Research Article

Melatonin and the heart circadian clock of euglycemic and type 2 diabetic male rats: a transcriptional evaluation

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ABSTRACT

Diabetes increases risk of various comorbidities, including retinopathy, neuropathy, and cardiovascular disease, comprising both ischemic and non-ischemic cardiomyopathy. Cardiac dysfunction during diabetes is associated with perturbations at histologic, metabolic, biochemical and molecular levels. The circadian clock is misaligned in multiple organs during diabetes, including the heart. Such alterations in clock function have been postulated to play a causal role in cardiac dysfunction even though the mechanisms leading to circadian misalignment are currently unknown. Melatonin has been reported to alter heart circadian clock components and its circulating levels are decreased during diabetes. These observations led to the hypothesis that decreased melatonin levels during diabetes could be related to misalignment of the heart clock. To evaluate this hypothesis, in the current study male Wistar and non-obese type 2 diabetic Goto-Kakizaki (GK) rats were given melatonin supplementation in their drinking water during the dark phase (for 12-wks), followed by assessment of clock component and the mRNA expression of the clock-controlled genes in the hearts of these animals. Melatonin supplementation significantly altered mRNA expression of targeted genes in both euglycemic and diabetic rat hearts. Collectively, under the condition of diabetes, the jeopardized pineal melatonin synthesis with misalignment of cardiac circadian clock components may likely mediate heart metabolic dysfunction, and/or even cause cardiovascular diseases.

Keywords: Melatonin, pineal gland, cardiac tissue, clock gene, metabolism, Goto-Kakizaki rats.

1. INTRODUCTION

The prevalence of diabetes *mellitus* (DM) is increasing at alarming rates worldwide. According to the International Diabetes Federation (IDF – 2017), the number of people aging between 20-79 years old that developed diabetes corresponds to 424.9 millions, while the projection for 2045 could reach up to 628.6 million (1). The diabetes is a primary risk factor for development of cardiovascular disease (2-4). During diabetes, some events are directly interlinked to precipitation of diabetic cardiomyopathy, such as: hyperglycemia, insulin resistance, hyperinsulinemia, oxidative stress as well as inflammatory processes (5).

Melatonin is one of the most versatile hormones within the organisms. It can interact with G protein-coupled membrane receptors or nuclear receptors (6-8). This molecule exhibits an amphiphilic property (9) and thus, it presents in any compartment of the cell to act directly as a potent antioxidant molecule and also to activate antioxidative enzymes (10). In addition, Melatonin has been reported to reduce blood pressure, decrease cardiac arrhythmias, attenuate cardiac ischemia-reperfusion injury, and protects against cardiac infarction (11-20).

It has been reported by our group that pineal melatonin synthesis can be further induced by insulin (21, 22), while streptozotocin (STZ)-induced diabetes leads to impairment of pineal melatonin synthesis, highlighting a possible role the rat pineal gland on hyperglycemic condition (23). Zhou and colleagues (2018) also reported that melatonin exerted the beneficial effects that counterbalance the diabetic cardiomyopathy through a mechanism that involves the Syk-mitochondrial complex I-SERCA pathway (24).

Cardiomyocyte circadian clock controls the heart expression of genes associated with metabolism and function (25, 26). The cardiac tissue exhibits a time-of-day dependency of many physiological events throughout the period of 24 h, including adequate response to fatty acid availability, hypertrophic stimuli, prevalence of heart attack in the early hours of the morning, myocardial infarction, and sudden death (27-30). Furthermore, the incidence of cardiovascular disease has been associated with conditions where the heart clock is in constant dyssynchrony (*e.g.* shift workers) (31, 32).

As an internal zeitgeber, melatonin signals to the cellular circadian clock, as described for suprachiasmatic nucleus (SCN), *pars tuberalis*, adipose tissue, liver, skeletal muscle, and the heart (33-39). In the cultured adult rat cardiomyocyte, melatonin treatment did not alter the expression pattern of *Bmal1*, *Nr1d1* and *Dbp*. However, 16 h after the coupling of circadian clock rhythmicity in the cell cultures, a challenge with melatonin avoids the reduction of *Nr1d1* mRNA expression, an effect that seems to be mediated by its interaction to nuclear receptors (8).

Considering the chronobiotic and cardioprotective effects of melatonin, its reduced synthesis during diabetes, and the major role of the heart circadian clock in cardiac physiology, the aim of this investigation is to evaluate whether melatonin supplementation affects the heart circadian clock gene expression in euglycemic (Wistar) and non-obese type 2 diabetic, Goto-Kakizaki (GK) rats, a reliable model in the study of type 2 DM (40).

2. MATERIAL AND METHODS

2.1. Animals.

GK male rats were obtained from Charles River Laboratories International, Inc. (Wilmington, MA, USA), and Wistar male rats from the animal facility of the Institute of Biomedical Sciences at University of São Paulo (ICB-USP), (São Paulo, SP, Brazil). The rodents were housed at the Institute's animal facility, maintained at 25 ± 2˚C under a light-dark cycle of 12 h/12 h (lights on 06:00 am= ZT0), being allowed free access to water and standard rodent chow (Nuvilab1, Curitiba, PR, Brazil) until the 10th week of age. The Committee of Ethics in Animal Experimentation of the ICB-USP (protocol # 86/2016) approved all experimental procedures of this study.

2.1.2. Experimental design.

The animals were then randomly divided into four groups: Wistar: Vehicle (W), Wistar:Melatonin (WMel), GK:Vehicle (GK), and GK:Melatonin (GKMel), in a total of 5-7 animals/ZT/group. Melatonin supplementation was given as previously described (41). In brief, melatonin (Sigma, St Louis, MO, USA) [3 mg/kg body weight] or Vehicle was supplemented in the drinking water of the rats during the dark phase in a daily basis for 12 weeks. At the end of the study, the rats were euthanized by decapitation and the heart ventricles were collected at ZTs 0, 4, 8, 12,16, and 20, respectively. The samples were frozen in liquid nitrogen and stored at -80˚C until further analysis.

