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Original Article

Effects of Pinealectomy on the Daily Profile of Circadian Clock and Apoptosis Components in Rat Retroperitoneal Adipose Tissue

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Abstract

Background and objectives: Visceral adipocytes, typically larger and more pro-inflammatory, are less sensitive to insulin action and more susceptible to apoptosis than subcutaneous adipocytes. Melatonin is an anti-inflammatory, anti-apoptotic, and temporal cue to several tissues, including adipose tissue. The modern lifestyle often involves irregular sleep-wake cycles, exposure to artificial light at night, and shift work, all of which suppress nocturnal melatonin secretion and could disrupt adipose tissue homeostasis. This study aimed to determine the effects of pinealectomy on the temporal expression of core clock components and apoptosis-related transcripts and proteins in rat retroperitoneal (RP) adipose tissue.

Methods: *In silico* analysis predicted putative binding sites of core clock components for *Caspase genes*. Eighty male Wistar rats were divided into either Sham-operated or Pinealectomized groups. Four weeks post-surgery, the animals were euthanized every 4 h over 24 h, and the RP tissue was processed for qRT-qPCR and ELISA.

Results: The promoter and regulatory regions of caspases 3, 8, and 9 were predicted. The expression of apoptosis-related genes exhibited circadian rhythmicity in control animals. Pinealectomy resulted in a loss of *Fas* rhythmicity, altered the cosinor parameters of *Bax*, *Bcl2*, *Casp3*, and *Casp9* expression, and increased the protein content of BAX, BCL2, CASP3, and CASP8 in the late dark phase. The daily expression of core clock components (*Clock*, *Bmal1*, *Per2*, *Cry1*, and *Nr1d1*) was significantly altered by the time main effect, while pinealectomy did not change their expression.

Conclusion: Melatonin plays important roles in the daily regulation of apoptosis in RP fat, suggesting that melatonin suppression due to a desynchronized modern lifestyle might contribute to the development of obesity and metabolic syndrome.

Keywords: Melatonin; Cell death; Clock genes; Visceral adipose tissue.

Abbreviations: ANOVA, Analysis of Variance; BCL2, B cell leukemia/lymphoma 2; BAX, BCL2 Associated X, Apoptosis Regulator; BID, BH3 interacting domain death agonist; CAAT, conserved consensus sequence of GGCCAATCT; CLOCKBMAL1, binding site for the heterodimer composed by CLOCK: Clock circadian regulator and BMAL1: basic helix-loop-helix ARNT like 1; *Csap*, caspase; EBOX, enhancer box; ELISA, enzyme-linked immunosorbent assay; PINX, Pinealectomized; RORA, RAR-related orphan receptor A; RP, retroperitoneal; RT-qPCR, real-time quantitative polymerase-chain-reaction; SEM, standard error of the mean; TATA, conserved consensus sequence of TATAWAW; TBP, TATA-binding protein; TNFRSF1A, Tumor Necrosis Factor Receptor Superfamily member 1A; ZT, Zeitgeber time.

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Introduction

Given its remarkable plasticity, adipose tissue has an unprecedented ability to expand and atrophy compared to other organs. These morphological changes involve coordinated actions of different cell types within adipose tissue.[1] Changes in adiposity are accompanied by adipose tissue remodeling, which seems to occur in a depot-specific manner and involves processes related not only to size but also to adipocyte cellularity.[2,3] In severe fat mass loss, a condition frequently studied in cancer-induced cachexia models, it has been shown that the adipocyte turnover, particularly of the

retroperitoneal depot, is markedly affected and may be the determining factor for the marked atrophy observed in this visceral deposit.[4,5] In contrast, excessive and chronic energy consumption is associated with obesity and adipose tissue expansion. The mechanisms by which adipocytes expand via hypertrophy or hyperplasia differentially affect cellular metabolism.[1,6]

Chronic hypertrophy makes the adipocytes more susceptible to apoptosis; this event is accompanied by increased macrophage infiltration and crown-like structures around the dead adipocytes, a condition more likely evident in visceral than subcutaneous fat.[2] Compared to subcutaneous adipose tissue, visceral adipocytes are typically larger, secrete more pro-inflammatory cytokines, and are less sensitive to insulin action, therefore being more lipolytic and susceptible to apoptosis.[7–9] *In vitro* studies using human and rodent adipocyte cultures have listed inflammation as one of the most effective signals for insulin resistance and concomitant apoptosis of adipocytes.[10–12]

Intrinsic and extrinsic pathways comprise the two major apoptosis pathways. The intrinsic, also known as the mitochondrial pathway, can be activated by different stressors stimuli, leading to an increase in the mitochondrial membrane permeabilization and the release of proteins from the intermembrane space into the cytosol, such as cytochrome c. Proteins belonging to the B cell leukemia/lymphoma 2 (BCL2)-family, such as the pro-apoptotic protein BAX (BCL2 associated X, apoptosis regulator) and the anti-apoptotic BCL2, regulate the permeability of the mitochondrial membrane. Together with other proteins [including Procaspase-9 and Apoptotic protease activating factor 1], cytochrome c composes the so-called apoptosome, activating the caspase 9 (CASP9), which signals to the executor caspases (CASP 3, 6, and 7). The extrinsic pathway is activated via ligand binding to death receptors, such as Fas and Tumor Necrosis Factor Receptor Superfamily Member 1A (TNFRSF1A), which, through adapter proteins, promotes the recruitment, dimerization, and activation of CASP8, directly initiating apoptosis by the cleavage, and activation of executioner caspases, or through activation of the intrinsic pathway, by cleavage of BH3 interacting domain death agonist (BID) proteins into tBID.[13,14]

Melatonin hormone is secreted by the pineal gland in response to the photoperiodic cues relayed from the retina-hypothalamic tract. Melatonin plays a relevant role in glucose homeostasis and insulin sensitivity,[15,16] exerts anti-inflammatory and anti-apoptotic effects,[17–20] and the pineal gland removal alters the expression of several genes in the white adipose tissue.[21,22] Melatonin also modulates the cellular circadian clock in a tissue-dependent manner,[23–26] including adipocytes,[27,28] increasing the core clock components' expression (*Clock* and *Per1*) and improving adipocytes metabolism and secretory function under culture conditions. In parallel, *in vitro* studies have also listed circadian clock oscillators as classic apoptosis regulators.[29–31] Thus, melatonin and the circadian clock seemed involved in the daily regulation of adipose tissue homeostasis.

