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Preprint del documento publicado en Journal of Pineal Research, 2010, 49, 342-348

Publicado en: Journal of Pineal Research 49:342–348; 2010

Melatonin effect on plasma adiponectin, leptin, insulin, glucose, triglycerides and cholesterol in normal and high-fat fed rats.

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Running title: melatonin and 24-h changes in adipocytokines

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Key words: melatonin; circadian rhythms; high-fat diet; adiponectin; leptin; insulin.

Abstract

Melatonin effect on body weight progression, mean levels and 24-hour pattern of circulating adiponectin, leptin, insulin, glucose, triglycerides and cholesterol were examined in rats fed a normal or a high-fat diet. In experiment 1, rats fed a normal diet were divided into two groups, receiving melatonin (25 μg/mL drinking water) or vehicle for 9 weeks. In experiment 2, animals were divided into 3 groups, two fed with a high-fat diet (35% fat) and melatonin (25 μg/mL) or vehicle in drinking water for 11 weeks, while a third group was given a normal diet (4% fat). At the end of experiments groups of 8 rats were killed at 6 different time intervals throughout a 24 hour period. Melatonin administration for 9 weeks decreased body weight gain from the 3rd week on without affecting food intake. A significant reduction of circulating insulin, glucose and triglyceride mean levels and disrupted daily patterns of plasma adiponectin, leptin and insulin were observed after melatonin. In high-fat fed rats, melatonin attenuated body weight increase, hyperglycemia and hyperinsulinemia, as well as the increase in mean plasma adiponectin, leptin, triglycerides and cholesterol levels. The high-fat diet disrupted normal 24 h patterns of circulating adiponectin, insulin and cholesterol, the effects on insulin and cholesterol being counteracted by melatonin. Nocturnal plasma melatonin concentration in control and obese rats receiving melatonin for 11 weeks attained values 21-24-fold greater than controls. The results indicate that melatonin counteracts some of the disrupting effects of diet-induced obesity in rats.

Introduction

Melatonin treatment has been reported to modify the circulating levels of a number of hormones related to the regulation of metabolism. Among these, insulin and leptin have been extensively studied. Melatonin increases insulin release and tends to normalize the insulin resistance seen in animal models of obesity (for a recent review, see [1]). In both islets of Langerhans and INS-1 insulinoma β-cells, evidence for the expression of MT_1 and MT_2 melatonin receptors was given (see for ref. [1,2]) and genomic studies of type 2 diabetes have indicated that two different single nucleotide polymorphisms in human gene *MTNR1B* (that corresponds to melatonin MT_2 receptors) are associated with the disease [3].

Regarding leptin, and depending on the experimental design employed, there is an indication of a stimulatory role [4-10], inhibition of release [11-21] or absence of effects [22-24] of melatonin. These controversial results may depend on the fact that, in most cases, the effects of melatonin on leptin were assessed at single time points, usually at morning hours. In the case of adiponectin, there is only one report from studies in vitro indicating an inhibitory influence of melatonin on adiponectin synthesis by adipocytes [25] and no information on the in vivo effects nor on the effect of melatonin on 24-h rhythmicity of adiponectin release. Therefore, one of the objectives of the present study was to examine whether melatonin, chronically administered in drinking water, would affect mean levels and 24-h pattern of circulating leptin, adiponectin and insulin in rats. Circulating glucose, triglycerides and cholesterol levels were also measured.

The effects of feeding regimens and food components on the circadian system have been well documented (see for ref. [26]). In rats fed a high-fat diet we reported a

significant disruption of 24-h pattern of plasma prolactin, luteinizing hormone, thyrotropin, testosterone and corticosterone [27]. Concomitantly, the amplitude of the nocturnal pineal melatonin peak decreased by about half. Since melatonin administration to middle-aged rats reduces body weight, intraabdominal adiposity and plasma leptin and insulin (independently of food intake [21,28]) and to effectively reduce adiposity in diet-induced obesity [19,29], we examined, in the second part of this study, whether chronic melatonin administration could restore the altered circulating adiponectin, leptin and insulin seen in high-fat fed rats. In these animals increased circulating levels of leptin and decreased plasma ghrelin occurred, together with signs of insulin resistance (i.e. hyperglycemia and increased insulin levels) (see for recent reviews [30,31]).

Materials and Methods

Animals and experimental design

Male Wistar rats (45 days of age) 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). Two experiments were performed.