2.1.3. RNA extraction and Real-Time qPCR (RT-PCR).

The total RNA extraction from rat heart ventricles using Trizol was performed according to the manufacturer's instruction. Single-strand DNA synthesis was accomplished by the use of MMLV-RT (Promega) from 1 μg total RNA. Gene expression analysis was performed by RT- qPCR using SYBR GREEN (Invitrogen) as fluorescent dye. The 2(-Delta Ct) method was applied as described by Livak and Schmittgen (2001) (42); the Ct value is the calculated cycle number where the fluorescence signal was emitted significantly above background levels. The efficiency/slope obtained values of all investigated genes were close to the optimal values required for the 2(-Delta Ct) analysis (43). The RT-qPCR data are normalized by the housekeeping gene *Peptidylprolyl isomerase A* (*Ppia*). The primer assays for *Adiponectin* (*Adpn*), *Peroxisome proliferator activated receptor alpha* (*Ppara*), and *Peptidylprolyl isomerase A* (*Ppia)* were designed from rat sequences available in the GenBank (Table 1). The primer sequences for all the other investigated genes have been published elsewhere (8, 26, 44-46).

Table1. Rat primer sequences used in RT-qPCR.

Gene/GenBank#	Primers	Sequences	Amp length (bp)
Adipoq (Adpn) NM_144744.3	Forward Reverse	5'-CTCCTGCTTCATTCCTCTCTTC-3' 5'-GTTGTCACTCACTCCTCCATC-3'	85
Ppara NM_013196.1	Forward Reverse	5'–ACTATGGAGTCCACGCATGTGA-3' 5'–GTACGCCAGCTTTAGCCGAA-3'	70
Ppia NM_017101.1	Forward Reverse	5'–AAGACTGAGTGGCTGGATGG-3' 5'–GGCTTCCACAATGCTCATGC-3'	72

2.1.4. Statistical analysis.

The data are plotted as the means ± SEM. One, two or three-way ANOVA was appropriately applied to evaluate the influence of variables such as time, melatonin treatment (referenced as treatment *per se*) and genotype, followed by Bonferroni's post-hoc analyses for pair-wise comparisons, using both GraphPad Prism (GraphPad Prism, version 7.0, San Diego, CA, USA) and Statgraphics Centurion (Statgraphics Centurion, version 17.1.06, Warrenton, VA, USA) software. The null hypothesis of no time, treatment, and genotype effect was rejected at $P < 0.05$. A second mathematical and statistical procedure was applied to investigate the presence of a daily 24 h rhythm (47). The method consists of adjusting a cosine curve to the actual 24 h time series (Cosinor method). The theoretical cosine curve fit was applied to each temporal series using the least-square calculation. The goodness of the fit using the F statistics was then estimated. The null hypothesis tested was that of zero amplitude, that is, no rhythmicity at the assumed frequency (24 h). A significant periodic fit was considered when the *P* value was <0.05. In addition, for each significant cosine fitted data series, the rhythmic parameters as acrophase (time of the peak), mesor (medium value of the adjusted curve) and amplitude (difference between peak value and mesor) of the adjusted curve were calculated (48, 49). When appropriate, Student's *t*-test was also applied to these parameters.

3. RESULTS

3.1 Effects of melatonin in the heart core circadian clock expression.

The mRNA expression of positive elements from the clockwork machinery presented time main effect for *Bmal1*, *Clock* and *Npas2* (Figs. 1A-C); genotype and time main effect for both *Clock* and *Npas2*, with interactions between these factors. In addition, *Npas2* exhibited a treatment-genotype interaction and two pair-wise differences at ZT20, related to genotype (Figures 1B-C). The cosinor analysis revealed an acrophase delay of *Bmal1* in type 2 diabetic (GK) versus euglycemic (W) rats. A phase advance was also observed in melatonin-treated diabetic animals (GKMel) compared to the GK group (Figure 2A, Table 2). As *Bmal1*, the cosinor curve adjustment of the gene *Clock* showed a 24-h rhythmic pattern for all groups, except for the melatonin treatment of Wistar animals (WMel), which had no rhythmicity. Both mesor and acrophase of type 2 diabetic animals of *Clock* had significant differences compared to euglycemic rats (increase and phase delay, respectively). The same pattern was observed for *Npas2*, except that in the latter case, GK had a reduction of the mesor instead an increase. Moreover, a phase delay was found for the acrophase of melatonin treated GK animals (GKMel) compared to euglycemic rats subjected to melatonin treatment (WMel) (Figures 2B-C, Table 2).

Three-way ANOVA of the core clock negative components analyzed in this study has shown a time main effect for expression of all genes, a genotype main effect for *Cry1* and *Cry2* expression and a treatment main effect only for *Nr1d1*. Genotype-time interaction was observed for *Per2* and *Cry1* expression, and treatment-genotype interaction was detected for *Per1* and *Cry1*, whereas interaction between treatment-time was observed for *Per2* and *Nr1d1*. The interaction between the three factors treatment-genotype-time was found only for *Per1* (Figures. 1D-H). Genotype pair-wise comparison for *Per1* at ZT12, and melatonin treatment in GK groups for *Per2* at ZT16 were observed (Figures 1D-E). For *Cry2* mRNA expression, pair-wise differences were found at ZT8

for melatonin treatment in GK (GKMel vs GK) and for genotype (GKMel vs WMel and W vs GK) at ZT12, whereas *Nr1d1* presented a melatonin-induced increase at ZT4 observed in the euglycemic rats (W vs WMel) (Figures 1G-H).