The circadian disruption imposed by artificial light exposure during the night, social jet lag, and shift works, conditions constantly observed in the modern lifestyle, is associated with melatonin suppression,[32] increased weight gain, particularly of visceral fat mass, and impairments in adipose insulin sensitivity,[33–37] which, together, may contribute to the development of obesity and other metabolic disorders.[32] This study unprecedentedly investigates the possible consequences of melatonin suppression for the daily regulation of apoptosis and the circadian clock in the rat retroperitoneal (RP) adipose tissue.

Materials and methods

In silico analysis

The *in silico* analysis was performed to identify possible consensus binding sequences for core clock components in the promoter and regulatory regions of apoptotic genes caspase 3 (*Casp3*), caspase 8 (*Casp8*), and *Casp9*, as previously described.[38] Briefly, the human gene sequence data were obtained from Genome Reference Consortium Human Build 37 (Hg19) on the NCBI gene website (https://www.ncbi.nlm.nih.gov/gene). The homology analysis, transcription factor targets selection, and final figure assemblies were performed using the Transfac professional V10.2 library available in the Evolutionary Conserved Region tools Browser (https://ecrbrowser.dcode.org/). The nucleotide sequences corresponding up to 5 Kb upstream of the first exon at 5′ untranslated region for each target gene were analyzed, and the identification of putative binding sites for CAAT/CAAT_C, CLOCK/BMAL1, enhancer box (EBOX), RAR-related orphan receptor A (RORA) 1, RORA2, TATA, TATA_C, and TATA-binding protein (TBP; transcription factors) was performed. The transcript nomenclature followed the MultiTF library pattern: Transfac professional V10.2 library.

Animals and treatments

Eighty male Wistar rats were obtained from the Central Animal Breeding House of the Institute of Biomedical Sciences, University of Sao Paulo (São Paulo, Brazil). The animals were kept on a 12 h:12 h light-dark cycle (lights on at 06:00 h = Zeitgeber time (ZT) 0; dark phase, red filter Kodak 1A, 0.5–1 lux) in a temperature-controlled room (25 ± 2 °C), with food (Nuvital S/A, Colombo, PR) and water *ad libitum*.

At 10 weeks of age, the animals were anesthetized with an intraperitoneal injection of Xilazin (Anazedan Vetbrands, Laranjal Paulista, SP, Brazil) and Ketamine (Dopalen Vetbrands, Laranjal Paulista, SP, Brazil) and randomly assigned into two experimental groups: control (CONTROL) or pinealectomized (PINX), as previously described.[39] Four weeks after the surgery, the animals were subdivided, and 6–7 animals/group were euthanized by decapitation under sodium thiopental anesthesia (20 mg/kg body weight) at 4 h intervals up to 24 h period (n = 6–7 animals/group/ZT). The retroperitoneal white adipose tissue (RP) was removed and stored at −80 °C for further real-time quantitative polymerase chain reaction (RT-qPCR) and enzyme-linked immunosorbent assay (ELISA) analysis. Some of the animals studied in this research were the same as used in the study published by de Farias *et al*. 2015, in which the serum levels of melatonin confirmed the effectiveness of the treatment.[22] All procedures were conducted according to the National Council for Control of Animals standards and approved by the Committee for Animal Experimentation of the University of Sao Paulo (03/10/2011, No. 129, book 2).

Total RNA extraction

Total RNA was extracted from 100 mg of RP tissue using the TRizol™ Plus RNA Purification Kit (ThermoFisher, Waltham, MA, USA), according to the manufacturer's instructions. The RNA concentration of each sample was determined by spectrophotometer reading (Epoch Microplate Spectrophotometer, Biotek Winooski VT) coupled to a Take3 Multi-Volume Plate (Biotek) and using Gen 5.0 software. RNA purity was evaluated by the 260 nm /280 nm ratio. Afterward, 1 µg of total RNA was plotted into a 1 % agarose gel to verify the integrity of RNA through the visualization of 28S and 18S RNA ribosomal bands.

Reverse transcription and RT-qPCR

Total RNA (2 µg) obtained from each sample was treated with DNAse (DNase I Amplification Grade (1 U/ µL; Invitrogen, ThermoFisher Scientific, Waltham, MA) and then submitted to reverse transcription reaction with (Superscript III Reverse Transcriptase; Invitrogen). The cDNA samples were stored at −20 °C until subsequent analysis. The *B2m, Bax*, *Bcl2*, *Casp3*, *Casp8*, *Casp9*, *Clock, Cry1, Fas*, *Per2*, and *Tnfrsf1a* transcript expression was quantified by RT-qPCR using gene-specific 20X TaqMan® hydrolysis probe. *Bmal1* and *Nr1d1* were quantified using specific oligonucleotide sequences and Platinum® Syber® qPCR Supermix-UDG, as shown in Table S1. The Platinum® Syber® reaction specificities were confirmed by the melting curve analysis, resulting in a single amplicon (peak) per sample.