In experiment 1, rats received melatonin (25 µg/mL) in drinking water or vehicle for 9 weeks. The stock solution of melatonin was prepared in 50 % ethanol; final ethanol concentration in drinking water was 0.015 %. Vehicle-treated controls received 0.015 % ethanol in drinking water. Water bottles were changed every other day. Since rats drank about 20 mL/day with 90-95% of this total daily water taken up during the dark period, the melatonin dosage used provided approximately 500 µg melatonin/day. The animals were weighed once a week for 9 weeks.

In experiment 2, rats were divided into 3 groups, two of them receiving a high-fat diet (35% fat) and melatonin (25 μg/mL) or vehicle in drinking water for 11 weeks while a third, control group was fed a normal diet (4% fat). Both control and high-fat diets were obtained from Harlan Iberica, Barcelona, Spain, and the characteristics of the diet employed are summarized in table 1.The animals were weighed once a week for 11 weeks.

Rats were sacrificed by decapitation under conditions of minimal stress at 6 different time intervals (8 rats per group), every 4 h throughout a 24-h cycle, starting at 09:00 h. At night intervals, animals were killed under dim red light. 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. EDTA (6 g/100 mL) was used as an anticoagulant. Samples were stored at –70 °C until further analysis.

In an independent group of normal and obese rats receiving melatonin (25 μg melatonin/mL of water) or vehicle for 11 weeks, the plasma levels of the methoxyindole were assayed in blood samples collected at 0100 h.

Biochemical assays

Plasma concentrations of, adiponectin, leptin 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 [32]. 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 manufacturers' protocols. Glucose, total cholesterol and total triglycerides were measured with commercially available kits (Biolabo Reagents, Maizy, France). Plasma glucose concentrations were measured by an automated glucose oxidase method with a Beckman Glucose Analyzer 2 (Beckman Instruments, Fullerton, CA, USA). Plasma melatonin levels were measured by ELISA (Immuno Biological Laboratories, Hamburg, Germany).

Statistical analysis

Afte verifying normality of distribution of data in a normal distribution probability plot, the statistical analysis of the results was performed by a Student´s t test, a one-way ANOVA or a two-way factorial ANOVA, as stated. Post-hoc Bonferroni´s multiple comparison tests were employed. P values lower than 0.05 were considered evidence for statistical significance.

Results

Figure 1 shows the time course of changes in body weight in animals receiving melatonin or vehicle during 9 weeks. The inhibitory effect of melatonin on the increase in body weight was already significant at the 3rd week of treatment. Individual daily food intake, as measured during the last two weeks of the experiment, was 18 ± 2 g (vehicle) and 19 ± 1 g (melatonin) (Student´s t= 0.45, p= 0.65). The percentage of food intake at night was 75.4 ± 7.9 % (vehicle) and 73.3 ± 6.8 % (Student´s t= 0.428, p= 0.84) (melatonin). Nocturnal water consumption did not differ among the experimental groups.

The effect of melatonin on 24-hour pattern of circulating adiponectin, leptin, insulin, glucose, triglycerides and cholesterol is depicted in figure 2. Melatonin decreased significantly mean values of circulating insulin, glucose and triglycerides ($F= 4.1$, 8.5 and 7.8, $p< 0.01$, factorial ANOVA). It also disrupted the normal circadian rhythmicity of adiponectin and leptin by shifting the maximum in adiponectin from afternoon to late night and by suppressing the late night nadir in plasma leptin ($p< 0.02$, Fig. 2).

The results of experiment 2 are summarized in figures 3 and 4. Individual daily food intake during the last two weeks of the experiment was 17 ± 1 g (normal diet), 19 ± 1 g (high-fat diet) and 18 ± 1 g (high-fat diet + melatonin) ($F= 0.99$, $p= 0.37$, analysis of variance, ANOVA). The percentage of food intake at night was 72.4 ± 7.6 % (normal diet), 69.9 ± 9.1 % (high-fat diet) and 71.3 ± 8.8 (high-fat diet + melatonin) ($F= 0.02$, $p= 0.97$, ANOVA). Nocturnal water consumption did not differ among the experimental groups.

As shown in figure 3 body weight of high-fat fed rats attained values 55% higher than controls after 77 days of treatment. The concomitant administration of melatonin significantly attenuated body weight increase in high-fat fed rats although the increase in body weight was not completely prevented (Fig. 3).

