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Melatonin Normalizes Clinical and Biochemical Parameters of Mild Inflammation in Diet-induced Metabolic Syndrome in Rats

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Running title: Melatonin and Mild Inflammation in High-Fat Fed Rats.

Abstract

The objective of the present study was to evaluate the efficacy of melatonin to affect mild inflammation in the metabolic syndrome (MS) induced by a high-fat diet in rats. Adult Wistar male rats were divided into four groups (n= 16/group): (i) control diet (3% fat); (ii) high-fat (35%) diet; (iii) high-fat diet + melatonin; (iv) melatonin. Rats had free access to high-fat or control chow and one of the following drinking solutions for 10 weeks: (a) tap water; (b) 25 μg/mL of melatonin. Plasma interleukin (IL)-1β, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and C-reactive protein (CRP) were measured at two time intervals, i.e. the middle of daylight period and the middle of the scotophase. In addition, a number of somatic and metabolic components employed clinically to monitor the MS were measured. Melatonin decreased the augmented circulating levels of IL-1β, IL-6, TNF-α, IFN-γ and CRP seen in obese rats and restored the depressed levels of IL-4 and IL-10. Rats fed with the high-fat diet showed significantly higher body weights and augmented systolic blood pressure from the 3rd and 4th week onwards, respectively, melatonin effectively preventing these changes. In high-fat fed rats circulating low-density lipoprotein-cholesterol, total cholesterol and triglyceride concentration augmented significantly, melatonin being effective to counteract these changes. Melatonin-treated rats showed a decreased insulin resistance, the highest values of plasma high-density lipoprotein-cholesterol and the lowest values of plasma uric acid. The results indicate that melatonin is able to normalize the altered biochemical pro-inflammatory profile seen in rats fed with a high-fat diet.

Key words:

cytokines; dyslipidemia; glucose tolerance; high fat diet; hypertension; inflammation; uric acid.

Introduction

The metabolic syndrome (MS), a cluster of cardiovascular disease risk factors including obesity, hypertension, hyperinsulinemia, glucose intolerance and dyslipidemia, is a major clinical challenge with a prevalence of 15-30% depending on the world region considered [1]. The MS increased overall cardiovascular mortality by 1.5- to 2.5-fold and together with neurodegenerative disorders like Alzheimer's disease, are the two greatest public health concerns of the 21st century [2,3].

There is impressive information indicating that obesity in MS is associated with a low-grade inflammation of the white adipose tissue that can subsequently lead to insulin resistance, impaired glucose tolerance and diabetes [4,5]. Adipocytes actively secrete leptin and proinflammatory cytokines and activate a vicious cycle leading to additional weight gain largely in the form of fat [2]. Inflammation in obesity is also indicated by the increased circulating levels of C-reactive protein (CRP) and other biological markers of inflammation.

Adipose tissue-produced pro-inflammatory molecules like tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 have both local and systemic effects [5]. The amounts of TNF-α, IL-1β and IL-6 are positively correlated with body fat and decrease in obese patients after weight loss. Therefore, fat cells are both a source of and a target for TNF-α, IL-1β and IL-6 effects [5].

In a previous study we assessed the effect of a high-fat diet (35% fat) on mean levels and 24-h pattern of circulating IL-1β, IL-6 and TNF-α in rats [6]. Mean levels of plasma cytokines augmented and the normal daily pattern seen in control became disrupted in high-fat fed rats compatible with the occurrence a mild inflammation. In a similar group of high-fat fed rats we also reported a significant decrease in amplitude of pineal melatonin rhythm [7] and the reversion by melatonin, administered orally for 9 weeks of body weight, dyslipidemia and insulin resistance [8].

The objective of the present study was to further evaluate the efficacy of melatonin to normalize clinical and biochemical signs of mild inflammation in the MS induced by a high-fat diet in rats. Plasma IL-1β, IL-4, IL-6, IL-10, TNF-α, interferon (IFN)-γ and CRP were measured at two time intervals, i.e., the middle of daylight period and the middle of scotophase. In addition a number of somatic and

metabolic components employed clinically to monitor the MS, i.e., body weight increase, systolic blood pressure (BP) and several circulating analytes including insulin, glucose, triglycerides, total cholesterol, high-density lipoprotein-cholesterol (HDL-c), low-density lipoprotein-cholesterol (LDL-c), total protein, creatinine, urea and uric acid were measured. At the time of completion of the present experiments a publication by Agil et al. [9]reported that melatonin was effective in ameliorating low-grade inflammation in young Zucker diabetic fatty rats as demonstrated by the decrease of plasma IL-6, TNF-α and CRP levels.

Materials and Methods

Animals and experimental design

Male Wistar rats (70 days of age, 230-260 g) were maintained under standard conditions with controlled light (12:12 h light/dark schedule; lights on at 08:00 h) and temperature (22 ± 2 C). Rats had ad libitum access to a normal or a high-fat diet. Normal rat chow contained 3% fat, 16% protein and 60% carbohydrate (mainly as starch with less than 0.4% fructose) providing a total caloric content of 2.9 Kcal/g. The high (35%) fat chow contained 35% carbohydrates and 20% proteins, providing a total caloric content of 5.4 Kcal/g.

Animals were randomly divided into four groups (n= 16/group) as follows: (i) control; (ii) high-fat diet (obese); (iii) obese + melatonin; (iv) melatonin. Rats had free access to high-fat or control chow and one of the following drinking solutions for 10 weeks: (a) tap water; (b) 25 μg/mL of melatonin. Since ethanol was used as a melatonin´s vehicle, drinking solutions in groups (i) and (iv) 0.015 % ethanol was added to the drinking solutions. Water bottles were changed every other day. Because rats drank about 30 mL/day with 90–95% of this total daily water taken up during the dark period, the daily melatonin dosage used provided approximately 2.3 mg/kg melatonin. The human equivalence dose, calculated by using the body surface area normalization method [10] was about 0.35 mg/kg (about 25-30 mg/day for a 75 kg human adult).

The animals were weighed once a week for 10 wk and were euthanized by decapitation under conditions of minimal stress at two time intervals: at the middle of the light period (13:00 h) and at the middle of the scotophase (01:00 h). All experiments were conducted in accordance with the guidelines of the

International Council for Laboratory Animal Science (ICLAS). Trunk blood was collected and plasma samples were obtained by centrifugation of blood at 1,500 x g for 15 min and were stored at –70 C until further analysis.

BP measurement

Systolic BP was measured by using a manometer-tachometer (Rat Tail NIBP System; ADInstruments Pty Ltd., Sydney, Australia) employing an inflatable tail-cuff connected to a MLT844 Physiological Pressure Transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments) as described previously [11]. Rats were placed in a plastic holder mounted on a thermostatically controlled warm plate that was maintained at 35°C during measurements. An average value from three-BP readings (that differed by no more than 2 mm Hg) was determined for each animal after they became acclimated to the environment. BP measurements were made weekly between 09:00 and 12:00 h.