The PCR was carried out by StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The qPCR efficiency and slope value for each probe or primer assay were evaluated, as described by Dussault and Pouliot (2006).[40] The respective values of slope, efficiency, R^2, and dilutions are presented in Table S2. The relative mRNA expression quantification of the target genes was performed by the $2^{\Delta CT}$ method,[41] and the data were normalized by *B2m* mRNA expression.[21]

Protein extraction and apoptotic proteins quantification by ELISA

The total daily content of apoptotic proteins BAX, BCL2, CASP3, 8, and 9 was quantified in RP fat from CONTROL and PINX rats (n = 4–7 animals/group/ZT). Briefly, 300 mg of RP adipose tissue was homogenized in 1 mL of 1X PBS and stored overnight at −20 °C. Subsequently, two freeze-thaw cycles were performed to break the cell membranes. The homogenates were then centrifuged for 5 min at 5,000 g at 4 °C, the supernatant was discarded, and the protein phase was transferred to a new tube. The total protein content was quantified using the Bradford method, and the sample aliquots were stored at −20 °C for further analysis.[42]

The apoptotic protein contents were quantified using commercial ELISA kits (CUSABIO, Biotech, Wuhan, China). The samples were diluted as follows: BAX (1:8), BCL2 (1:10), CASP3 (1:4), CASP8 (1:8), and CASP9 (1:16). The absorbance at 450 nm was assessed by spectrophotometer reading (Epoch Microplate Spectrophotometer, Biotek Winooski VT) and Gen 5.0 software. The quantification was calculated by analyzing the absorbance values at 450 nm compared to the standard curve for each assay, following the manufacturer's instructions.

Statistical analysis

The data were submitted to the Shapiro-Wilk test for normality assessment and Barlett's for homoscedasticity. Afterward, a one-way Analysis of Variance (ANOVA) was performed to evaluate the influence of time in each experimental group. Two-way ANOVA followed by pairwise comparisons using Bonferroni's post hoc test were performed to assess the main effects of time-treatment (PINX) in both groups. The statistical analysis was performed using GraphPad Prism version 8.02 for Windows, GraphPad Software, Boston, Massachusetts, USA (www.graphpad.com).

The presence of circadian rhythms was assessed by the cosinor method, which consists of fitting the data to a 24 h-period cosine curve, using the least squares calculation for each temporal series that presented statistical differences in the one-way ANOVA test.[43] Mesor, amplitude, and acrophase cosinor function parameters were calculated for each gene/experimental group and compared by a two-tailed, unpaired Student's t-test.[44] All the data are expressed as means ± standard error of the mean (SEM). Results were considered significant when $p < 0.05$.

Results

In silico analysis of the promoter and regulatory regions of caspases 3, 8, and 9

The computational binding site predictions performed in the regions corresponding to 5 Kb upstream to the first exon in the 5′ untranslated region of *CASP3*, *CASP8*, and *CASP9* human gene sequences revealed the presence of putative binding sites for core clock components [EBOX, RORA1 and RORA2, and binding site for the heterodimer composed by CLOCK: Clock circadian regulator and BMAL1: basic helix-loop-helix ARNT like 1 (CLOCKBMAL1) in conserved regions compared to mice (mm10) and monkey (rheMac2) genome sequences (Fig. 1). The presence of canonic eukaryotic transcription binding sites for TBP, TATA (conserved consensus sequence of TATAWAW), and CAAT (conserved consensus sequence of GGCCAATCT) strongly suggested that the selected regions correspond to the regulatory and promoter regions of the target genes. RORA1 and EBOX consensus binding sites were depicted in the *CASP3* gene sequence (Fig. 1a). The *CASP8* gene exhibits possible binding sites for CLOCKBMAL1 and EBOX (Fig. 1b). The *CASP10* gene is encoded in the opposite direction of the screened region of *CASP8,* and putative binding sites for RORA2 and EBOX were also noticed. In the *CASP9* gene sequence, CLOCKBMAL1 and EBOX binding sites were pointed out (Fig. 1c).

Effects of pinealectomy in daily apoptosis-related gene expression of retroperitoneal adipose tissue

The expression of apoptosis-related genes was investigated in RP adipose tissue of CONTROL and PINX rats (Fig. 2, panel 1). Both time and treatment (pinealectomy) main effects significantly altered the content of *Bcl2*, *Casp3*, and *Casp8* transcript over the 24 h period investigated by the two-way ANOVA (Fig. 2b-d, panel 1), while only the time main effect was observed for *Casp9*, *Fas*, and *Tnfrsf1a* (Fig. 2e-g, panel 1) and, time main effect and interaction were observed for *Bax* expression (Fig. 2a, panel 1). Pairwise comparisons showed that pinealectomy increased the mRNA content of *Casp8* at ZT0/24 and 12 compared to the CONTROL group.

The rhythmic analysis of apoptosis-related gene expression was assessed by cosinor (Table 1 and Fig. 2, panel 2). The expression of all apoptotic genes (*Bax*, *Bcl2*, *Casp3*, *Casp8, Casp9*, *Fas*, and *Tnfrsf1a*) exhibited circadian rhythmicity in CONTROL animals with all acrophase expressed in the middle of the dark phase (ZTs 15–17) (Fig. 2a-g, panel 2; Table 1). The pinealectomy induced a loss in the daily rhythmicity of *Fas* mRNA expression in RP adipose tissue (Fig. 2f, panel 2), delayed the acrophase of *Bax* (Fig. 2a, panel 2) and *Casp9* (Fig. 2e, panel 2) mRNA content, and increased the mesor of *Bcl2* (Fig. 2b, panel 2) and *Casp3* (Fig. 2c, panel 2) transcript expression. No effects were observed for the daily *Tnfrsf1a* mRNA expression induced by PINX (Fig. 2g, panel 2).