The effect of a high-fat diet together with melatonin or vehicle on 24-hour pattern of circulating adiponectin, leptin, insulin, glucose, triglycerides and cholesterol is depicted in Fig. 4. A factorial ANOVA indicated a significant experimental group effect of for each parameter when tested as a main factor ($F= 48.3$, 54.7, 24.9, 92.4, 12.7 and 45.6. $p< 0.0001$, for adiponectin, leptin, insulin, glucose, triglycerides and cholesterol, respectively). Post-hoc Bonferroni tests indicated that the high-fat diet

brought about significant increases of mean plasma levels of every parameter tested, whereas the concomitant administration of melatonin blunted the increase in circulating adiponectin, insulin and glucose and partially counteracted that of leptin, triglycerides and cholesterol ($p < 0.01$). The high-fat diet disrupted the normal 24 h pattern of circulating adiponectin, insulin and cholesterol, the effects on insulin and cholesterol being counteracted by the concomitant melatonin administration ($p < 0.02$).

In an independent group of control and obese rats receiving melatonin or vehicle in the drinking water for 11 weeks plasma concentration of melatonin was measured at 01:00 h (Table 2). After exogenous melatonin administration the values obtained in control and obese rats were 21-24-fold greater than those found in animals receiving vehicle. Melatonin concentrations attained in control and obese rats did no differ significantly.

Discussion

The foregoing results indicate that the chronic administration of 25 µg melatonin/mL of drinking water for 9 weeks to 45 days-old rats (experiment 1) brought about a significant impairment in body weight increase, together with a significant decrease of mean values of circulating insulin, glucose and triglycerides as well as a disrupted daily pattern of plasma adiponectin, leptin and insulin. This occurred in the absence of any significant effect of melatonin on food intake.

The reduction of insulin secretion (and concomitantly, in circulating glucose) seen in experiment 1 is consistent with the bulk of data supporting a negative regulation of insulin release and improvement in insulin sensitivity by melatonin [1],

while the lack of effect on mean values of leptin in face of a disrupted 24-h rhythmicity may explain some of the contradictory results on leptin levels cited reported in the literature [4-24], i.e., they could depend on sample timing.

Concerning adiponectin, the results herein reported provide the first published evidence on the effect of melatonin on circulating adiponectin levels, and indicate that the effect of a chronic melatonin treatment is mainly exerted of the 24 h rhythmicity of adiponectin secretion. Therefore, the reduced adiponectin protein expression in preadipocytes in the presence of millimolar concentrations of melatonin [25] does not reflect in changes in the circulating levels of adiponectin.

Rasmussen and co-workers [18,21,28] convincingly demonstrated that the daily administration of a physiological amount of melatonin (0.2 μg melatonin/mL of drinking water), started at middle age (12 months), suppressed intra-abdominal fat deposition and decreased plasma leptin and insulin to youthful levels. The administration of the same physiological dosage of melatonin starting at 3 months of age did not significantly alter any of the parameters tested [20]. The melatonin-induced changes in leptin and insulin levels reported herein in 45-days-old rats could be partly explained by the pharmacological amounts of the methoxyindole given, which produced plasma melatonin levels at a single time point (0100 h) in the same range that those achieved by the usual 3 mg dose employed in humans [33]. Whether similar circulating melatonin levels are found at the remaining time intervals examined awaits further elucidation.

In a previous study we reported that after 4 days of melatonin administration to rats fed a normal diet, the circulating levels of free cholesterol decreased in the absence of significant effects on total cholesterol levels, presumably by augmentation

of lecithin-cholesterol acyltransferase-mediated cholesterol esterification [34]. Similarly, the administration of 4 μg melatonin/mL drinking water for 12 weeks to 6-months-old rats under a normal diet did not affect total cholesterol [35]. Our foregoing results are in agreement to those previous observations in that the chronic melatonin administration for 9 weeks to rats under a normal diet did not affect mean levels or 24-h rhythmicity of plasma total cholesterol. Melatonin treatment did decrease plasma triglycerides levels.