Biochemical assays

Plasma concentrations of IL-1β, IL-6, TNF-α, IFN-γ and insulin were measured in a multianalyte profiling by using the Luminex-100 system and the XY Platform (Luminex Corporation, Oosterhout, The Netherlands) as described elsewhere [6]. Calibration microspheres for classification and reporter readings as well as sheath fluid were also purchased from Luminex Corporation. Acquired fluorescence data were analyzed by the MasterPlexTM QT software. All analyses were performed according to the manufacturer' protocol. Plasma concentrations of IL-4, IL-10 and CRP were measured by ELISA assays (Elabscience Biotechnology Co., Wuhan, Hubei Province, China) following manufacturers' instructions. Concentrations were estimated from a standard curve and expressed as pg/mL (IL-4 and IL-10) or μg/mL (CRP). The plasma concentrations of glucose, cholesterol, triglycerides, HDL-c, LDL-c, urea, uric acid, total protein and creatinine were measured by commercially available kits according to the manufacturers' instructions (Randox Laboratories, Antrim, Northern Ireland, UK).

Statistical analysis

After verifying normality of distribution of data, the statistical analysis was performed by a three-factor analysis of variance (ANOVA) or a one-way ANOVA followed by a Holm-Sidak multiple comparisons test, as stated. The three-factor ANOVA was used to test for differences between individual data grouped according to the levels of each factor, i.e. treatment, diet, and time period, and for interactions between the factors. Four hypotheses were tested: (a) there was no difference between the levels of treatment (melatonin or vehicle); (b) there was no difference between the levels of diet (normal or high-fat diet); (c) there was no difference between the levels of time period (weeks or middle of day or middle of night, as stated); (d) there was no interaction between the factors. P values lower than 0.05 were taken as evidence of statistical significance.

Results

Individual daily chow and water consumption were similar for controls (17 ± 3 g and 27 ± 3 ml) and high-fat-fed rats (16 ± 3 g and 29 ± 2 ml). The percentage of food intake at night was 75.7 ± 6.5 % (normal diet), 79.8 ± 8.1% (high-fat diet), 81.1± 6.7% (high-fat diet + melatonin) and 71.0 ± 8.9 (melatonin; F= 0.03, P= 0.98, ANOVA). Melatonin administration did not affect significantly chow or water consumption.

Figure 1 depicts the changes in body weight and systolic BP in the four experimental groups. Body weight of high-fat–fed rats receiving vehicle attained values 44 % higher than controls after 70 days of treatment (Panel A). The concomitant administration of melatonin significantly attenuated body weight increase in high-fat–fed rats. In the three-factor ANOVA all factors were significant (F= 50.4, P< 0.001 for treatment; F= 103.9, P< 0.001 for diet; F= 43.9, P<0.001 for week interval) as well as the interactions “treatment x diet” (F= 35.1, P< 0.001) and “diet x week interval”, F= 5.6, P= 0.023). The significant interactions found supported the conclusion that the effect of melatonin on body weight increase is seen mainly in obese rats and is dependent on the elapsed time of treatment.

Systolic BP was significantly higher in the obese rats from the 4th week on, melatonin being effective to counteract this effect (Fig. 1, Panel B). In the three-factor ANOVA the significant factors detected were treatment (F= 26.2, P< 0.001)

and diet (F= 36.3) with a significant interaction “treatment x diet” (F= 35.1, P< 0.001) indicating that the effect of melatonin was mainly observed in obese rats.

Figure 2 summarizes the changes in plasma IL-1β and IL-6 levels at the two examined time intervals after the 10-week experiment. Obesity generally augmented both cytokines and melatonin decreased them. In the three-way ANOVA the three factors were significant for IL-1β results (F= 94.6, P< 0.001 for treatment; F= 12.7, P< 0.001 for diet; F= 72.6, P<0.001 for time of day) as well as the interactions “treatment x diet” (F= 12.1, P= 0.001), “treatment x time of day” (F= 56.6, P< 0.001) and “diet x time of day”, F= 5.6, P= 0.023). In the case of IL-6 two factors were significant (F= 29.1, P< 0.001 for treatment; F= 69.1, P<0.001 for time of day) with significant interactions “treatment x diet” (F= 4.67, P= 0.03) and “treatment x time of day” (F= 18.7, P< 0.001). Collectively this analysis supported the view that melatonin decreased IL-1β and IL-6 mainly in obese rats. Melatonin treatment was effective to decrease both cytokine levels at day hours.

Plasma levels of TNF-α and IFN-γ in rats fed a normal or a high-fat diet and receiving melatonin or vehicle in drinking water are shown in figure 3. As results in figure 2, the high-fat diet brought about an increase in both cytokines melatonin being effective to reverse the changes observed. The three factors were statistically significant for TNF-α and IFN-γ (F= 29.4 and F= 14.1, P< 0.001 for treatment; F= 11.7 and 10.9, P= 0.002 for diet; F= 171.2 and 12.1, P<0.001 for time of day, respectively). The interactions were significant for TNF-α only (“treatment x diet”: F= 15.5, P< 0.001); “treatment x time of day”: F= 17.8, P< 0.001; “diet x time of day”: F= 10.9, P= 0.002). In the case of IFN-γ the three-factors were significant (F= 14.1, P< 0.001 for treatment; F= 10.9, P= 0.002 for diet; F= 12.1 P= 0.001 for time of day). Collectively this indicated that melatonin decreased TNF-α and IFN-γ in obese rats. Melatonin treatment was globally effective to decrease IFN-γ at day hours. The circulating levels of IL-1β, IL-6 and TNF-α detected during the day were significantly greatly than those found in daylight (Figs. 2 and 3).

Figure 4 depicts the changes in plasma IL-4 and IL-10 levels. The picture for both anti-inflammatory cytokines was similar, the high-fat diet decreasing and melatonin restoring the levels to controls. In the three-factor ANOVA the only factor significant was treatment (F= 16.1 and 12.9 for IL-4 and IL-10, P <0.001,

respectively) with a significant interaction “treatment x diet” (F= 32.2 and 26.7, P< 0.001, respectively), the effect of melatonin being observed in the obese rats only.

As shown in table 1 plasma CRP levels augmented 40-57% in obese rats, an effect blunted by concomitant melatonin administration. In the three-way ANOVA the only factor significant was treatment (F= 26.3, P <0.001) with a significant interaction “treatment x diet” (F= 15.5, P< 0.001, respectively) the effect of melatonin being observed in the obese rats only.