Effects of pinealectomy in the daily apoptosis-related protein content in the retroperitoneal adipose tissue

The total content of BAX, BCL2, CASP3, CASP8, and CASP9 was quantified in RP adipose tissue of CONTROL and PINX rats (Fig. 3). The main effects of time, treatment, and time-treatment interaction were observed for BAX and CASP3 protein contents

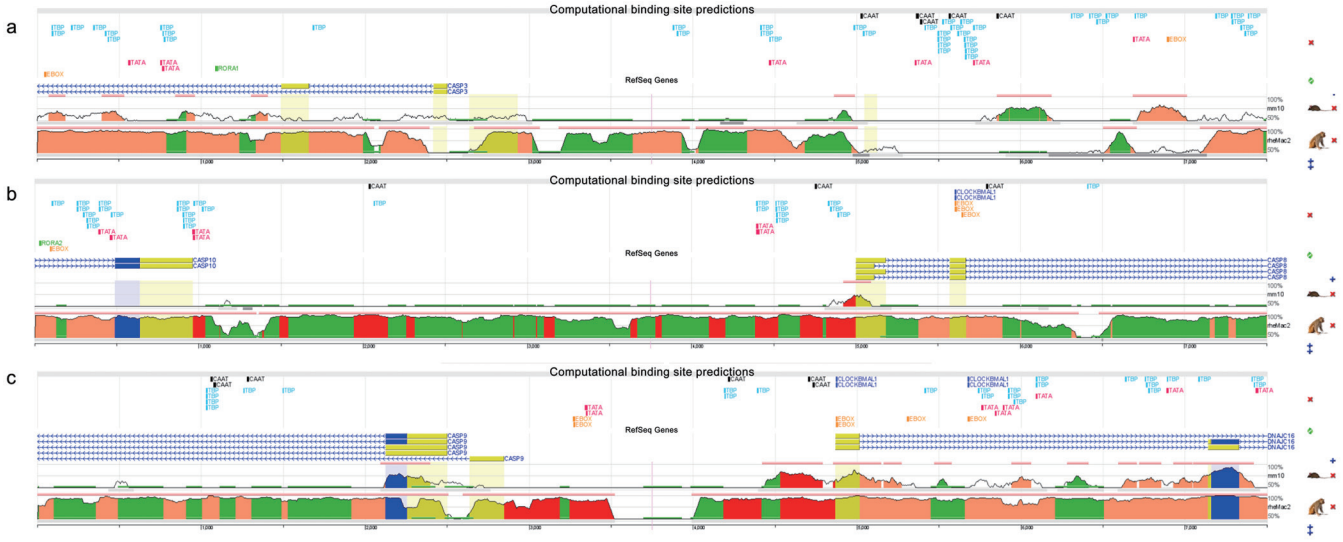

Fig. 1. *In silico* analysis performed in the regulatory and promoter regions of *CASP3* (a), *CASP8* (b), and *CASP9* (c) in human genome (Hg19). The transcription factors screening was carried out in the region corresponding to 5 Kb upstream to the first exon at 5′ untranslated region (shown in yellow). The computational binding site predictions for TBP (light blue), TATA (pink), CAAT (black), EBOX (orange), CLOCKBMAL1 (dark blue) and RORA1, and RORA 2 (green) for each target gene are displayed above the gene sequence. The conserved regions between the aligned species (monkey: rheMAc2 and mice: mm10) are highlighted in pink below the target sequence. The percentage of homology between sequences is shown on the right. Below each gene sequence is shown: the intronic regions in salmon color, transposons and simple repeats in dark green, and intergenic regions in red. The images were customized according to the Evolutionary Conserved Regions browser instructions. CAAT, conserved consensus sequence of GGCCAATCT; CLOCKBMAL1, binding site for the heterodimer composed by CLOCK: Clock circadian regulator and BMAL1: basic helix-loop-helix ARNT like 1; *Csap*, caspase; EBOX, enhancer box; RORA, RAR-related orphan receptor A; TATA, conserved consensus sequence of TATAWAW; TBP, TATA-binding protein.

in the 24 h period according to the two-way ANOVA (Fig. 3a and 3c), while the total content of BCL2 showed time main effect (Fig. 3b), and treatment main effect and time-treatment interaction were observed for CASP8 protein content (Fig. 3d). For the CASP9 protein, neither time nor treatment main effects were found. The PINX group showed increased content of BAX at ZTs 0/24 (Fig. 3a) and BCL2, CASP3, and CASP8 at ZT20 compared to the CONTROL group (Fig. 3b-d), while a reduction in the CASP8 protein content was observed at ZT4. No differences between groups were observed for CASP9 protein content in RP adipose tissue.

Effects of pinealectomy in the expression of circadian core clock components of retroperitoneal adipose tissue

Next, the daily profile of circadian core clock gene (*Clock*, *Bmal1*, *Per2*, *Cry1*, and *Nr1d1*) expression in RP tissue was evaluated (Fig. 4 and Table 1). According to two-way ANOVA analysis, the daily expression of all investigated core clock components was significantly altered by the time main effect, while the treatment – pinealectomy – did not impact their expression (Fig. 4a-e, panel 1). Additionally, pairwise comparisons showed a punctual increase in the *Nr1d1* mRNA content at ZT8 (Fig. 4e, panel 1).

Cosinor analysis revealed circadian rhythmicity for *Bmal1*, *Per2*, *Cry1*, and *Nr1d1* genes in the RP adipose tissue of CONTROL animals, while the expression of *Clock* mRNA did not exhibit an oscillatory pattern (Fig. 4b-e, panel 2). The PINX did not affect the oscillatory pattern of circadian clock components in RP adipose tissue nor alter the cosinor function parameters (Fig. 4b-e, panel 2; Table 1).

Discussion

Apoptosis is a crucial physiological phenomenon involved in several processes, such as embryonic development, removal of damaged or potentially harmful cells, and tissue turnover.[45] Understanding the temporal processes involved in adipose tissue cellularity and its regulatory mechanisms can enhance the effectiveness of therapeutic strategies for clinical conditions related to altered adiposity and insulin resistance. In this study, we demonstrated: a) the presence of consensus binding sequences for circadian core clock components in the promoter and regulatory regions of the initiator (*Casp8* and *Casp9*) and effector (*Casp3*) caspase encoding genes through *in silico* analysis, suggesting a potential regulation of apoptosis by the circadian clock; b) the circadian oscillatory profile of apoptosis-related and core clock components expression in the RP adipose tissue; c) the daily oscillation of apoptosis-related protein content in RP fat; and d) the influence of pinealectomy on the daily expression of the circadian clock and apoptosis pathway components in the RP adipose depot.