Cosinor analysis depicted the loss of circadian rhythmicity of *Per1* expression in GK animals challenged with melatonin (GKMel), while *Per2* expression exhibited a phase advance in the acrophase in both melatonin-treated groups (WMel and GKMel) compared to their respective controls (W and GK, respectively). The acrophase of *Cry1* expression was phase delayed in GK melatonin-treated rats compared to WMel rats. In addition, both melatonin-treated groups presented no circadian rhythmicity for *Cry2*. No statistical differences were observed in the cosinor analysis of *Nr1d1* mRNA expression (Figures 2D-H, Table 2).

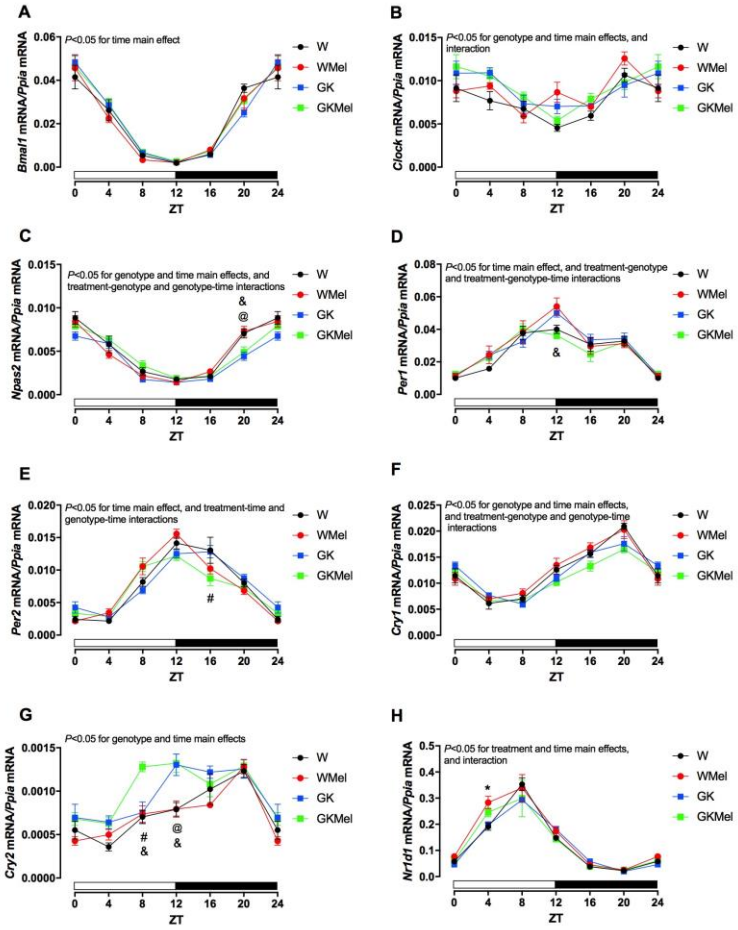

Figure 1. Effects of melatonin on circadian core clock components mRNA expression in euglycemic and type 2 diabetic rat hearts.

*ZT24 was double plotted as ZT0. A-H) mRNA expression of Bmal1, Clock, Npas2, Per1, Per2, Cry1, Cry2 and Nr1d1, respectively, normalized by Ppia mRNA molecules and presented as absolute values of means ± SEM. Three-way ANOVA (P value, time, treatment and interactions are reported within respective graphs). Bonferroni's post hoc test for pair-wise comparisons (respective ZTs) *,@,&,#P<0.05 for W vs WMel, W vs GK, WMel vs GKMel and GK vs GK Mel, respectively. n=5/ZT/group. ZT=Zeitgeber Time. Wistar (W); Melatonin (Mel); Goto-Kakizaki (GK).*