Results shown in figures 5 to 8 summarize the efficacy of melatonin to prevent a number of biochemical changes typical of MS. The high-fat diet induced insulin resistance as shown by the significantly augmented plasma insulin and glucose levels, the effect being prevented by melatonin (Fig. 5). In the three-factor ANOVA, two factors were significant for insulin (F= 54.3, P< 0.001 for treatment; F= 23.8, P< 0.002 for diet) as well as the interaction “treatment x diet” (F= 14.4, P< 0.001). In the case of glucose the three-factors were significant (F= 41.6, P< 0.001 for treatment; F= 35.1, P< 0.001 for diet; F= 65.4 P< 0.001 for time of day) with a significant interaction “treatment x diet” (F= 23.9, P<0.001). Collectively the results indicate that melatonin decreased insulin resistance in obese rats (Fig. 5).

The changes in plasma lipid profile are summarized in figures 6 and 7. The increase in total cholesterol, triglycerides and LDL-c brought about by the high-fat diet was reversed by melatonin whereas the highest value of HDL-c was seen in melatonin-treated rats. This was further supported by the three-way ANOVA. Two factors were significant for cholesterol and LDL-c, i.e. treatment (F= 71.3 and F= 32.9, P< 0.001, respectively) and diet (F= 6.72, P= 0.03 and F= 42.1, P< 0.001, respectively). In the case of triglycerides the three factors were significant (F= 16.3, P< 0.001 for treatment; F= 58.8, P< 0.001 for diet; F= 4.11, P= 0.046 for time of day) as well as the interaction “treatment x diet” (F= 4.65, P= 0.034). For HDL-c the only significant factor detected was treatment (F= 71.3, P< 0.001).

The plasma levels of urea remained unchanged in rats fed a normal or a high-fat diet with melatonin or vehicle while the lowest value of plasma uric acid was seen in melatonin-treated animals, as indicated by both by the one-way ANOVA shown in the Fig. 8 well as by the significance of the factor treatment in the three-way ANOVA (F= 114.8, P< 0.001). Neither total plasma protein nor plasma creatinine changed significantly in the four examined groups (results not shown).

Discussion

The foregoing results support the view that melatonin is able to normalize the altered biochemical pro-inflammatory profile seen in obese rats fed a high-fat diet. Melatonin decreased the augmented circulating levels of IL-1β, IL-6, TNF-α, IFN-γ and CRP, all markers of inflammation, and restored the depressed circulating levels of the anti-inflammatory cytokines IL-4 and IL-10. Rats fed with the high-fat diet showed significantly higher body weights and augmented systolic BP from the 3rd and 4th week on, respectively, melatonin effectively preventing these changes. Additionally, in high-fat fed rats circulating LDL-c, cholesterol and triglyceride concentration augmented significantly, melatonin having the opposite effect. Melatonin administration counteracted the increased insulin resistance seen in obese rats and resulted in the highest values of plasma HDL-c and the lowest values of circulating uric acid.

TNF-α, IL-1β and IL-6 are major soluble factors of the innate response and are considered as indicators of inflammation, like that occurring in high-fat fed rats [4]. In the case of the specific immune response, and based on the cytokine milieu, T helper (Th) lymphocytes can differentiate primarily into several major phenotypes. One of these, Th1 cells, play a key role in the development of inflammatory processes through the production of IFN-γ, a cytokine which showed increased circulating values in the group of high-fed rats studied herein. Th2 cells produce cytokines such as IL-4 and IL-10 that mediate, among other, anti-inflammatory responses and tended to be impaired under the predominance of a pro-inflammatory condition like obesity [4]. Therefore, the increased in circulating levels of IL-1β, IL-6, TNF-α and IFN-γ with the concomitant decrease of IL-4 and IL-10 reported in obese rats in the present study all support the conclusion that a mild inflammation happened in rats after a high-fat diet administration. Such a conclusion is further supported by the increase of another marker of inflammation, i.e. plasma CRP in high-fat fed rats.

Among several substances with the capacity to curtail the MS, melatonin has received attention because of its very low or absent toxicity that turns it potentially appropriate for human use. Concerning the immune system, Carrillo-Vico and co-workers have put forth the idea that melatonin acts as a buffer for the

immune system, displaying stimulant effects under basal or immunosuppressive conditions and acting as an anti-inflammatory signal in situations when there is an exacerbated immune response [12]. Indeed, melatonin neutralized the exacerbated production of pro-inflammatory cytokines in a large number of animal models of inflammation (for ref see [12]). *In vitro* melatonin inhibited lipopolyssacharide-stimulated TNF-α, IL-1β, IL-6 and IL-8 production through a mechanism involving the attenuation of nuclear factor (NF)-κB activation [13,14]. Melatonin also decreased both the methamphetamine-induced up regulation of TNF-α, IL-1β and IL-6 in a rat microglial cell line [15] and the amyloid-beta-induced overproduction of TNF-α and IL-6 in organotypic hippocampal cultures [16]. Agil et al. recently showed that melatonin attenuated low-grade inflammation in young Zucker diabetic fatty rats as indicated by normalizing the augmented plasma IL-6, TNF-α and CRP levels [9]. Collectively the results obtained in the present study are compatible with the idea that melatonin behaves as an anti-inflammatory signal in rats fed a high-fat diet.

Several in vivo studies have shown the capacity of melatonin to promote a Th2 response, high doses of melatonin enhancing the production of the Th2 cytokine IL-4 in bone marrow lymphocytes [17]. Chronic administration of melatonin to antigen-primed mice increased the production of IL-10 and decreased that of TNF-α, also indicative of a Th2 response [18]. Melatonin increased IL-10 production in a murine model of septic shock [19] and significantly reversed aging- and pancreatitis-induced reduction of IL-10 levels in rats [20,21]. Thus the increase in IL-4 and IL-10 levels reported in the present study after melatonin administration is compatible with a promoting effect of melatonin on Th2 response.

The endothelial dysfunction and increased BP play an important role in the development of secondary cardiovascular complications in MS. The results depicted in Fig. 1, panel B, indicate that melatonin is very effective to prevent systolic BP increase since an early phase of MS development (i.e. from the 4th week on). To our knowledge this is the first detailed description of the hypotensive effect of melatonin in high-fat fed rats. Leibowitz et al. reported those changes in another model of MS, the fructose-fed rat [22].

At a high dose melatonin protects against several comorbilities of the experimental MS, including diabetes and concomitant oxyradical-mediated

damage, inflammation, microvascular disease and atherothrombotic risk [23-25]. Vascular production of both excessive reactive oxygen and nitrogen species contribute to endothelial dysfunction by directly damaging macromolecules and by activating several cellular stress-sensitive pathways, e.g. NF-κB, which play a key role in the development of type 1 and type 2 diabetes complications as well as in the insulin resistance and impaired insulin secretion occurring type 2 diabetes [26,27]. Since melatonin provides both *in vivo* and *in vitro* protection at the level of cell membrane, mitochondria and nucleus, partly due to its free-radical scavenging and antioxidant properties [24], the involvement of these mechanisms in melatonin´s prevention of vascular sequels and insulin resistance in the diet-induced model of rodent MS herein examined seems warranted. Remarkably the effect of melatonin in rodent models of hyperadiposity [8,28-35] is exerted in the absence of significant differences in food intake. To what extent the weight-loss-promoting effect of melatonin is attributable to an increase in energy expenditure by brown adipose tissue deserves further exploration (see for ref. [36]).