The existence of EBOX, CLOCKBMAL1, and RORA binding sites in caspase-encoding genes corroborates previous findings showing the influence of the circadian clock on the regulation of apoptosis in distinct cell lines.[29–31] In accordance, the expression of core clock and apoptosis-related genes exhibit a marked circadian oscillatory profile in RP adipose tissue of CONTROL rats and, for all anti (*Bcl2*) and pro-apoptotic (*Bax*, *Fas*, *Tnfrsf1a, Casp3*, *8* and *9*) encoding genes evaluated, the peak of gene expression was observed in the middle of the dark phase, corresponding to the period of higher activity in rodents. Moreover, the putative binding sites for RORA1 and 2 also suggest that melatonin could regulate the expression of apoptosis-related components through its interaction with nuclear receptors and binding to RORA cis-elements on caspase gene sequences. Notably, the modulation of circadian core clock components by melatonin via RORA was already demonstrated in cultured cardiomyocytes.[25]

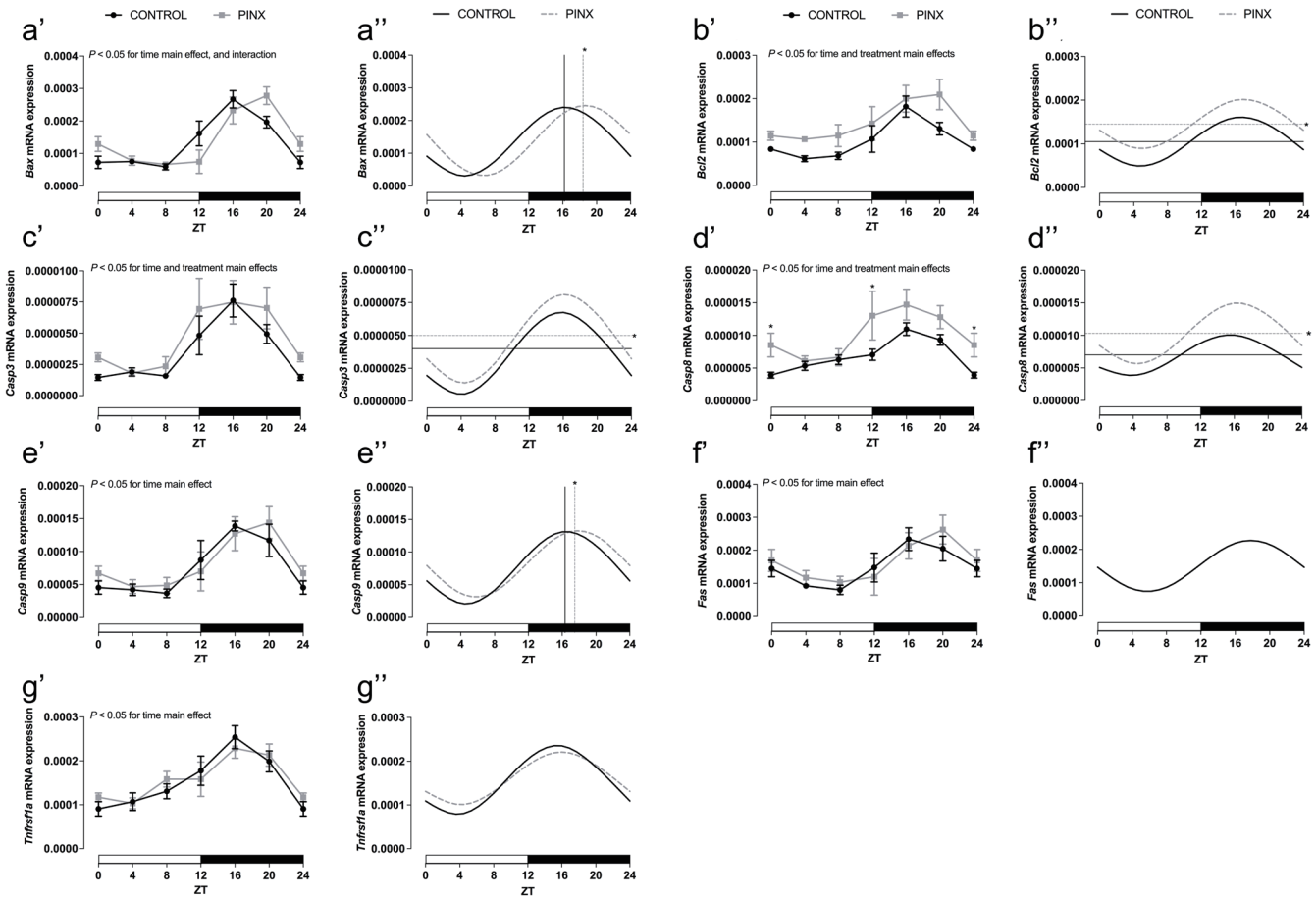

Fig. 2. Effects of pinealectomy in the mRNA expression of apoptosis-related genes in rat retroperitoneal adipose tissue (RP). (a-g) Daily expression of *Bax, Bcl2, Casp3, Casp8, Casp9, Fas*, and *Tnfrsf1a*, respectively. Data presented as means ± SEM. (1) Two-way ANOVA analysis for assessments of time and treatment (pinealectomy) main effects and time-treatment interaction; CONTROL: solid black lines and PINX: solid gray lines. Bonferroni post hoc test, $*p < 0.05$ for paired comparisons *vs.* the respective controls. (2) Cosinor analysis (CONTROL: solid black lines and PINX: dashed gray lines). Differences in mesor and acrophase are depicted as horizontal or vertical straight lines compared to the Student's test. The white horizontal bar corresponds to ZTs 0–12 period. The black horizontal bar represents the period of ZTs 12–24. *n* = 6–7/ZT/group. PINX, Pinealectomized. ANOVA, Analysis of Variance; *Bax*, *Bcl2* Associated X, Apoptosis Regulator; *Bcl2*, B cell leukemia/lymphoma 2; *Csap*, caspase; PINX, Pinealectomized; RP, retroperitoneal; SEM, standard error of the mean; ZT, Zeitgeber time.