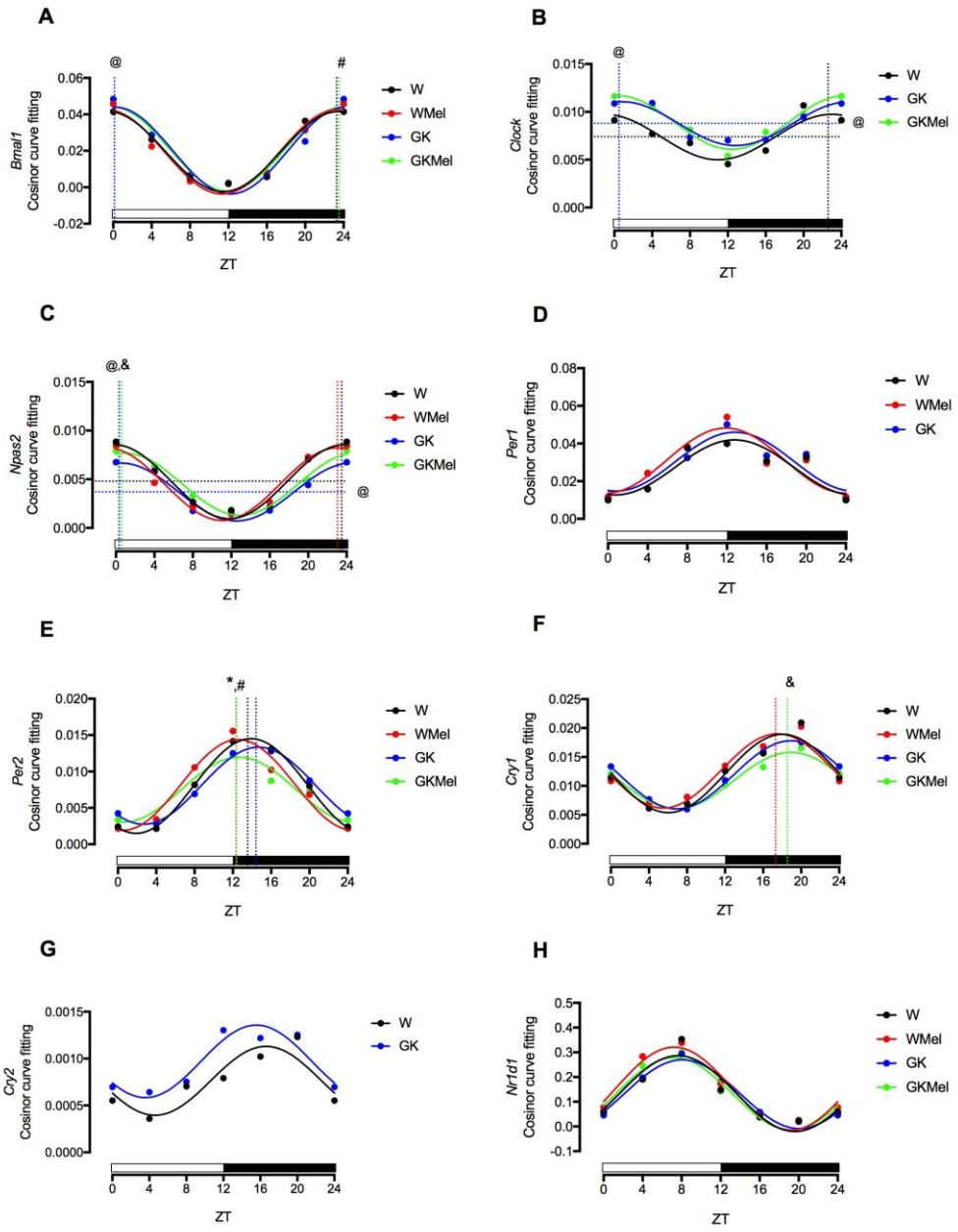

Figure 2. Cosinor analysis of melatonin-mediated effects on circadian core clock components mRNA expression in euglycemic and type 2 diabetic rat hearts.

ZT24 was double plotted as ZT0. A-H) Cosinor curve fit of Bmal1, Clock, Npas2, Per1, Per2, Cry1, Cry2 and Nr1d1, respectively. One-way ANOVA and Cosinor were independently calculated for the experimental groups. Each of the cosinor parameters (mesor, amplitude and acrophase) was compared by Two-way ANOVA or Student's t-test (when appropriate). Mesor (dashed horizontal lines) and acrophase (dashed vertical lines). Bonferroni's post hoc test, $^{*,@,\&,\#}P<0.05$ for W vs WMel, W vs GK, WMel vs GKMel and GK vs GK Mel, respectively. ZT=Zeitgeber Time. Wistar (W); Melatonin (Mel); Goto-Kakizaki (GK).

Table 2. Cosinor analysis (plotted as means ± SEM), periodicity of 24 h.

	W			WMel			GK			GKMel		
	Mesor	Amplitude	Acrophase	Mesor	Amplitude	Acrophase	Mesor	Amplitude	Acrophase	Mesor	Amplitude	Acrophase
Bmal1^{c}	1.96±0.15	2.20±0.20	23.27±0.23	1.94±0.19	2.31±0.25	23.20±0.28	2.01±0.23	2.38±0.30	0.12±0.33$^{@}$	2.09±0.15	2.31±0.19	23.53±0.22$^{\#}$
Clock^{a,c}	0.74±0.04	0.24±0.06	22.59±1.04	-	-	-	0.88±0.02$^{@}$	0.23±0.03	0.49±0.32$^{@}$	0.89±0.02	0.28±0.03	0.23±0.25
Npas2^{a,c}	0.48±0.03	0.38±0.04	23.47±0.26	0.45±0.03	0.37±0.04	23.04±0.26	0.37±0.02$^{@}$	0.30±0.03	0.31±0.27$^{@}$	0.45±0.02	0.32±0.02	0.56±0.20$^{\&}$
Per1	2.74±0.27	1.46±0.36	12.46±1.04	3.10±0.29	1.72±0.39	11.53±0.58	3.04±0.27	1.56±0.36	12.48±1.00	-	-	-
Per2^{b,c}	0.80±0.01	0.65±0.02	13.54±0.08	0.82±0.05	0.62±0.07	12.33±0.27*	0.80±0.01	0.53±0.02	14.41±0.09	0.75±0.06	0.45±0.08	12.38±0.47$^{\#}$
Cry1^{c}	1.22±0.08	0.68±0.11	18.03±0.34	1.26±0.07	0.64±0.10	17.31±0.31	1.19±0.01	0.59±0.02	18.54±0.06	1.09±0.04	0.49±0.05	18.54±0.22$^{\&}$
Cry2	0.08±0.01	0.04±0.01	16.37±0.56	-	-	-	0.10±0.00	0.04±0.01	15.34±0.38	-	-	-
Nr1d1	13.32±1.92	15.33±2.82	7.38±0.39	15.32±1.26	16.77±1.88	7.13±0.23	13.06±0.89	13.92±1.30	8.04±0.20	13.16±1.32	14.66±1.96	7.14±0.28
Pdk4^{a,b}	23.59±0.58	13.65±0.86	17.03±0.13	-	-	-	20.53±0.78$^{@}$	15.96±1.17	17.29±0.15	15.65±0.35$^{\#}$	8.66±0.52$^{\#}$	17.20±0.12
Ucp3^{a,b}	0.83±0.05	0.49±0.08	19.06±0.34	-	-	-	0.98±0.02$^{@}$	0.82±0.06$^{@}$	19.21±0.16	0.98±0.02	0.67±0.03	19.26±0.10
Pparα	-	-	-	x	x	x	x	x	x	x	x	x
Acot1	0.43±0.07	0.37±0.10	18.30±0.54	x	x	x	0.45±0.03	0.19±0.04	19.00±0.48	-	-	-
Cd36	x	x	x	x	x	x	x	x	x	x	x	x
Adpn^{a,b}	0.22±0.03	0.14±0.03	22.11±1.02	0.31±0.02*	0.17±0.03	21.43±0.44	0.12±0.01$^{@}$	0.05±0.01	21.53±0.51	0.13±0.00$^{\&}$	0.05±0.01$^{\&}$	22.25±0.31

