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Cadmium as an endocrine disruptor. Correlation with anterior pituitary redox and circadian clock mechanisms and prevention by melatonin.

Vanesa Jiménez-Ortega,* Pilar Cano Barquilla,* Pilar Fernández-Mateos,*† Daniel P. Cardinali,‡§ and Ana I. Esquifino.*

* Department of Biochemistry and Molecular Biology III, Faculty of Medicine, Universidad Complutense, Madrid 28040, Spain.

† Department of Cellular Biology, Faculty of Medicine, Universidad Complutense, Madrid 28040, Spain

‡ Department of Teaching & Research, Faculty of Medical Sciences, Pontificia Universidad Católica Argentina, 1107 Buenos Aires, Argentina.

§ Department of Physiology, Faculty of Medicine, University of Buenos Aires, 1121 Buenos Aires, Argentina.

Corresponding Author:

Dr. D.P. Cardinali,
Director, Departamento de Docencia e Investigación,
Facultad de Ciencias Médicas,
Pontificia Universidad Católica Argentina,
Av. Alicia Moreau de Justo 1500, 4º piso
1107 Buenos Aires, Argentina.
Tel: +54 11 43490200 ext 2310
E-mail: danielcardinali@uca.edu.ar; danielcardinali@fibertel.com.ar

Running title: Cadmium effects in rat adenohypophysis

Abstract

To examine the effect a low dose of Cd as an endocrine disruptor, male Wistar rats received CdCl₂ (5 ppm Cd) in drinking water or drinking water alone. After 1 month, rats were euthanized at one of six time intervals around the clock and the 24-h pattern of adenohypophysial PRL synthesis and release, lipid peroxidation and redox enzyme and metallothionein (MT) gene expression was examined. Cd suppressed 24-h rhythmicity in expression of PRL gene and in circulating PRL by increasing them at early photophase only, in correlation with an augmented pituitary lipid peroxidation and redox enzyme expression. CdCl₂ treatment effectively disrupted the 24-h variation in expression of every pituitary parameter tested except for MT-3. In a second experiment the effect of melatonin (3 µg/mL drinking water) was assessed at early photophase, the time of maximal endocrine disrupting effect of Cd. Melatonin treatment blunted the effect of Cd on PRL synthesis and release, decreased Cd-induced lipid peroxidation and counteracted the effect of Cd on expression of most redox enzymes. A third experiment was performed to examine whether melatonin could counteract Cd-induced changes in the 24-h pattern of pituitary circadian clock gene expression and plasma PRL, LH, TSH and corticosterone levels. Rats receiving CdCl₂ exhibited a suppressed daily rhythm of *Clock* expression and a significant disruption in daily rhythms of pituitary *Bmal1*, *Per1*, *Per2*, *Cry1* and *Cry2*. The co-administration of melatonin restored rhythmicity in *Clock* and *Bmal1* expression but shifted the maxima in pituitary *Per1*, *Cry1* and *Cry2* expression to the scotophase. Melatonin also counteracted the effect of Cd on 24-h rhythmicity of circulating PRL, LH, TSH and corticosterone. The results underline the occurrence of a significant endocrine disruptor effect of a low dose of Cd. Generally melatonin counteracted the effects of Cd and ameliorated partly the circadian disruption caused by the pollutant.

Keywords: cadmium; prolactin; redox state; circadian rhythms; gene expression; melatonin; LH; TSH; corticosterone

Introduction

The heavy metal cadmium (Cd) is one of the most toxic industrial and environmental metals and acts as an endocrine disruptor in humans and rodents [1]. Cd is ranked eighth in the top 20 hazardous substances; it is released into water as a by-product of smelting, into air by combustion of coal and oil, and into soils as impurities. Cd main uses are for nickel–cadmium battery manufacture, pigments and plastic stabilizers [2].

The neuroendocrine and neurobehavioral disturbances in animals and humans caused by endocrine disruptors are suspected to be implicated in the recent declining fertility in developed countries [1,3]. Cd is recognized as an endocrine disruptor that modifies, among other, prolactin (PRL) secretion in a number of species including humans [4-11]. Cd is readily absorbed and retained in the pituitary gland of rats [12,13] and affects lactotroph cell activity causing biochemical, genomic and morphological changes [7]. In the rat, the effect of orally administered CdCl₂ on PRL release is dose- and time-dependent

[14]. A high dose of Cd inhibits PRL release both in vivo and in vitro [8,14-16] while low doses of Cd are stimulatory at early morning hours and inhibitory later on [9].

The objective of the present study was to examine the effect a low dose of Cd as an endocrine disruptor in male rats. Specifically we aimed to answer the following questions: (i) is the 24-h variation in pituitary PRL synthesis and release affected by Cd? (ii) does the effect of Cd on PRL synthesis correlate with pituitary redox status as assessed by measuring lipid peroxidation and redox enzyme and metallothionein (MT) gene expression; (iii) is the 24-h expression of circadian clock genes in the anterior pituitary modified by CdCl₂ treatment? (iv) can Cd act as an endocrine disruptor to affect 24-h variations in plasma LH, TSH and corticosterone levels? (v) is melatonin effective to palliate the activity of Cd as an endocrine disruptor? The rationale to employ melatonin relied on its demonstrable chronobiotic [17,18] and cytoprotective activities [19]. We had previously reported melatonin efficacy to prevent the stimulatory effect of Cd on anterior pituitary lipid peroxidation and mRNA levels for nitric oxide synthase (NOS)-1 and -2 and heme oxygenase-1 (HO-1), when examined at two time intervals in the 24-h span [20].