Thus, the influence of melatonin on the daily expression of core clock components, anti (*Bcl2*), and pro-apoptotic (*Bax*, *Fas*, *Tnfrsf1a, Casp3*, *8*, and *9*) encoding genes was investigated in RP adipose tissue of PINX animals. Our data reveal that melatonin exerts a direct and slight modulatory effect on the synchronization of the circadian apoptosis-related gene expression in RP fat, except for *Tnfrsf1a* mRNA content, which was unaltered by pinealectomy. The modulatory effect of melatonin on the circadian pattern of apoptosis components was evidenced by the alterations in the parameters of cosinor analysis, such as delayed acrophase on *Bax* and *Casp9* expression and increased mesor for *Bcl2* and *Casp8* mRNA contents in the visceral RP adipose tissue of PINX animals and reinforced by the disruption of *Fas* circadian rhythmicity.

Following the circadian oscillatory pattern observed on apoptosis-related gene expression, the total protein content of BAX, BLC2, and CASP3 showed a time-of-day influence, and the treatment (pinealectomy) affected BAX, CASP3, and CASP8 content in RP fat. Besides that, BAX, BLC2, CASP3, and CASP8 contents were higher at the end of the dark phase (ZTs 20–24) in the RP fat of PINX animals compared to their respective controls. Thus, melatonin is possibly involved in the physiological balance of apoptosis in RP adipose tissue since the reduction in melatonin serum concentration induced by PINX led to increased levels of proteins involved in both anti- and pro-apoptotic pathways. Collectively, these findings achieved in RP fat tissue corroborate the well-characterized effect of melatonin as a modulator of apoptosis described in the literature,[17,46] an effect that might be mediated by membrane melatonin receptors,[47] or yet by transcriptional regulation of caspase gene expression through interaction with RORA cis-element.

The existence of an oscillatory circadian clock in the RP adipose tissue was also characterized in this investigation. The melatonin effects as a Zeitgeber for circadian clocks are well described in various tissues, including other types of white adipose tissues.[22,28] Interestingly, the circadian clock expression pattern in RP tissue is not altered by PINX. This suggests that the melatonin-induced effects on transcript and protein content of apoptosis pathways components were independent of core clock modulation. Interestingly, a previous study conducted by our group showed that pinealec-

Table 1. One-way and cosinor analysis for the investigated genes in retroperitoneal adipose tissue of CONTROL and PINX animals

Gene	ONE-WAY ANOVA		COSINOR ANALYSIS							
	CONTROL	PINX	CONTROL				PINX			
	p-value	*p*-value	Mesor	Amplitude	Acrophase	*p*-value	Mesor	Amplitude	Acrophase	*p*-value
Bax	0.0003	0.0015	0.000136 ± 0.000012	0.000105 ± 0.000018	16.19 ± 0.36	0.010	0.000138 ± 0.000016	0.000107 ± 0.000023	18.40 ± 0.44*	0.025
Bcl2	<0.0001	0.0164	0.000105 ± 0.000006	0.000056 ± 0.000009	16.43 ± 0.34	0.009	0.000145 ± 0.000008*	0.000056 ± 0.000012	17.00 ± 0.44	0.023
Bmal1	<0.0001	<0.0001	0.0007 ± 0.0001	0.0007 ± 0.0001	22.29 ± 0.33	0.004	0.0008 ± 0.0001	0.0009 ± 0.0001	22.52 ± 0.29	0.002
Casp3	0.0022	0.0121	0.0000036 ± 0.0000004	0.0000031 ± 0.0000005	15.49 ± 0.38	0.012	0.0000048 ± 0.0000002*	0.0000034 ± 0.0000004	16.12 ± 0.22	0.002
Casp8	<0.0001	0.0336	0.000007 ± 0.0000009	0.0000031 ± 0.0000009	15.33 ± 1.04	0.050	0.0000103 ± 0.0000003*	0.0000047 ± 0.0000004	16.25 ± 0.17	0.001
Casp9	0.0085	0.0297	0.000076 ± 0.000006	0.000055 ± 0.000008	16.34 ± 0.32	0.007	0.000082 ± 0.000006	0.000051 ± 0.00001	17.48 ± 0.38*	0.015
Clock	0.0002	0.0031	–	–	–	0.181	–	–	–	0.265
Cry1	<0.0001	<0.0001	0.0002 ± 0.00000	0.0002 ± 0.0000	18.11 ± 0.25	0.003	0.0002 ± 0.00000	0.0002 ± 0.000000	18.31 ± 0.2	0.001
Fas	0.0419	0.0186	0.000151 ± 0.000004	0.000077 ± 0.000007	17.47 ± 0.17	0.001	–	–	–	–
Nr1d1	<0.0001	<0.0001	0.0029 ± 0.0003	0.0033 ± 0.0004	9.36 ± 0.25	0.002	0.0031 ± 0.0006	0.0036 ± 0.0008	8.43 ± 0.51	0.029
Per2	<0.0001	<0.0001	0.0003 ± 0.0000	0.0004 ± 0.0000	15.36 ± 0.3	0.004	0.0003 ± 0.0000	0.0003 ± 0.0001	15.48 ± 0.36	0.009
Tnfrsf6	<0.0001	0.0004	0.000157 ± 0.00001	0.000079 ± 0.000014	15.29 ± 0.41	0.014	0.000161 ± 0.00001	0.00006 ± 0.000015	16.00 ± 0.53	0.037

One-way ANOVA for each experimental condition (CONTROL and PINX). Cosinor parameters (mesor, amplitude and acrophase) are plotted as means ± SEM; periodicity of 24 h. Student's unpaired, two-tailed *t*-test for cosinor analysis. * $p < 0.05$ *vs* respective Control. No 24 h rhythmicity for cosinor analysis (-). CONTROL and pinealectomized (PINX) animals. *n* = 6–7/ZT/group. ANOVA, Analysis of Variance; *Bax*, *Bcl2* Associated X, Apoptosis Regulator; *Bcl2*, B cell leukemia/lymphoma 2; *Casp*, caspase; SEM, standard error of the mean; ZT, Zeitgeber time.

tomy significantly affects the mRNA content of some circadian clock components in epididymal fat.[22] Together, these findings point out a melatonin-tissue-dependent effect on the oscillatory pattern of the adipocyte circadian clocks. Differences in the expression and distribution of melatonin membrane and/or nuclear receptors might contribute to this differential response between adipose depots.[47] Besides, distinct Zeitgebers (feeding, insulin, i.e.,[48,49]) could maintain circadian clock rhythmicity in RP tissue, even in the absence or reduced melatonin levels.

