:143–147.

Scalera G, Benassi C, Bigiani A (2008) Daytime melatonin treatment influences food carrying (hoarding) behavior in rats. Endocr Regul 42:3–11.

Seiva FR, Berbert CM, Souza GA, Rocha KK, Ebaid GM, Burneiko RC, Novelli EL (2010) Energy expenditure, lipid profile, oxidative stress, and

cardiac energy metabolism after growth hormone treatment in obese young rats. Horm Metab Res 42:496–501.

Seiva FR, Ebaid GM, Castro AV, Okoshi K, Nascimento A, Rocha KK, Padovani CR, Cicogna AC, Novelli EL (2008) Growth hormone and heart failure: oxidative stress and energetic metabolism in rats. Growth Horm IGF Res 18:275–283.

She M, Deng X, Guo Z, Laudon M, Hu Z, Liao D, Hu X, Luo Y, Shen Q, Su Z, Yin W (2009) NEU-P11, a novel melatonin agonist, inhibits weight gain and improves insulin sensitivity in high-fat/high-sucrose-fed rats. Pharmacol Res 59:248–253.

Smith RR, Hong J, Harvey AE, Lewis T, Diaz D, Núñez NP (2008) Ethanol consumption does not promote weight gain in female mice. Ann Nutr Metab 53:252–259.

Strbák V, Benický J, Macho L, Jezová D, Nikodémová M (1998) Four-week ethanol intake decreases food intake and body weight but does not affect plasma leptin, corticosterone, and insulin levels in pubertal rats. Metabolism 47:1269–1273.

Subramanian P, Mirunalini S, Pandi-Perumal SR, Trakht I, Cardinali DP (2007) Melatonin treatment improves the antioxidant status and decreases lipid content in brain and liver of rats. Eur J Pharmacol 571:116–119.

Tahsili-Fahadan P, Yahyavi-Firouz-Abadi N, Ghahremani MH, Dehpour AR (2005) Effect of light/dark cycle alteration on morphine-induced conditioned place preference. Neuroreport 16:2051–2056.

Tampier L, Quintanilla ME, Israel Y (2008) Tolerance to disulfiram induced by chronic alcohol intake in the rat. Alcohol Clin Exp Res 32:937–941.

Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, Taketani T, Matsuoka A, Yamagata Y, Shimamura K, Morioka H, Ishikawa H, Reiter RJ, Sugino N (2008) Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. J Pineal Res 44:280–287.

Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ (2007) One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? J Pineal Res 42:28–42.

Tomas-Zapico C, Coto-Montes A (2005) A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. J Pineal Res 39:99–104.

Vázquez N, Díaz E, Fernández C, Jiménez V, Esquifino A, Díaz B (2007) Seasonal variations of gonadotropins and prolactin in the laboratory rat. Role of maternal pineal gland. Physiol Res 56:79–88.

Voordouw BC, Euser R, Verdonk RE, Alberda BT, De Jong FH, Drogenduk AC, Fauser BC, Cohen M (1992) Melatonin and melatonin-progestin combinations alter pituitary-ovarian function in women and can inhibit ovulation. Clin Endocrinol Metab 74:108–117.

Wheeler GL, Trotter EW, Dawes IW, Grant CM (2003) Coupling of the transcriptional regulation of glutathione biosynthesis to the availability of glutathione and methionine via the Met4 and Yap1 transcription factors. J Biol Chem 278:8435–8441.

Yeomans MR (2004) Effects of alcohol on food and energy intake in human subjects: evidence for passive and active over-consumption of energy. Br J Nutr 92:531–534.

Yeomans MR, Hails NJ, Nesic JS (1999) Alcohol and the appetizer effect. Behav Pharmacol 10:151–161.

Yu CX, Zhu CB, Xu SF, Cao XD, Wu GC (2000) The analgesic effects of peripheral and central administration of melatonin in rats. Eur J Pharmacol 403:49–53.

Zhao H, Poon AMS, Pang SF (2000) Pharmacological characterization, molecular subtyping, and autoradiographic localization of putative melatonin receptors in uterine endometrium of estrous rats. Life Sci 66:1581–1591.