Materials and methods

Animals and experimental design

Male Wistar rats (45 days of age) were kept under standard conditions of controlled light (12:12 h light/dark schedule; lights on at 0800 h: Zeitgeber time, ZT, 00:00) and temperature (22 ± 2 C). Three experiments were carried out as follows.

In Experiment 1 rats received CdCl₂ (8 µg/ml, 5 ppm Cd) in drinking water or drinking water alone for 1 month (control). The dose of Cd was calculated by using the body surface area (BAS) normalization method [21] taking in consideration the tolerable limit in humans proposed by the World Health Organization (WHO) (1 µg/day) [22]. After 1 month, groups of 6-8 rats were euthanized by decapitation under conditions of minimal stress at 6 different time intervals (every 4 h) throughout a 24-h cycle, starting at ZT 01:00. At night intervals, animals were killed under red dim light. The brains were rapidly removed and the adenohypophysis was quickly dissected out. Trunk blood was collected and the plasma was frozen at -70C until further processing.

In Experiment 2 rats were divided into 4 groups and treated for 1 month as follows: (a) CdCl₂ (8 µg/ml drinking water); (b) CdCl₂ (8 µg) plus melatonin (3 µg) per ml of drinking water; (c) melatonin (3 µg/ml drinking water); (d) drinking water alone (control). The stock solution of melatonin was prepared in 50 % ethanol, the final ethanol concentration in drinking water being 0.015 %. Cd-administered animals and controls received 0.015 % ethanol in drinking water. Nocturnal water consumption did not differ among the experimental groups in any of the experiments. After 1 month, groups of 6-8 rats were euthanized by decapitation under conditions of minimal stress at ZT 01:00. The brains were rapidly removed and the adenohypophysis was quickly dissected out. Trunk blood was collected and the plasma was frozen at -70C until further processing.

In Experiment 3, four groups of rats treated for 1 month as for Experiment 2 were euthanized at the same 6 time intervals as in Experiment 1. The brains were rapidly removed and the adenohypophysis was quickly dissected out. Trunk blood was collected and the plasma was frozen at -70 C until further processing.

The care and use as well as all procedures involving animals were approved by the Institutional Animal Care Committee, Faculty of Medicine, Complutense University, Madrid. The study was in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health and the Guide for the Care and Use of Laboratory Animals [23].

Real-time Quantitative Polymerase Chain Reaction (qPCR)

Total RNA extraction was performed using the RNeasy protect mini kit and was analyzed using QuantiTec SYBR green kit (Qiagen, Hilden, Germany). The iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories SA; Madrid) was used to synthesize cDNA from 1 µg of total RNA, according to the manufacturer's protocol. The house keeping gene β -actin was used as a constitutive control for normalization. Reactions were carried out in the presence of 200 nM of specific primers for genes of PRL, NOS-1 and -2, heme oxygenase (HO)-1 and -2, Cu/Zn and Mn superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GRd) and metallothionein (MT)-1 and -3, as well as for the circadian genes *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1* and *Cry2*. Primers were designed using Primer3 software (The Whitehead Institute, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are shown in Table 1.

PCR reactions were carried out in an Eppendorf RealPlex Mastercycler (Eppendorf AG, Hamburg, Germany). The real-time qPCR reaction program included a 94 C enzyme activation step for 2 min followed by 40 cycles of 95 C denaturation for 15 s, 60 C annealing for 30 s and 72 C extension for 30 s. Detection of fluorescent product was carried out at the end of the 72 C extension period.

Serial dilutions of cDNA from control adenohypophysis were used to perform calibration curves in order to determine amplification efficiencies. For the primers used there were no differences between transcription efficiencies, the amount of initial cDNA in each sample being calculated by the $2^{-\Delta\Delta Ct}$ method [24]. All samples were analyzed in triplicate and in three different measures. Fractional cycle at which the amount of amplified target becomes significant (Ct) was automatically calculated by the PCR device.

To estimate whether treatment or time of day modified the expression of anterior pituitary β -actin, PCR employing serial dilutions of this housekeeping gene was performed. In this study Ct did not vary significantly as a function of treatment or of time of day, indicating the validity to employ β -actin as a housekeeping gene.

Lipid peroxidation

Lipid peroxidation was measured in the anterior pituitary by the thiobarbituric acid reactive substances (TBARS) assay as described elsewhere [20]. Supernatant absorbance (535 nm) was measured. Results were expressed as (absorbance/mg of protein in treated sample)/(absorbance/mg of protein in control sample) x 100.