The adipose tissue participates in the regulation of body weight homeostasis, glucose and lipid metabolism via a number of secreted proteins (adipocytokines) that include hormones, cytokines, growth factors, complement factors and matrix proteins (see for ref. [30]). Indeed, the circadian oscillation of many hormones involved in metabolism, such as corticosterone, insulin, glucagon, adiponectin, leptin, ghrelin and melatonin, becomes disrupted in the development of the metabolic syndrome and obesity. We recently reported in high-fat fed rats increased circulating levels of leptin and decreased plasma ghrelin, together with signs of insulin resistance (i.e. hyperglycemia and increased insulin levels) [32]. Mean levels of plasma adiponectin, interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)α and leptin augmented, and ghrelin decreased, in high-fat fed rats. The normal daily pattern of plasma insulin, adiponectin, IL-1, IL-6, TNFα, leptin, ghrelin and monocyte chemoattractant protein-1 became disrupted in high-fat fed rats [32]. This prompted us to examine in experiment 2 whether melatonin treatment could counteract some of the effects of a high-fat diet seen in rats.

A number of studies were addressed to assess whether melatonin could effectively reduce adiposity in obese rats. In one of them [17] rats fed from weaning

with a high-fat diet until they were overweight were then treated for 3 weeks with melatonin (30 mg/kg) 1 h before lights out. The treatment decreased body weight gain and feed efficiency by about half. Melatonin had no effect on plasma insulin levels, but it decreased plasma glucose, leptin and triglyceride levels [17].

Puchalski and co-workers [19] examined in middle-aged rats whether melatonin altered consumption of a liquid diet with high-fat content. Ten-month-old rats received this high caloric liquid diet containing either melatonin (0.2 μg/mL) or vehicle. The animals receiving melatonin gained 4% body weight during the first 2 weeks and then stabilized, whereas rats receiving vehicle continued to gain for an additional week. In melatonin-treated rats, night but not daytime plasma leptin levels, and daytime but not night plasma insulin levels, decreased. Melatonin treatment did not alter cumulative food consumption [19].

In a study performed in a diet-induced murine model of obesity the effects of an 8-week oral treatment with melatonin on insulin and glucose tolerance were assessed [29]. In high-fat diet-fed mice, but not in normal chow-fed control mice, melatonin significantly improved insulin sensitivity and glucose tolerance, as evidenced by a higher rate of glucose infusion to maintain euglycemia during hyperinsulinemic clamp studies and an attenuated hyperglycemic response to a glucose challenge [29]. Other animal models in which melatonin was shown to be effective to reduce obesity include the ovariectomized rat [24,36], the type 2 diabetic (OLETF) rat [15], high-fat fed rabbits [37] and olanzapine-treated rats [38]. Not only melatonin but also its analog NEU-P11 inhibited weight gain and improves insulin sensitivity in high-fat fed rats [39].

The foregoing results indicate that the concomitant administration of melatonin and a high-fat diet for 11 weeks prevented the significant hyperglycemia and

hyperinsulinemia given by the high-fat diet alone. Likewise, the increase in mean levels of plasma adiponectin, leptin, triglycerides and total cholesterol seen in high-fat fed rats was significantly decreased by melatonin. These changes coexisted with a significant reduction of body weight increase in the rats receiving melatonin in the absence of any significant effect of melatonin on food intake. By comparing the results of experiment 1 and 2 in the present study it seems clear that the effect of melatonin was more dramatic in rats on the high caloric intake than in rats kept under a normal diet.

The reasons for the decrease in body weight by melatonin in the absence of significant differences in food intake deserve to be further explored. A key piece of evidence in this respect is the observation that melatonin plays a fundamental role in the seasonal changes of adiposity of Siberian hamsters by increasing the activity of the sympathetic nervous system innervating white fat, thereby increasing lipolysis [40]. Whether or not a similar mechanism is also operative in a non-seasonal species like the laboratory rat remains to be defined.

Most studies show a reduction in plasma concentration of adiponectin in obesity (see for ref. [30,31]) with only one exception [41]. Our present and previous results [32] indicate an increase in mean levels of adiponectin in high-fat fed rats as well as a significant modification in its daily pattern in circulation. Further studies are needed to explain why under certain circumstances the decrease in adiponectin mRNA levels reported in fat of obese rats does not translate to a parallel decrease in plasma adiponectin concentration [41].

The high-fat diet disrupted the normal 24 h pattern of circulating adiponectin, insulin and cholesterol, the effects on insulin and cholesterol being reversed by the

concomitant melatonin administration. Our results indicate that the administration of melatonin effectively counteracts some of the disrupting effects seen in diet-induced obesity in rats, in particular, insulin resistance, dyslipidemia and overweight.