In the present study melatonin decreased plasma uric acid levels both in normal and high-fat fed rats. This effect could be of a potential therapeutic value in human MS since hyperuricemia is considered a cardiovascular and renal risk factor in MS. Mild hyperuricemia in normal rats induces systemic hypertension, renal vasoconstriction, glomerular hypertension and hypertrophy, as well as tubulointerstitial injury independent of intrarenal crystal formation [37]. Therefore lowering uric acid in high-fat fed rats may help to ameliorate much of the MS, including a reduction in BP, serum triglycerides, hyperinsulinemia, and weight gain.

Collectively the present study supports the view that melatonin limits body weight increase, reduces BP and normalizes cytokine levels in high-fat fed rats. These physiological findings must be complemented with the analysis of the molecular mechanisms involved in order to define their possible clinical implications. As well as in animal models, clinical studies have shown that melatonin provides benefits on lipid profiles. Melatonin treatment (1 mg/kg for 30 days) elevated HDL-c levels in peri- and postmenopausal women [38]. In an open-label study which included 33 healthy volunteers and 30 MS patients treated with melatonin, patients with MS had significantly higher values than controls in total

cholesterol, LDL-c, triglycerides, systolic and diastolic BP, glycemia, fibrinogen, and erythrocyte thiobarbituric acid-reactive substrate levels [39]. They also had lower levels of HDL-c and reduced activities of catalase, glutathione peroxidase and superoxide dismutase in erythrocytes. Melatonin (5 mg/day) decreased significantly hypertension and improved the serum lipid profile and the antioxidative status [39]. In another open label study comprising 100 elderly hypertensive patients the simultaneous application of melatonin together with lisinopril or amlodipine had the normalizing effect on BP and metabolic parameters [40]. Melatonin administration improved the enzymatic profile in patients with non alcoholic hepatic esteatosis [41,42]. Treatment with melatonin improved the MS occurring after treatment of schizophrenic or bipolar patients with 2nd generation antipsychotics [43,44].

Collectively, the results suggest that melatonin therapy can be of benefit for patients with MS, particularly with arterial hypertension. Further studies employing melatonin doses in the 30-50 mg/day range are needed to clarify its potential therapeutical implications on the MS in humans. If one expects melatonin to be an effective cytoprotector, especially in aged people, it is likely that the low doses of melatonin employed so far (2-5 mg) are not very beneficial.

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Conflict of Interest

The authors declare no conflict of interest.

Author contribution

P.C.B., E.S.P, V.J-O. and M.P.F-M were responsible of acquisition of data, data analysis and initial data interpretation. A.I.E. and D.P.C. contribute to the concept

and design of the experiments, to the drafting of the manuscript, and to the critical revision of the manuscript and approval of the article.

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FIGURE LEGENDS

Figure 1. (A) Percent increase in body weight of rats fed a normal or a high-fat diet and melatonin (25 μg/mL) or vehicle in drinking water for 10 weeks. (B) Systolic blood pressure difference as compared to the previous week. Shown are the mean + S.E.M. (n = 12-16/group). Asterisks indicate the existence of significant differences with the remaining groups after a one-way ANOVA followed by a Holm-Sidak multiple comparisons test at a given week of treatment.

Figure 2. Plasma levels of IL-1β and IL-6 in rats fed a normal or a high-fat diet and melatonin (25 μg/mL) or vehicle in drinking water for 10 weeks. Groups of rats were euthanized at the middle of the light period or at the middle of the scotophase. Shown are the means ± SEM (n= 7-8 per group). Letters indicate significant differences in a one-way ANOVA followed by a Holm-Sidak multiple comparisons test, as follows: (a) $P< 0.01$ as compared to the melatonin-treated groups; (b) $P< 0.05$ as compared to the remaining groups; (c) $P< 0.05$ as compared to the melatonin-treated groups; (d) $P< 0.05$ as compared to the obese + melatonin group. For further statistical analysis, see text.

Figure 3. Plasma levels of TNF-α and IFN-γ in rats fed a normal or a high-fat diet and melatonin (25 μg/mL) or vehicle in drinking water for 10 weeks. Groups of rats were euthanized at the middle of the light period or at the middle of the scotophase. Shown are the means ± SEM (n= 7-8 per group). Letters indicate significant differences in a one-way ANOVA followed by a Holm-Sidak multiple comparisons test, as follows: (a) $P< 0.01$ as compared to the remaining groups; (b) $P< 0.05$ as compared to the melatonin-treated groups. For further statistical analysis, see text.

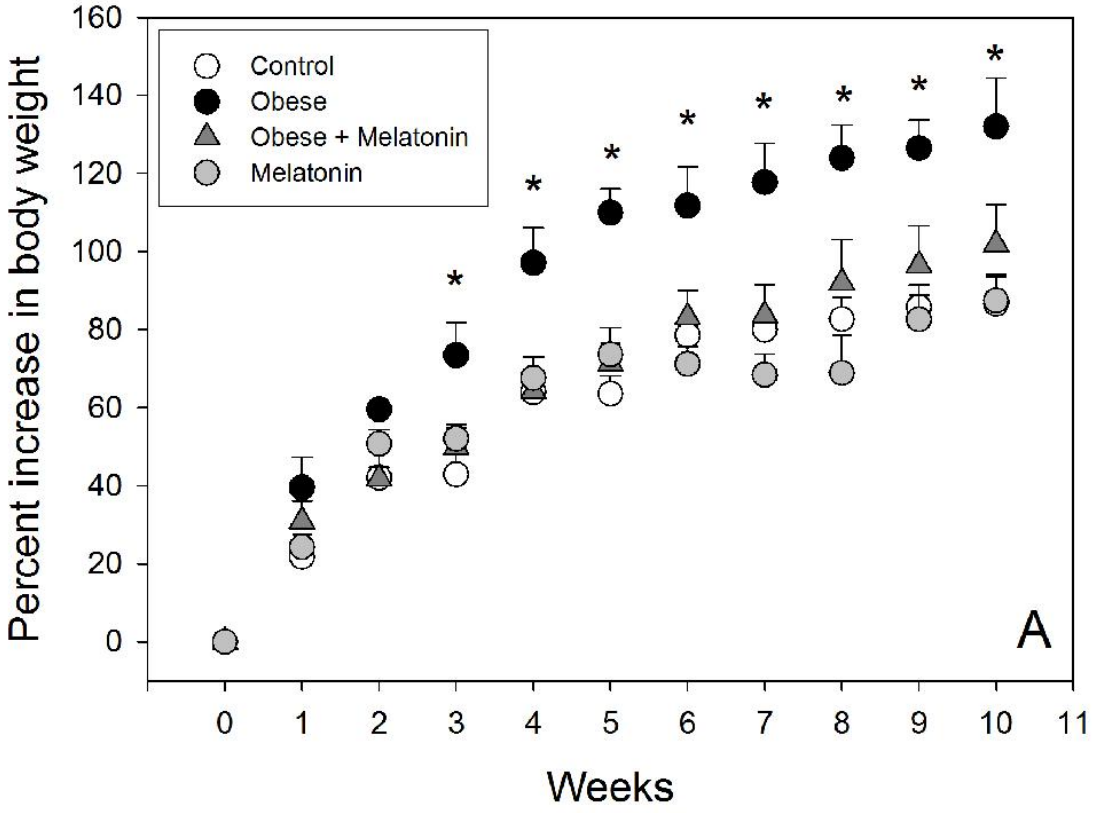
Figure 4. Plasma levels of IL-4 and IL-10 in rats fed a normal or a high-fat diet and melatonin (25 μg/mL) or vehicle in drinking water for 10 weeks. Groups of rats were euthanized at the middle of the light period or at the middle of the scotophase. Shown are the means ± SEM (n= 7-8 per group). Asterisks indicate significant differences in a one-way ANOVA followed by a Holm-Sidak multiple comparisons test, $P< 0.01$ as compared to the remaining groups. For further statistical analysis, see text.