*Two-way ANOVA or Student's t-test (when appropriated), P<0.05 for each of the parameters: mesor (a), amplitude (b) and acrophase (c). Bonferroni's post hoc test, *P<0.05 vs W, $^{@}$P<0.05 vs W, $^{\&}$P<0.05 vs WMel, $^{\#}$P<0.05 vs GK. (x) no period variation of the means in One-way ANOVA. (-) no circadian rhythmicity in the cosinor analysis. Wistar (W); Melatonin (Mel); Goto-Kakizaki (GK). For optimization of cosinor statistical analysis, the mesor and amplitude values of all groups are multiplied by 100. n=5.*

3.2 Melatonin-induced alterations in metabolism clock-controlled gene expression in the heart.

The mRNA content of clock-controlled genes involved in glucose and fatty acid metabolism, and uncoupling of ADP oxidative phosphorylation in the heart was also investigated in euglycemic and non-obese type 2 diabetic animals treated or not with melatonin (Figure 3). *Pdk4*, *Acot1*, and *Adpn* showed treatment, genotype and time main effects in the three-way ANOVA with interactions of treatment-time for *Pdk4*, genotype-time, treatment-time and treatment-genotype-time for *Acot1* and genotype-time and treatment-genotype for *Adpn* expression (Figures 3A-C). Moreover, *Pdk4* mRNA expression also revealed melatonin treatment induced pair-wise differences at ZT16 regardless the genotype, and at ZT20 for GK animals (GKMel vs GK). *Ucp3* and *Cd36* expression presented genotype and time main effects, with treatment-time and genotype-time interactions for *Ucp3*, whereas *Cd36* showed a treatment-genotype interaction. The pair-wise comparison revealed genotype differences for both genes (ZT 16 for *Ucp3* and ZTs 4 and 12 for *Cd36*), but only in the euglycemic rats (Figures 3D-E). The content of *Pparα* mRNA presented time main effect and treatment-genotype-time interaction (Figure 3F).

The cosinor analysis of metabolic clock-controlled genes showed a melatonin-induced arrhythmic circadian expression of *Pdk4*, *Acot1* and *Ucp3* in the hearts of euglycemic rats (WMel) (Figures 4A-B, and D, Table 2). Melatonin also induced a loss of the circadian rhythmicity of *Acot1* in the hearts of type 2 diabetic rats (GKMel) (Figure 4B, Table 2). The melatonin supplementation evoked significant alterations in the mesor values of *Adpn* (increase, Figure 4C, Table 2) and *Pdk4* (reduction, Figure 4A, Table 2) expression in euglycemic and GK rats (WMel and GKMel), respectively. In the latter, the altered amplitude in cosine curve was observed for GK animals supplemented with melatonin (GKMel) (Figure 4A, Table 2). Genotype-induced alteration of mesor was also seen in *Pdk4* and *Adpn*, as well as, mesor and amplitude of *Ucp3* (Figure 4, Table 2). *Pparα* and *Cd36* mRNA expression did not present a circadian pattern of oscillation in the heart of animals subjected to the experimental conditions (Table 2).

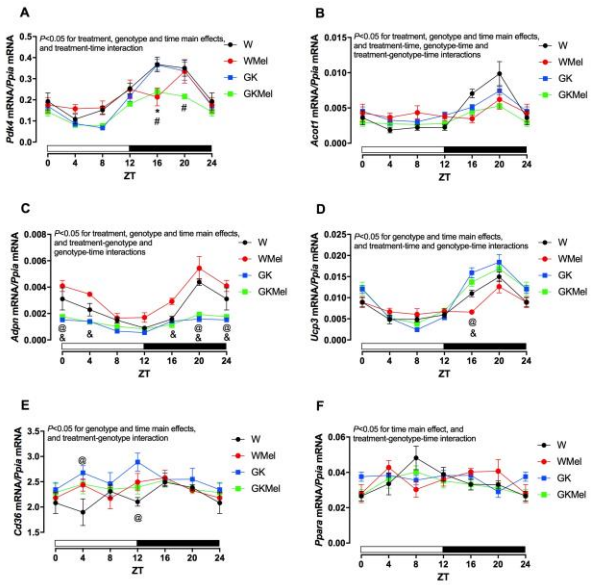

Figure 3. Effects of melatonin on clock-controlled genes mRNA expression in euglycemic and type 2 diabetic rat hearts.

ZT24 was double plotted as ZT0. A-F) mRNA expression of Pdk4, Acot1, Adpn, Ucp3, Cd36 and Ppara, respectively, normalized by Ppia mRNA molecules and presented as absolute values of means ± SEM. Three-way ANOVA (P value, time, treatment and interactions are reported within respective graphs). Bonferroni's post hoc test for pair-wise comparisons (respective ZTs) $*,@,\&,\#P<0.05$ for W vs WMel, W vs GK, WMel vs GKMel and GK vs GK Mel, respectively. n=5/ZT/group. ZT=Zeitgeber Time. Wistar (W); Melatonin (Mel); Goto-Kakizaki (GK).

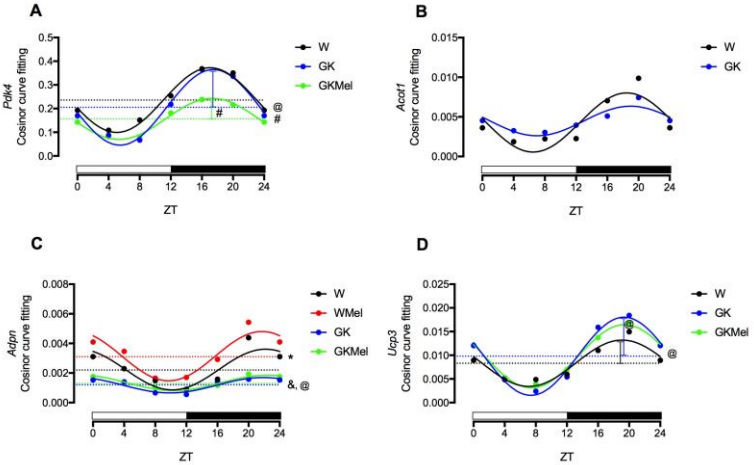

Figure 4. Cosinor analysis of melatonin-mediated effects on clock-controlled genes mRNA expression in euglycemic and type 2 diabetic rat hearts.