In summary, obesity and insulin resistance represent a problem of utmost clinical significance worldwide. Insulin-resistant states are characterized by the inability of insulin to induce proper signal transduction leading to defective glucose uptake in skeletal muscle tissue and impaired insulin-mediated effects. A high-fat diet, that contributes to insulin resistance, aggravates type 2 diabetes mellitus, stroke, and coronary artery disease and can feed back to influence the biological clock. The results of the present study support the concept that melatonin can be a useful ad-on therapy to curtail insulin resistance, dyslipidemia and overweight in obese individuals.

Acknowledgements

This work was supported by grants from Ministerio de Educación y Ciencia, Spain (SAF2008-00442) and Agencia Nacional de Promoción Científica y Tecnológica, Argentina (PICT 2007-01045). and the University of Buenos Aires (M 006). DPC is a Research Career Awardee from the Argentine Research Council (CONICET) and Professor Emeritus, University of Buenos Aires.

Disclosure statement

The authors declared no conflict of interest.

Author contribution

M.J.R-L, P.C., V.J-O., M.P.F-M, and P.A.S. were responsible of acquisition of data, data analysis and initial data interpretation. D.P.C. and A.I.E. 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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Table 1. Diet composition and consumption in experiment 2.

	Control	**High-fat diet**
% Fat	3	35
% Carbohydrates	60	35
% Protein	16	20
% Vitamins and minerals	21	10
Caloric content (Kcal/g)	2.9	5.4
Consumed fat (Kcal/day)	4.86	56.7
Consumed carbohydrates (Kcal/day)	43.2	25.2
Consumed protein (Kcal/day)	11.5	14.4

Table 2. Plasma values of melatonin at 01.00 h in control and obese rats receiving melatonin dissolved in the drinking water at a concentration of 25 μg/ml or vehicle for 11 weeks (means ± SEM, n= 6/group)

	Plasma melatonin concentration (pg/ml)
Control + vehicle	201 ± 38
Obese + vehicle	197 ± 22
Control + melatonin	4319 ± 645**
Obese + melatonin	4821 ± 622**

Plasma melatonin concentration was measured by ELISA.

** $p < 0.0001$, as compared to rats receiving vehicle (one-way ANOVA, Bonferroni´s multiple comparison test).

Fig. 1. Percent increase in body weight of rats receiving melatonin (25 µg/mL) in drinking water or vehicle for 9 weeks. Shown are the means ± S.E.M. (n= 48/group). * p< 0.01 vs. control, Student´s t test.