Figure 5. Plasma levels of insulin and glucose in rats fed a normal or a high-fat diet and melatonin (25 μg/mL) or vehicle in drinking water for 10 weeks. Groups of rats were euthanized at the middle of the light period or at the middle of the scotophase. Shown are the means ± SEM (n= 7-8 per group). Asterisks indicate significant differences in a one-way ANOVA followed by a Holm-Sidak multiple comparisons test, $P< 0.01$ as compared to the remaining groups. For further statistical analysis, see text.

Figure 6. Plasma levels of cholesterol and triglycerides in rats fed a normal or a high-fat diet and melatonin (25 μg/mL) or vehicle in drinking water for 10 weeks. Groups of rats were euthanized at the middle of the light period or at the middle of the scotophase. Shown are the means ± SEM (n= 7-8 per group). Letters indicate significant differences in a one-way ANOVA followed by a Holm-Sidak multiple comparisons test, as follows: (a) $P< 0.01$ as compared to the remaining groups; (b) $P< 0.01$ as compared to animals fed a high fat diet. For further statistical analysis, see text.

Figure 7. Plasma levels of LDL-c and HDL-c in rats fed a normal or a high-fat diet and melatonin (25 μg/mL) or vehicle in drinking water for 10 weeks. Groups of rats were euthanized at the middle of the light period or at the middle of the scotophase. Shown are the means ± SEM (n= 7-8 per group). Letters indicate significant differences in a one-way ANOVA followed by a Holm-Sidak multiple comparisons test, as follows: (a) $P< 0.01$ as compared to the remaining groups; (c) (b) $P< 0.01$ as compared to the melatonin-treated groups. For further statistical analysis, see text.

Figure 8. Plasma levels of urea and uric acid in rats fed a normal or a high-fat diet and melatonin (25 μg/mL) or vehicle in drinking water for 10 weeks. Groups of rats were euthanized at the middle of the light period or at the middle of the scotophase. Shown are the means ± SEM (n= 7-8 per group). Letters indicate significant differences in a one-way ANOVA followed by a Holm-Sidak multiple comparisons test, as follows: (a) $P< 0.01$ as compared to the melatonin-treated groups. For further statistical analysis, see text.


Control
Obese
Obese + Melatonin
Melatonin
Percent increase in body weight
Weeks
A
*