Notably, the modern lifestyle imposes constant desynchronized conditions that suppress melatonin production by the pineal gland.[32] Sleep deprivation and shift work are associated with increased adiposity and impaired insulin signaling,[33,35,37] alterations that precede the development of diabetes *mellitus*. This disease affects 537 million adults worldwide and is expected to increase to 783 million people in 2045 (https://diabetesatlas.org/atlas/tenth-edition/).

Conclusion

Our findings underscore the importance of melatonin in the daily regulation of apoptosis in RP visceral adipose tissue. This suggests that the desynchronization associated with the modern lifestyle such as sleep deprivation and shift work may negatively impact metabolism, inducing a pro-inflammatory state associated with obesity and metabolic syndrome.

Supporting information

Supplementary material for this article is available at https://doi.org/10.14218/GE.2023.00072.

Table S1. Rat TaqMan® and primer sequences used for Sybr Green® assays for the RT-qPCR analysis.

Table S2. Slope values, efficiencies, correlation coefficients (R^2) and dilutions used for each gene in the rat retroperitoneal tissue.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Conceptualization (TNFC, FBL, and PBS); methodology (TNFC, DSGM, SA, FBL, and PBS); investigation (TNFC, DSGM, SA, and PBS); validation (TNFC, RAPG, FBL, and PBS); formal analysis (TNFC, RAPG, and PBS); visualization (TNFC, RAPG, and

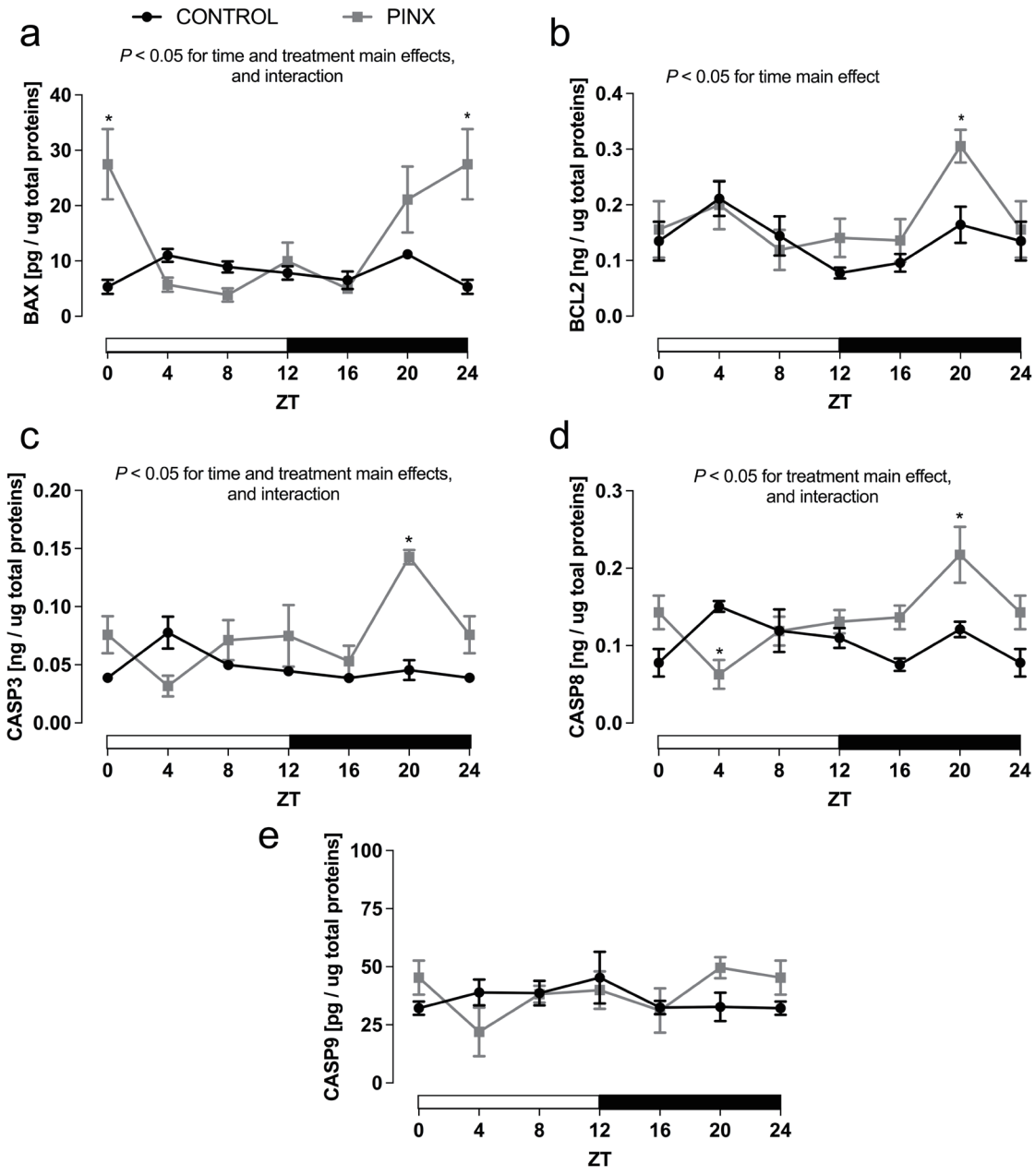

Fig. 3. Effects of pinealectomy in apoptotic-related protein content in rat retroperitoneal adipose tissue (RP). (a-e) BAX, BCL2, CASP3, CASP8, and CASP9 protein content over 24 h. Data presented as means ± SEM. Two-way ANOVA analysis for assessments of time and treatment (pinealectomy) main effects and time-treatment interaction; CONTROL: solid black lines and PINX: solid gray lines. Bonferroni post hoc test, **p* < 0.05 for pairwise comparisons *vs.* the respective controls. The white horizontal bar corresponds to ZTs 0–12 period. The black horizontal bar represents the period of ZTs 12–24. *n* = 4–7/ZT/group. ANOVA, Analysis of Variance; *Bax*, *Bcl2* Associated X, Apoptosis Regulator; *Bcl2*, B cell leukemia/lymphoma 2; *Csap*, caspase; PINX, Pinealectomized; RP, retroperitoneal; SEM, standard error of the mean; ZT, Zeitgeber time.