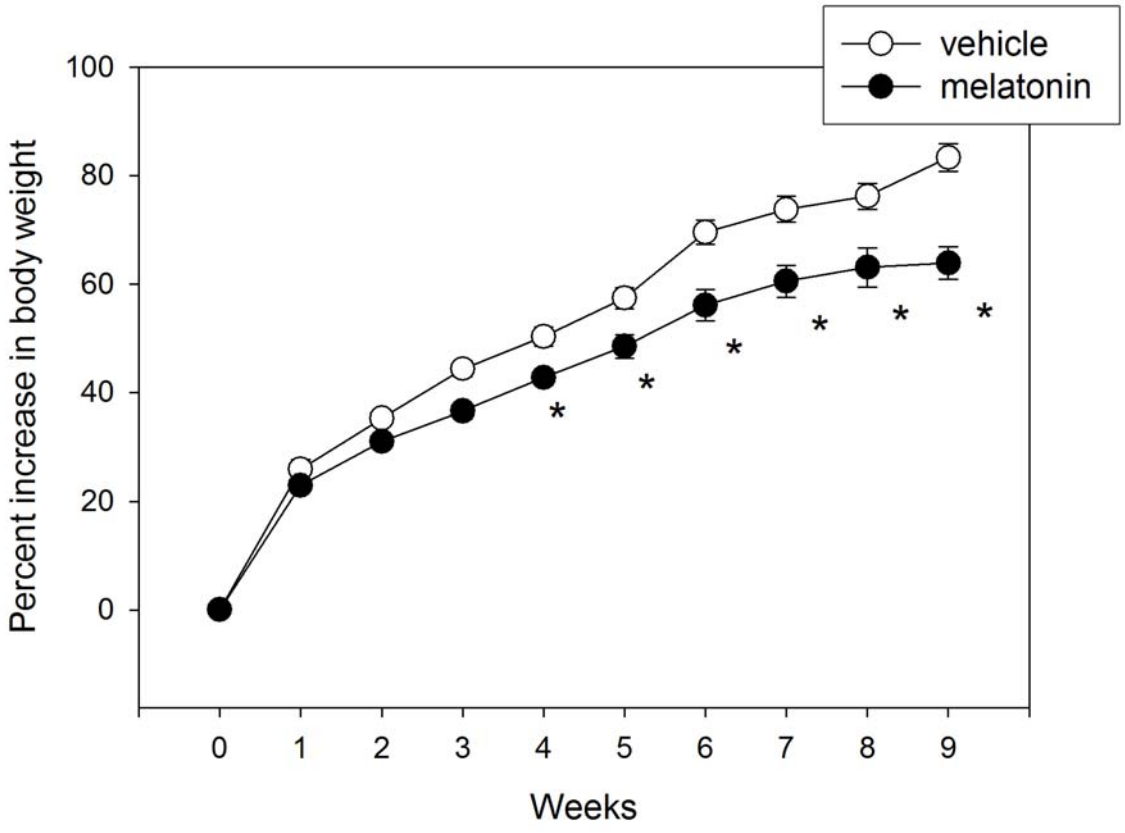

Fig. 2. Twenty-four hour changes in circulating levels of adiponectin, leptin , insulin, glucose, triglycerides and cholesterol of Wistar male rats receiving melatonin (25 µg/mL drinking water) or vehicle for 9 weeks. Groups of 8 rats were killed by decapitation at 6 different time intervals throughout a 24-h cycle. Bars indicate scotophase duration. Shown are the means ± S.E.M. Letters indicate the existence of significant differences between time points within each experimental group after a one-way ANOVA followed by a Bonferroni´s multiple comparisons test, [a] $p< 0.05$ vs. 0900 h. [b] $p< 0.05$ vs. 1700 h. [c] $p< 0.02$ vs. all groups. [d] $p< 0.05$ vs. 0900 and 1300 h. [e] $p< 0.05$ vs. 0100 h.[f] $p< 0.01$ vs. 0900 and 1300 h. [g] $p< 0.02$ vs. 0900 h and 1300 h, $p< 0.05$ vs.1700 h. For further statistical analysis, see text.