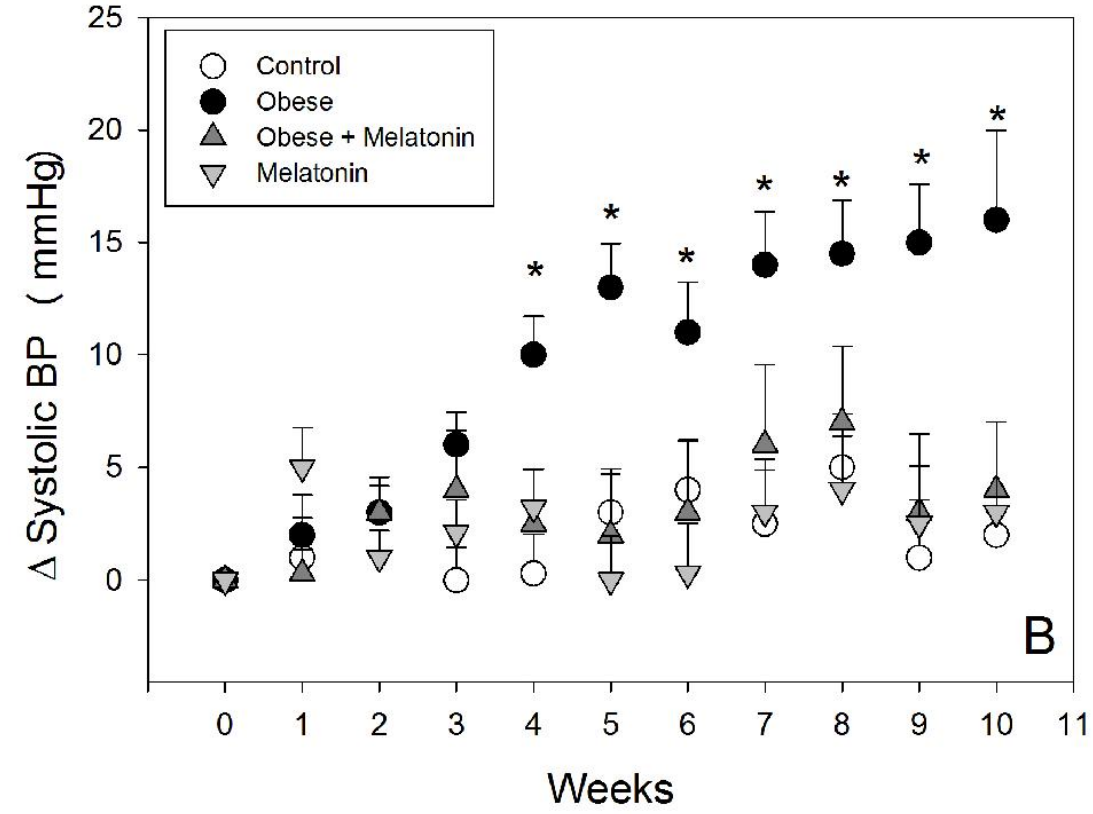

Control
Obese
Obese + Melatonin
Melatonin
Δ Systolic BP (mmHg)
Weeks
B
*

Figure 1

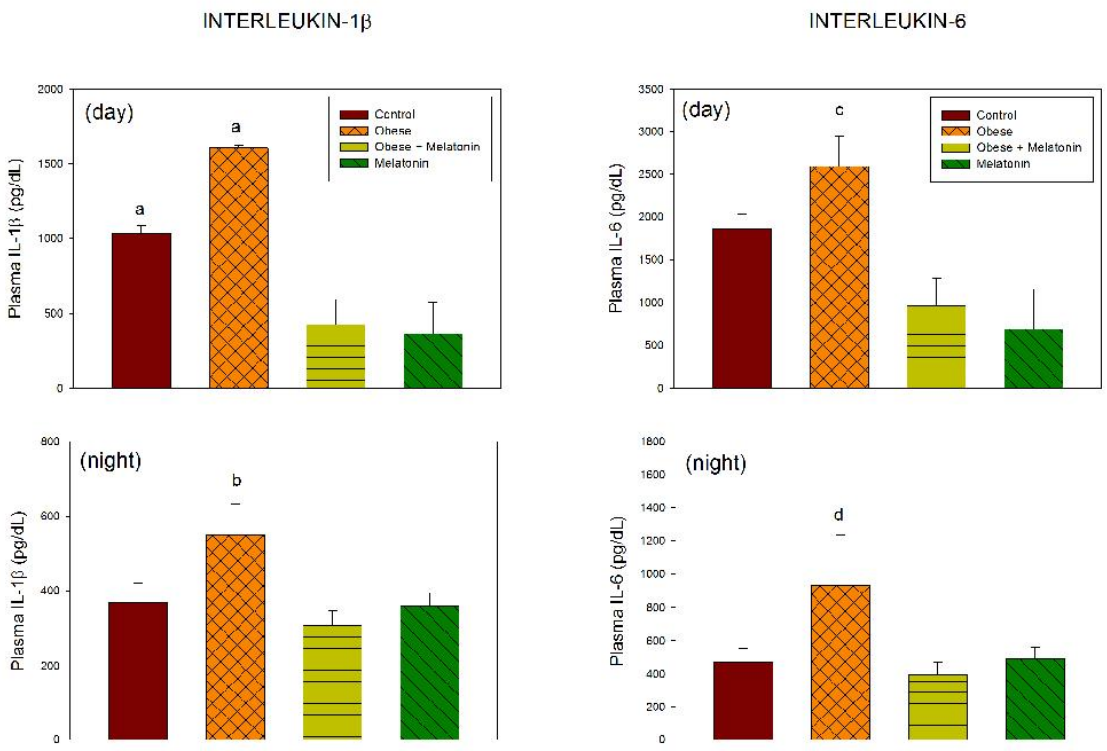

INTERLEUKIN-1β
INTERLEUKIN-6
(day)
(night)
Control
Obese
Obese + Melatonin
Melatonin
Plasma IL-1β (pg/dL)
Plasma IL-6 (pg/dL)
a
b
c
d

Figure 2

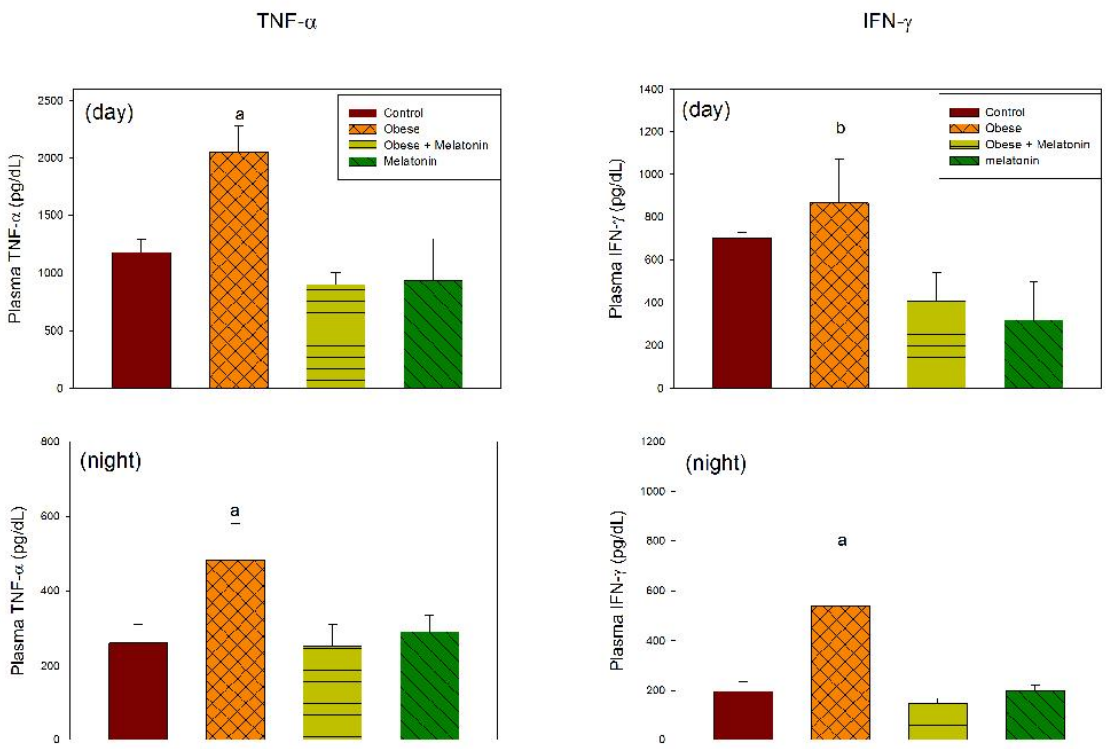

TNF-α
IFN-γ
(day)
(night)
Control
Obese
Obese + Melatonin
Melatonin
melatonin
Plasma TNF-α (pg/dL)
Plasma IFN-γ (pg/dL)
a
b