ZT24 was double plotted as ZT0. A-D) Cosinor curve fit of Pdk4, Acot1, Adpn, Ucp3, respectively. One-way ANOVA and Cosinor were independently calculated for the experimental groups. Each of the cosinor parameters (mesor, amplitude and acrophase) was compared by Two-way ANOVA or Student's t-test (when appropriate). Mesor (dashed horizontal lines), amplitude (vertical line interval) and acrophase (dashed vertical lines). Bonferroni's post hoc test,

**,@,&,#P<0.05 for W vs WMel, W vs GK, WMel vs GKMel and GK vs GK Mel, respectively. ZT=Zeitgeber Time. Wistar (W); Melatonin (Mel); Goto-Kakizaki (GK).*

4. DISCUSSION

Several studies reported the effects of melatonin in the cardiovascular system ranging from reduction of blood pressure to protective effects of the heart after ischemia-reperfusion events (17-20, 50). The therapeutic benefits of melatonin in pathological conditions as the diabetic cardiomyopathy have also been described (24). During diabetes, the pineal melatonin synthesis is reduced as demonstrated in STZ-induced diabetic rats (23), as well as, in type 2 diabetic Goto-Kakizaki rats (51). This reduction might directly impair the systemic effects of melatonin, including signaling to the heart, which could lead to dyssynchrony of the cardiac clockwork machinery.

Indeed, a phase advance of core clock and metabolic clock-controlled genes were reported in STZ-induced diabetes rat heart (52). Moreover, an increase in the mRNA expression of fatty acid and glucose-related metabolic genes as *Acot1*, *Cd36*, *Pdk4*, and *Ucp3* has been also reported in STZ-induced diabetic mice hearts (53). The few data related to the heart circadian clock and type 2 DM was obtained by a transcriptome analysis, which revealed significant alterations of core clock and clock-controlled genes in the heart of GK rats including *Bmal1*, *Npas2*, *Per2*, *Dbp* and *Tef* (54). Our results partially reinforce the correlation between type 2 diabetes and core clock/clock-regulated genes mRNA expression in the heart, since genotype main effects or genotype interactions between time and/or treatment were significant for all targeted genes, except *Bmal1* and *Nr1d1*. These data contrast the overexpression of *Ucp3*, *Pdk4*, *Acot1* and the acrophase alteration of core clock components observed in type 1 DM (52, 53), suggesting a differential metabolic adaptation of the heart according to the diabetes features of type 1 or 2.

The effects of melatonin in the heart and cardiovascular system circadian clocks of hypertensive rats induced a phase-shift of *Per2* and *Bmal1* mRNA expression (37, 38). In cultured rat cardiomyocytes, we reported that administration of melatonin did not alter the mRNA expression of *Bmal1*, *Nr1d1* and *Dbp*, but specifically at time 16 h of the culture it avoided the decrease of *Nr1d1* and induced its expression for up to 12 h after the melatonin challenge. The blockage of melatonin membrane receptors (MT1 and MT2) did not alter the melatonin-induced alterations mentioned above, suggesting the non participation of melatonin membrane receptors in this response (8). The differences between whole heart and cell type-specific melatonin effects might be associated with cell types (cardiomyocytes, fibroblasts and endothelial cells) and by 3D structure interaction of the organ, which is not the case when cells are in the culture. In fact, the 3D dependence in organ function was already reported in central nervous system structures, as the pituitary (55, 56). However, the relationship between heart function and its cellular organization for the melatonin effects requires further investigation.

Melatonin supplementation abolished or altered the rhythmic expression pattern of core clock components (observed through cosinor analysis) including *Clock, Cry2* and *Per2*, metabolic clock-controlled genes involved in glucose, fatty acid metabolism and energy generation through the electron transport chain oxidative phosphorylation including *Pdk4*, *Ucp3* and *Acot1*, and increased the mesor of *Adpn* in the heart of euglycemic (W) rats. Although *Nr1d1* did not present any significant alteration in the cosinor analysis, an obvious effect of melatonin treatment was observed for this gene. Once again, cellular and whole heart responses to melatonin should be taken into consideration. The type 2 diabetic animals also reflected the effects of melatonin

supplementation observed in the core circadian clock and clock-controlled metabolic genes. GKMel group showed a phase advance for *Bmal1* and *Per2*, an abolishment of *Per1* and *Cry2* circadian rhythmicities, whereas *Pdk4* mesor had a significant reduction. Collectively, these data suggest that at least some of the heart circadian clock components as well as relevant glucose and fatty acid clock-related genes are independent targets for melatonin, which corroborates the literature (cell and heart), either in euglycemic or type 2 DM conditions.

As already mentioned, the heart circadian clock has a crucial role on the regulation of the heart metabolism and function (57, 58). The elevated fatty acid availability found in diabetes can modulate the heart metabolism, which in turn is regulated by the heart circadian clock (53). Thus, misalignment of heart metabolism and circadian clock, additionally influenced by melatonin reduction, might be partially related to development of cardiac dysfunction, which is often observed during diabetes.

In summary, melatonin-mediated alterations in the mRNA expression of some core clock and metabolic clock-related genes in hearts of euglycemic and type 2 diabetic rats were observed in the current study. Melatonin treatment altered or even evoked loss of the transcript's circadian rhythmicity. Genotype-related differences in mRNA expression of genes in the cardiac tissue of both Wistar and Goto-Kakizaki rats were also identified with or without melatonin supplementation. The results provide additional information on the potential associations of melatonin and diabetic cardiomyopathy, particularly related to the circadian genes as well as their regulatory genes expression in heart locally. This information is valuable to use melatonin as a potential therapeutic agent for increased cases of diabetes.