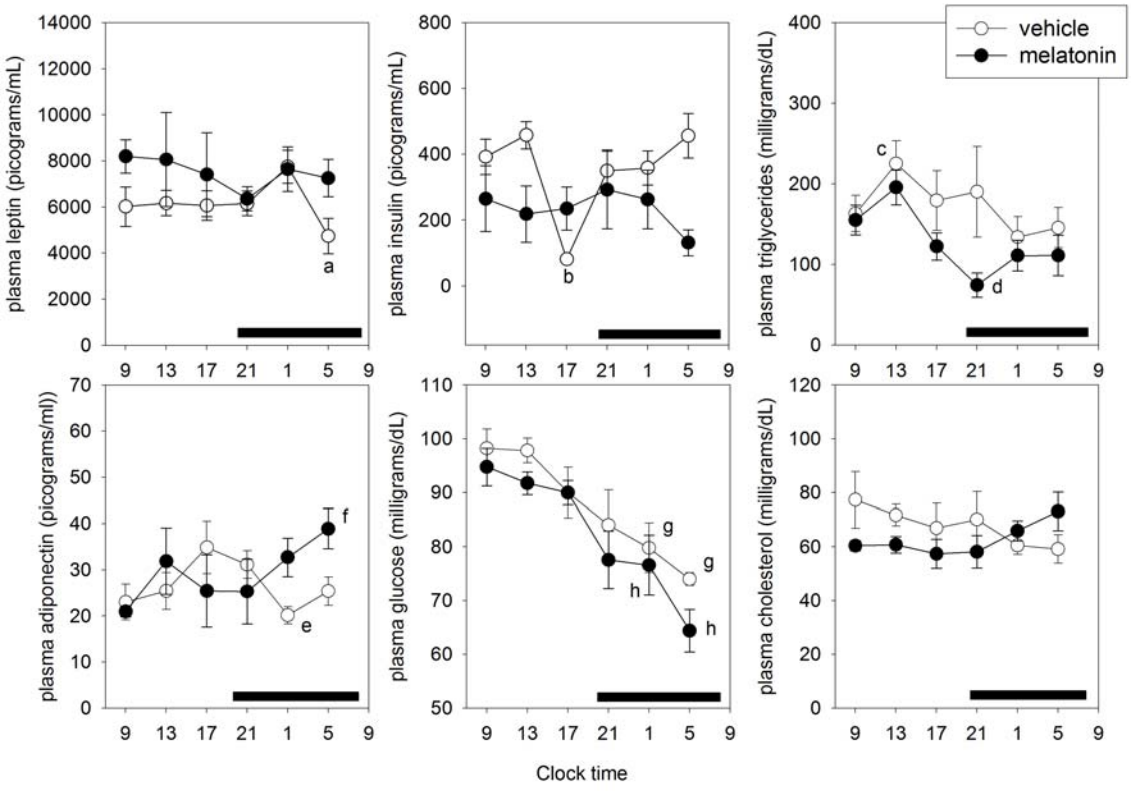

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Fig. 3. Percent increase in body weight of rats fed a normal diet (4% fat) or a high-fat diet (35% fat) and melatonin (25 μg/mL) or vehicle in drinking water for 11 weeks. Shown are the means ± S.E.M. (n= 48/group). Letters indicate the existence of significant differences after a one-way ANOVA followed by a Bonferroni´s multiple comparisons test and a given week of tratment, [a] $p < 0.01$ vs. the remaining groups. [b] $p < 0.01$ vs. high-fat fed rats receiving melatonin.