Figure 3

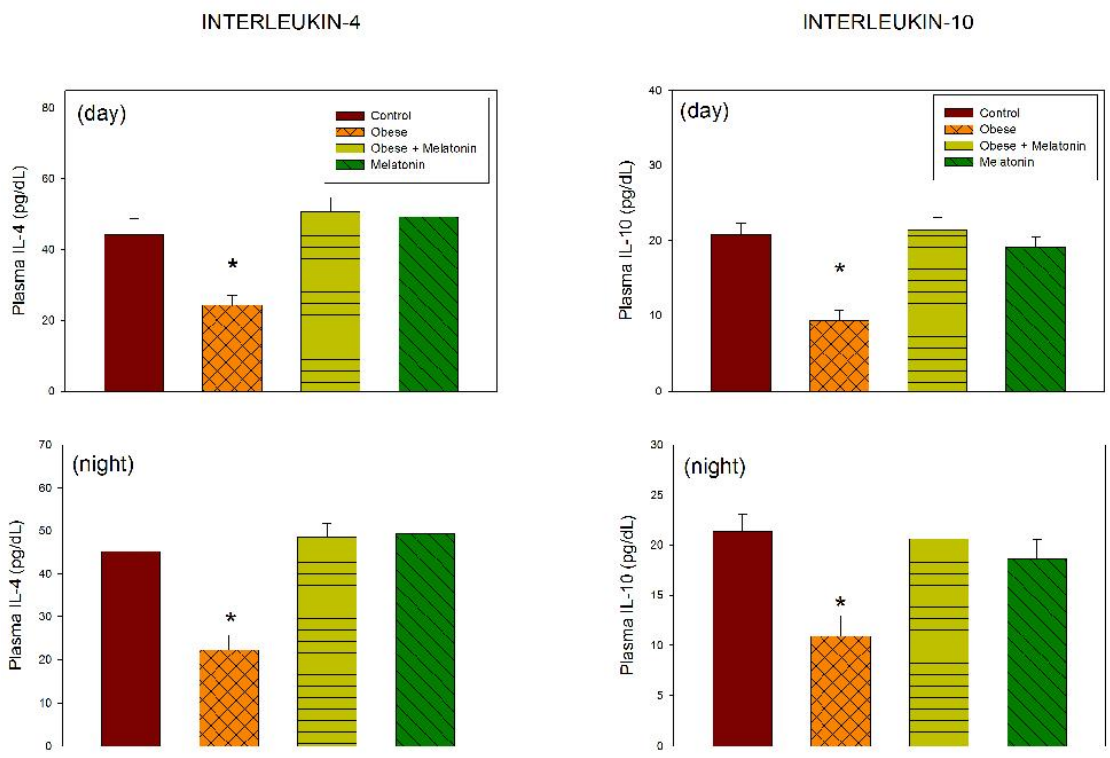

INTERLEUKIN-4
INTERLEUKIN-10
(day)
(night)
Control
Obese
Obese + Melatonin
Melatonin
Me atonin
Plasma IL-4 (pg/dL)
Plasma IL-10 (pg/dL)
*

Figure 4

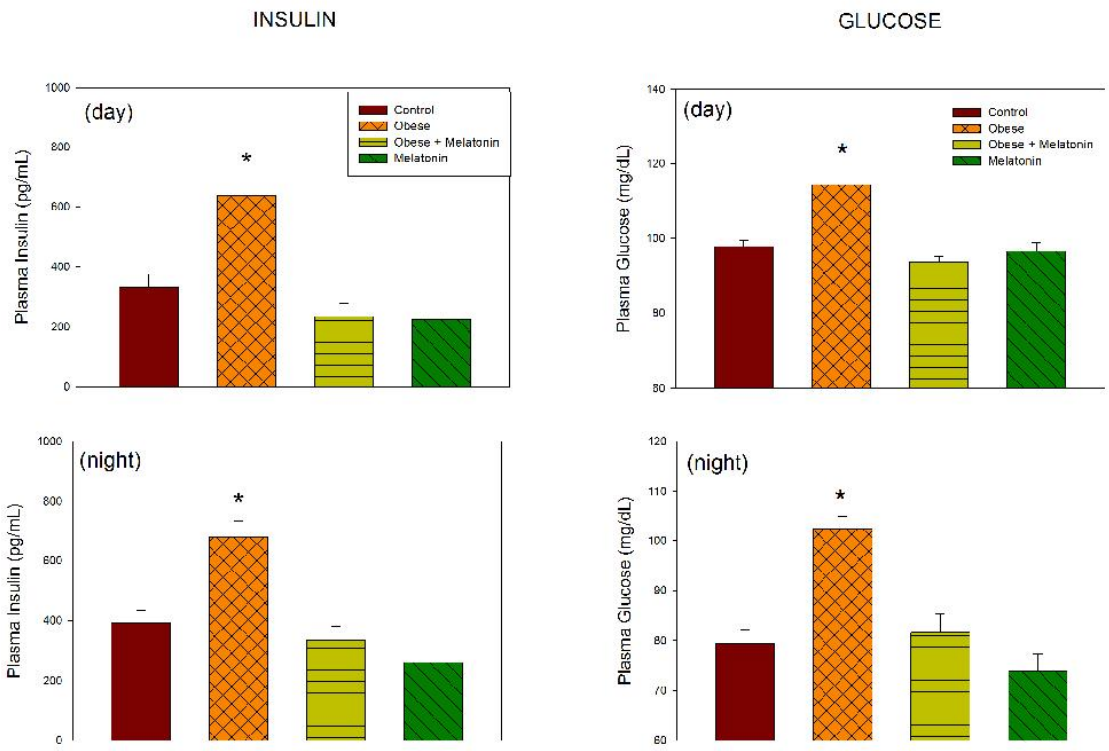

INSULIN
GLUCOSE
(day)
(night)
Control
Obese
Obese + Melatonin
Melatonin
Plasma Insulin (pg/mL)
Plasma Glucose (mg/dL)
*

Figure 5

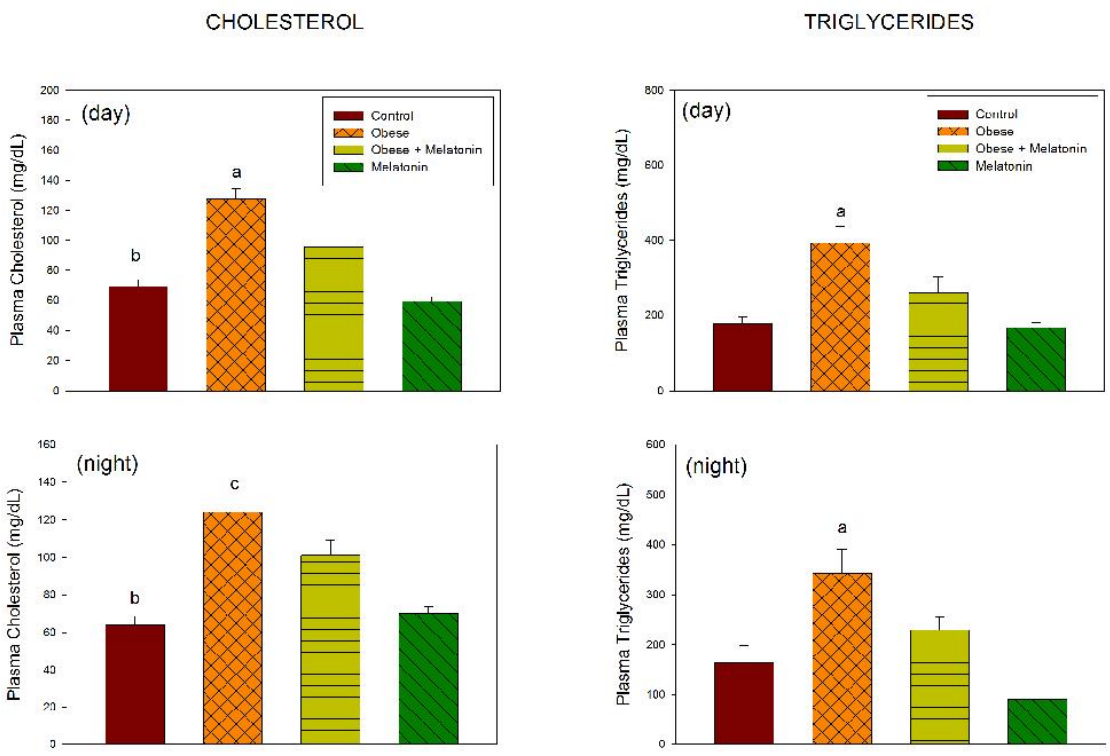

CHOLESTEROL
TRIGLYCERIDES
(day)
(night)
Control
Obese
Obese + Melatonin
Melatonin
Plasma Cholesterol (mg/dL)
Plasma Triglycerides (mg/dL)
a
b
c