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AUTHORSHIP

José Sinésio-Jr – melatonin supplementation, data acquisition/analysis; Paula Bargi-Souza – data acquisition/analysis/interpretation, drafting of the manuscript, critical revision of the manuscript; Raphael Afonso Matos, Eduardo de Almeida Leite, Daniella do Carmo Buonfiglio, Jéssica Andrade-Silva and Lívia Clemente Motta-Teixeira – melatonin supplementation; Rui Curi, Martin Elliot Young and José Cipolla-Neto – critical revision of the manuscript; Rodrigo Antonio Peliciari-Garcia – data acquisition/analysis/interpretation, drafting of the manuscript, critical revision of the manuscript and approval of the article.

CONFLICT INTEREST

The authors declare no conflict of interest.

REFERENCES

1. IDF (2017) International diabetes federation atlas.
2. Battiprolu PK, *et al.* (2010) Diabetic cardiomyopathy: Mechanisms and therapeutic targets. *Drug Discov. Today Dis. Mech.* **7** (2): e135-e143.
3. Echouffo-Tcheugui JB, *et al.* (2018) Diabetes *mellitus* and cardiogenic shock complicating acute myocardial infarction. *Am. J. Med.* **131** (7): 778-786 e771.
4. Bening C, *et al.* (2018) Impact of diabetes *mellitus* on the contractile properties of the left and right atrial myofilaments. *Eur. J. Cardiothorac. Surg.* **54** (5): 826-831.
5. Palomer X, *et al.* (2018) Emerging actors in diabetic cardiomyopathy: Heartbreaker biomarkers or therapeutic targets? *Trends Pharmacol. Sci.* **39** (5): 452-467.
6. Dubocovich ML, *et al.* (2003) Molecular pharmacology, regulation and function of mammalian melatonin receptors. *Front. Biosci.* **8**: d1093-1108.
7. Witt-Enderby PA, *et al.* (2003) Melatonin receptors and their regulation: Biochemical and structural mechanisms. *Life Sci.* **72** (20): 2183-2198.
8. Peliciari-Garcia RA, *et al.* (2011) Expression of circadian clock and melatonin receptors within cultured rat cardiomyocytes. *Chronobiol. Int.* **28** (1): 21-30.
9. Kanikkannan N, *et al.* (2001) Evaluation of skin sensitization potential of melatonin and nimesulide by murine local lymph node assay. *Eur. J. Pharm. Sci.* **14** (3): 217-220.
10. Reiter RJ, *et al.* (2016) Melatonin as an antioxidant: Under promises but over delivers. *J. Pineal Res.* **61** (3): 253-278.
11. Baker J & Kimpinski K (2018) Role of melatonin in blood pressure regulation: An adjunct anti-hypertensive agent. *Clin. Exp. Pharmacol. Physiol.* **45** (8): 755-766.
12. Bertuglia S & Reiter RJ (2007) Melatonin reduces ventricular arrhythmias and preserves capillary perfusion during ischemia-reperfusion events in cardiomyopathic hamsters. *J. Pineal Res.* **42** (1): 55-63.
13. Genade S, *et al.* (2008) Melatonin receptor-mediated protection against myocardial ischaemia/reperfusion injury: Role of its anti-adrenergic actions. *J. Pineal Res.* **45** (4): 449-458.
14. Grossman E, *et al.* (2006) Melatonin reduces night blood pressure in patients with nocturnal hypertension. *Am. J. Med.* **119** (10): 898-902.
15. Lochner A, *et al.* (2018) Melatonin and cardioprotection against ischaemia/reperfusion injury: What's new? A review. *J. Pineal Res.* **65** (1):e 12490.
16. Sahna E, *et al.* (2008) Melatonin protects myocardium from ischemia-reperfusion injury in hypertensive rats: Role of myeloperoxidase activity. *Clin. Exp. Hypertens.* **30** (7): 673-681.
17. Sallinen P, *et al.* (2007) The effect of myocardial infarction on the synthesis, concentration and receptor expression of endogenous melatonin. *J. Pineal Res.* **42** (3): 254-260.
18. Simko F & Paulis L (2007) Melatonin as a potential antihypertensive treatment. *J. Pineal Res.* **42** (4): 319-322.
19. Yang Y, *et al.* (2014) A review of melatonin as a suitable antioxidant against myocardial ischemia-reperfusion injury and clinical heart diseases. *J. Pineal Res.* **5 7**(4): 357-366.
20. Zhou H, *et al.* (2018) Protective role of melatonin in cardiac ischemia-reperfusion injury: From pathogenesis to targeted therapy. *J. Pineal Res.* **64** : e12471: 1-21.
21. Garcia RA, *et al.* (2008) Insulin modulates norepinephrine-mediated melatonin synthesis in cultured rat pineal gland. *Life Sci.* **82** (1-2):108-114.