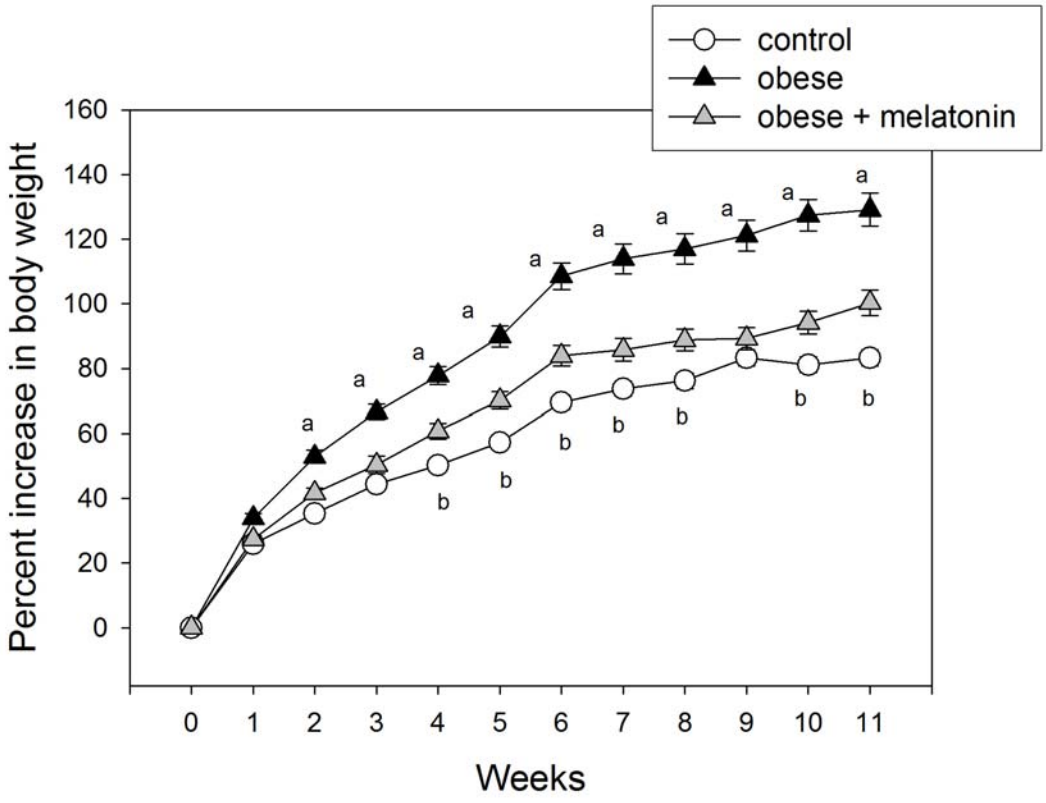
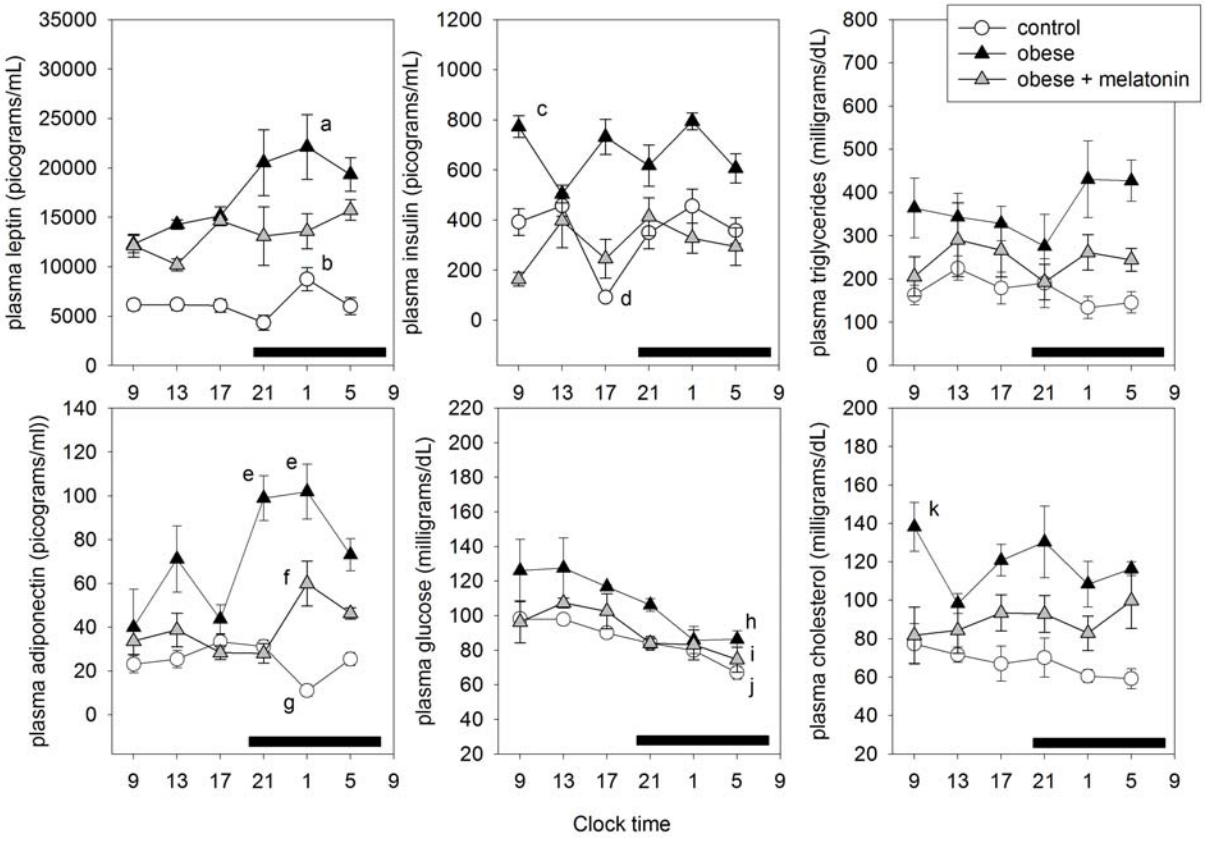


Fig. 4. Twenty-four hour changes in circulating levels of adiponectin, leptin, insulin, glucose, triglycerides and cholesterol of Wistar male rats fed a normal diet (4% fat) or a high-fat diet (35% fat) and melatonin (25 µg/mL) or vehicle in drinking water for 11 weeks. Groups of 8 rats were killed by decapitation at 6 different time intervals throughout a 24-h cycle. Bars indicate scotophase duration. Shown are the means ± S.E.M. Letters indicate the existence of significant differences between time points within each experimental group after a one-way ANOVA followed by a Bonferroni´s multiple comparisons test, [a] p< 0.02 vs. 0900 and 1700 h. [b] p< 0.05 vs. 1700 and 2100 h. [c] p< 0.02 vs. 1700 and 2100 h. [d] p< 0.02 vs. 1300 h. [e] p< 0.05 vs. 0900 h. [f] p< 0.02 vs. 2100 h. [g] p< 0.05 vs. 1300 h. For further statistical analysis, see text.



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