Figure 6

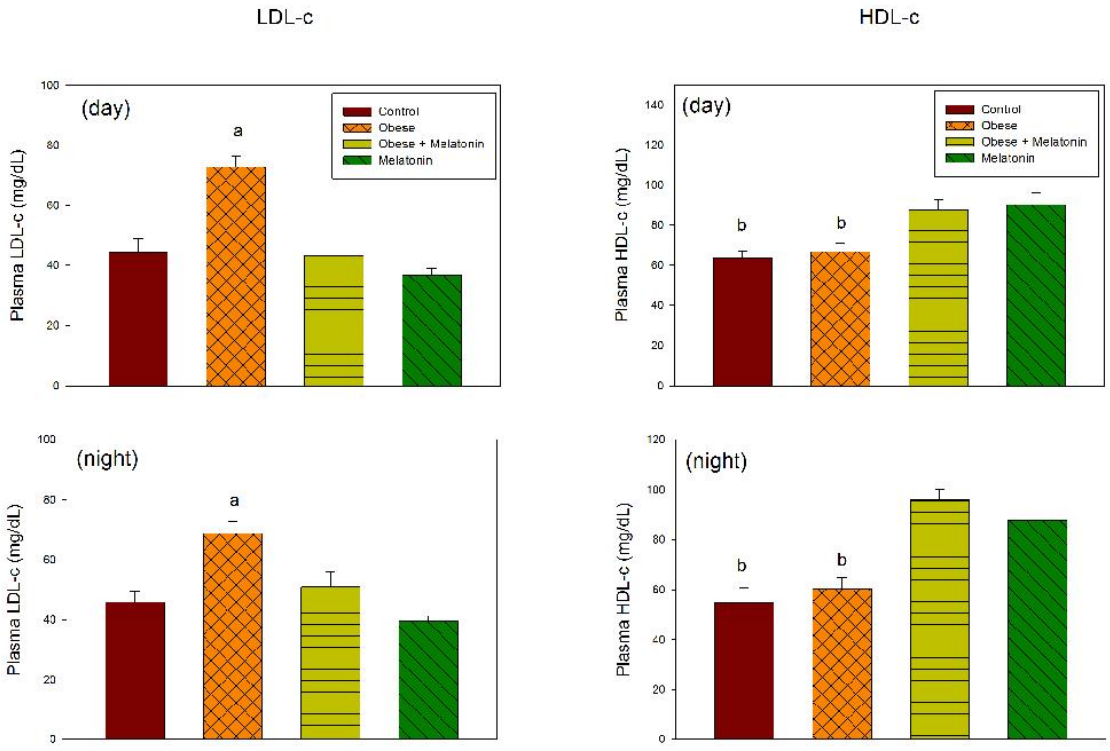

LDL-c
HDL-c
(day)
(night)
Control
Obese
Obese + Melatonin
Melatonin
Plasma LDL-c (mg/dL)
Plasma HDL-c (mg/dL)
a
b

Figure 7

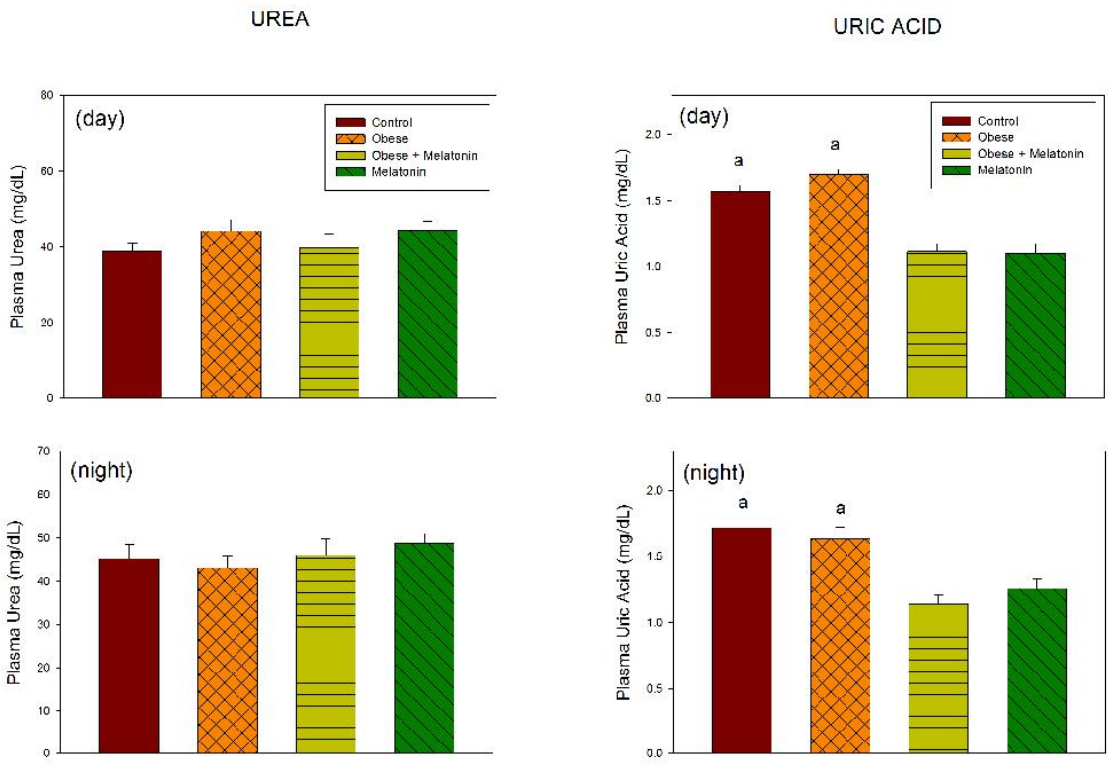

UREA
URIC ACID
(day)
(night)
Control
Obese
Obese + Melatonin
Melatonin
Plasma Urea (mg/dL)
Plasma Uric Acid (mg/dL)
a
a

Figure 8