DSGM); resources (FBL); Funding acquisition (FBL); supervision and project administration (PBS); writing the original draft (TNFC and RAPG); editing the revised version (TNFC, RAPG, FBL, and PBS). All authors read and approved the final manuscript.

Ethics statement

All procedures were conducted according to the National Council for Control of Animals standards and approved by the Committee for Animal Experimentation of the University of Sao Paulo (03/10/2011, No. 129, book 2).

Data sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

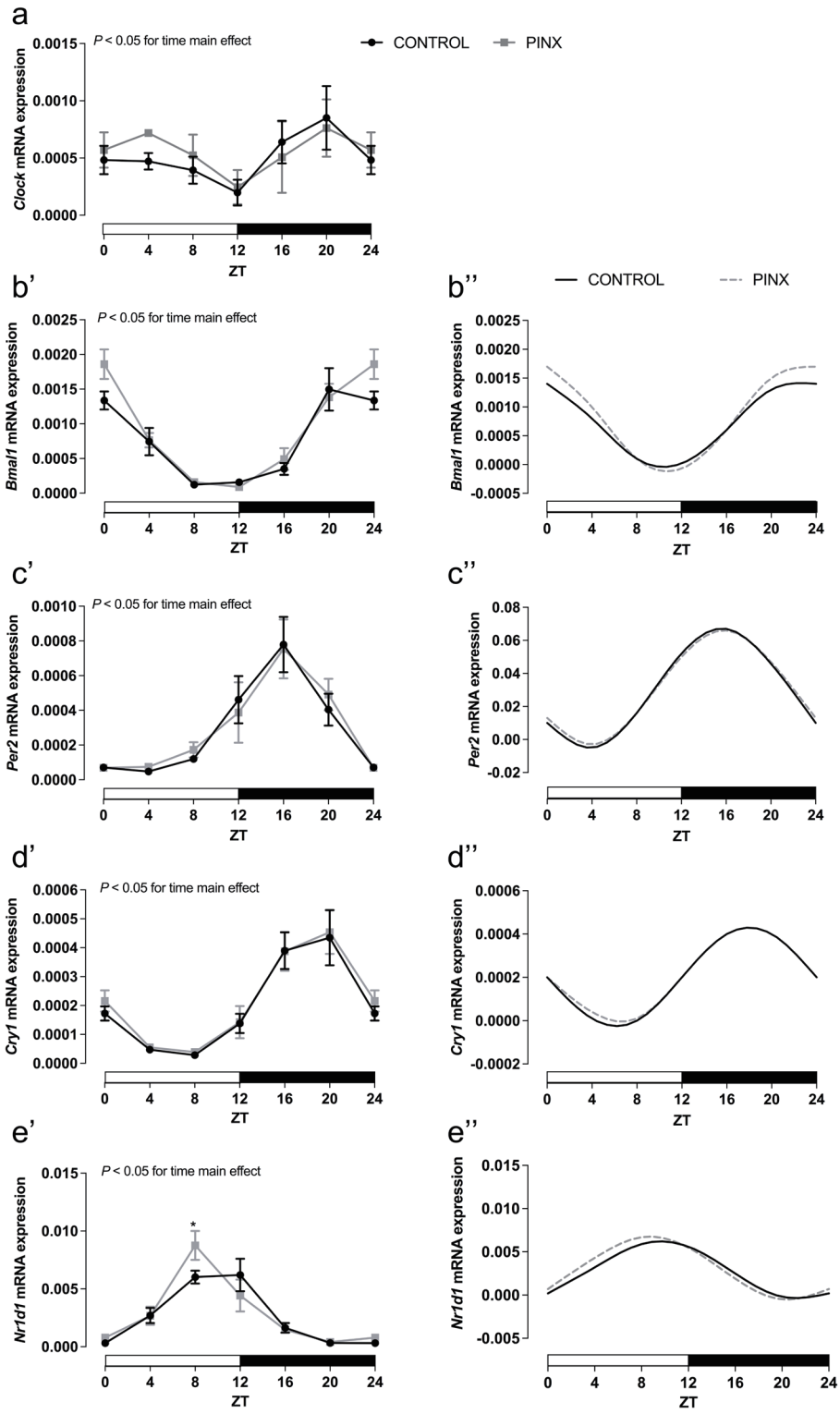

Fig. 4. Effects of pinealectomy in the mRNA expression of circadian clock components of rat retroperitoneal adipose tissue (RP). (a-e) Daily expression of *Clock*, *Bmal1*, *Per2*, *Cry1*, and *Nr1d1*, respectively. Data presented as means ± SEM. (1) Two-way ANOVA analysis for assessments of time and treatment (pinealectomy) main effects and time-treatment interaction; CONTROL: solid black lines and PINX: solid gray lines. Bonferroni post hoc test, **p* < 0.05 for paired comparisons *vs.* respective controls. (2) Cosinor analysis (CONTROL: solid black lines and PINX: dashed gray lines). The white horizontal bar corresponds to ZTs 0–12 period. The black horizontal bar represents the period of ZTs 12–24. *n* = 6–7/ZT/group. ANOVA, Analysis of Variance; PINX, Pinealectomized; RP, retroperitoneal; SEM, standard error of the mean; ZT, Zeitgeber time.

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