22. Peliciari-Garcia RA, *et al.* (2010) Insulin temporal sensitivity and its signaling pathway in the rat pineal gland. *Life Sci.* **87** (5-6):169-174.
23. Amaral FG, *et al.* (2014) Melatonin synthesis impairment as a new deleterious outcome of diabetes-derived hyperglycemia. *J. Pineal Res.* **57** (1): 67-79.
24. Zhou H, *et al.* (2018) Melatonin therapy for diabetic cardiomyopathy: A mechanism involving syk-mitochondrial complex i-serca pathway. *Cell Signal.* **47**: 88-100.
25. Bray MS, *et al.* (2008) Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression. *Am. J. Physiol. Heart Circ. Physiol.* **294** (2): H1036-1047.
26. Young ME, *et al.* (2001) Intrinsic diurnal variations in cardiac metabolism and contractile function. *Circulation Res.* **89** (12): 1199-1208.
27. Durgan DJ & Young ME (2010) The cardiomyocyte circadian clock: Emerging roles in health and disease. *Circulation Res.* **106** (4): 647-658.
28. Durgan DJ, *et al.* (2006) The circadian clock within the cardiomyocyte is essential for responsiveness of the heart to fatty acids. *J. Biol. Chem.* **281** (34): 24254-24269.
29. Durgan DJ, *et al.* (2011) Evidence suggesting that the cardiomyocyte circadian clock modulates responsiveness of the heart to hypertrophic stimuli in mice. *Chronobiol. Int.* **28** (3): 187-203.
30. Young ME (2016) Temporal partitioning of cardiac metabolism by the cardiomyocyte circadian clock. *Exp. Physiol.* **101** (8): 1035-1039.
31. Chellappa SL, *et al.* (2019) Impact of circadian disruption on cardiovascular function and disease. *Trends Endocrinol. Metab.* **S1043-2760** (19): 30131-30136. https://doi.org/10.1016/j.tem.2019.07.008
32. Young ME & Bray MS (2007) Potential role for peripheral circadian clock dyssynchrony in the pathogenesis of cardiovascular dysfunction. *Sleep Med.* **8** (6): 656-667.
33. Agez L, *et al.* (2007) Melatonin affects nuclear orphan receptors mRNA in the rat suprachiasmatic nuclei. *Neuroscience.* **144** (2): 522-530.
34. Dardente H, *et al.* (2003) Melatonin induces cry1 expression in the *pars tuberalis* of the rat. *Brain Res. Mol. Brain Res.* **114** (2): 101-106.
35. Owino S, *et al.* (2016) Melatonin signaling controls the daily rhythm in blood glucose levels independent of peripheral clocks. *PloS one.* **11** (1): e0148214.
36. Wagner GC, *et al.* (2007) Melatonin induces gene-specific effects on rhythmic mRNA expression in the *pars tuberalis* of the siberian hamster (phodopus sungorus). *Eur. J. Neurosci.* **25** (2): 485-490.
37. Zeman M & Herichova I (2013) Melatonin and clock genes expression in the cardiovascular system. *Front. Biosci. (Schol. Ed).* **5**: 743-753.
38. Zeman M, *et al.* (2009) Effect of rhythmic melatonin administration on clock gene expression in the suprachiasmatic nucleus and the heart of hypertensive TGR(mRen2)27 rats. *J. Hypertens. Suppl.* **27** (6): S21-26.
39. Vriend J & Reiter RJ (2015) Melatonin feedback on clock genes: A theory involving the proteasome. *J. Pineal Res.* **58** (1): 1-11.
40. Kuwabara WMT, *et al.* (2017) Comparison of Goto-Kakizaki rats and high fat diet-induced obese rats: Are they reliable models to study type 2 diabetes *mellitus*? *PloS one* **12** (12): e0189622.
41. Mendes C, *et al.* (2013) Adaptations of the aging animal to exercise: Role of daily supplementation with melatonin. *J. Pineal Res.* **55** (3): 229-239.

42. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* **25** (4): 402-408.
43. Dussault AA & Pouliot M (2006) Rapid and simple comparison of messenger RNA levels using real-time PCR. *Biol. Proced. Online.* **8**: 1-10.
44. Peliciari-Garcia RA, *et al.* (2018) Repercussions of hypo and hyperthyroidism on the heart circadian clock. *Chronobiol. Int.* **35** (2): 147-159.
45. Peliciari-Garcia RA, *et al.* (2016) Interrelationship between 3,5,3 -triiodothyronine and the circadian clock in the rodent heart. *Chronobiol. Int.* **33** (10): 1444-1454.
46. Young ME, *et al.* (2001) Clock genes in the heart: Characterization and attenuation with hypertrophy. *Circulation Res.* **88** (11):1142-1150.
47. Nelson W, *et al.* (1979) Methods for cosinor-rhythmometry. *Chronobiologia* **6** (4): 305-323.
48. Cornelissen G (2014) Cosinor-based rhythmometry. *Theor. Biol. Med. Model.* **11**: 16.
49. Cornelissen G, *et al.* (1980) Chronobiometry with pocket calculators and computer systems. *Ric. Clin. Lab.* **10** (2): 333-385.
50. Baker J & Kimpinski K (2018) Role of melatonin in blood pressure regulation: An adjunct anti-hypertensive agent. *Clin. Exp. Pharmacol. Physiol.* **45**: 755-766.
51. Frese T, *et al.* (2009) Pineal melatonin synthesis is decreased in type 2 diabetic Goto-Kakizaki rats. *Life Sci.* **85** (13-14): 526-533.
52. Young ME, *et al.* (2002) Alterations of the circadian clock in the heart by streptozotocin-induced diabetes. *J. Mol. Cell. Cardiol.* **34** (2): 223-231.
53. Peliciari-Garcia RA, *et al.* (2016) Altered myocardial metabolic adaptation to increased fatty acid availability in cardiomyocyte-specific clock mutant mice. *Biochim. Biophys. Acta.* **1861** (10): 1579-1595.
54. Sarkozy M, *et al.* (2016) Transcriptomic alterations in the heart of non-obese type 2 diabetic Goto-Kakizaki rats. *Cardiovasc. Diabetol.* **15** (1): 110.
55. Bargi-Souza P, *et al.* (2015) Loss of basal and TRH-stimulated Tshb expression in dispersed pituitary cells. *Endocrinology* **156** (1): 242-254.
56. Mollard P, *et al.* (2012) A tridimensional view of pituitary development and function. *Trends Endocrinol. Metab.* **23** (6): 261-269.
57. Martino TA & Young ME (2015) Influence of the cardiomyocyte circadian clock on cardiac physiology and pathophysiology. *J. Biol. Rhythms.* **30** (3): 183-205.
58. Young ME (2017) Circadian control of cardiac metabolism: Physiologic roles and pathologic implications. *Methodist Debakey Cardiovasc. J.* **13** (1): 15-19.

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