Plasma hormone levels

Plasma PRL and LH levels were measured by a homologous specific double antibody RIA, using materials kindly supplied by the NIDDK's National Hormone and Pituitary Program. The intra- and interassay coefficients of variations were 6-9%. Sensitivities of the RIAs were 45 pg/mL for both hormones using the NIDDK rat PRL RP-3 and rat LH-RP-3, respectively. Results were expressed as ng/mL (PRL) or pg/mL (LH) [25,26]. Plasma testosterone levels were measured using a commercial kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA). Sensitivity of the assay was 0.2 ng/mL and the intraassay coefficient of variation was 5%, as previously described [25]; results were expressed as ng/mL. Plasma corticosterone was assayed by a specific RIA obtained from Labor Diagnostika Nord GmbH & Co., Nordhorn, Germany. The intra- and inter-assay coefficients of variation were 6 and 8%, respectively. Sensitivity of the RIA was 25 ng of corticosterone/ml; results were expressed as ng/mL.

Data analysis

After verifying normality of distribution of data, the statistical analysis of the results was performed by a one-way or a two-way factorial analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests or by Student's t tests, as stated. A Cosinor analysis of the mean values at each time series (n= 6) was performed to calculate the acrophase (the maximum of the cosine function fit to the experimental data) and amplitude (half the difference between maximal and minimal values of the derived cosine curve) of the 24-h rhythms. Statistical significance of the derived cosine curves was tested against the null hypothesis (i.e. amplitude = 0) [27]. To calculate the mesor (the statistical estimate of the 24-h time series mean) and R^2 statistical validity the total number of individual values was considered. P values lower than 0.05 were taken as evidence for statistical significance.

Results

Figure 1 depicts the effect of Cd on 24-h pattern in expression of anterior pituitary PRL gene and of plasma PRL levels in male rats. When analyzed as a main factor in a factorial ANOVA, Cd augmented expression of PRL gene by 24% ($p < 0.03$). A significant interaction "Cd x time of day" was found ($p < 0.01$), i.e., the stimulatory effect of Cd on gene expression was seen during the photophase, in particular at morning hours (Fig. 1). The changes in gene expression brought about by Cd became reflected in a significant increase of circulating PRL at early morning only. In controls only a Cosinor analysis indicated that the 24 h changes in PRL gene expression and plasma PRL followed a significant sinusoidal pattern, with acrophases at ZT 09:36 and 14:23, respectively (Table 2).

Data from Fig. 2 and 3 summarize the daily changes in gene expression of enzymes in the redox pathway and in lipid peroxidation in rat adenohypophysis after the administration of Cd. When analyzed as a main factor in the factorial ANOVA, CdCl₂ treatment augmented expression of adenohypophysial NOS-1, HO-2 and Cu/Zn SOD by 40,

22 and 25% ($p < 0.01$) and of lipid peroxidation by 24% ($p < 0.05$) whereas it decreased expression of GPx by 20% ($p < 0.01$). Significant interactions “Cd treatment x time of day” were found for pituitary expression of every redox parameter tested ($p < 0.01$) except for MT-3 expression, indicating that Cd effectively disrupted 24-h variation of redox enzyme expression. Generally, Cd treatment augmented expression of redox enzymes and lipid peroxidation at early morning with varying effects on gene expression and absence of significant changes in lipid peroxidation at other time intervals (Fig. 2 and 3). In the case of MT-1 gene expression, CdCl₂ treatment decreased it at late scotophase (Fig. 3).

The data were further analyzed by the Cosinor analysis summarized in Tables 3 and 4. CdCl₂ treatment phase-advanced by 6-9 h (from late to early photophase) HO-1 and GRd mRNA expression (Table 3) whereas it phase delay by 9-10 h (from early to late scotophase) that of Cu/Zn SOD and Mn SOD (Table 4). CdCl₂ increased significantly the amplitude of NOS-1, NOS-2 and Cu/Zn SOD expression. The 24-h variations in HO-2 (Table 3) and MT-1 expression (Table 4) did not follow a sinusoidal pattern in controls while lipid peroxidation did not follow a sinusoidal pattern in either group (Table 4).

Figures 4 and 5 summarize the results of Experiment 2, designed to assess whether the concomitant administration of melatonin could counteract CdCl₂ effects on PRL synthesis and release and redox parameters when assessed at ZT 0100, i.e., the time interval of maximal effects of Cd, as per Experiment 1. As shown in Fig. 4, melatonin treatment was effective to blunt the effect of Cd on PRL synthesis and circulating levels. Moreover, a significant effect of melatonin in decreasing PRL synthesis and release was shown when it was examined as a main factor in a factorial ANOVA ($p < 0.001$, Fig. 1). Melatonin was also effective to counteract the effect of Cd on expression of NOS-1, NOS-2, Cu/Zn SOD, Mn SOD and catalase and on lipid peroxidation when assessed at ZT 0100 (Fig. 5). The analysis of melatonin as a main factor in the factorial ANOVA indicated a significant effect in decreasing NOS-2 expression and in augmenting HO-2, catalase, GRd, MT-1 and MT-3 expression ($p < 0.02$) (Fig. 5).

Figures 6 and 7 and Tables 5 and 6 summarize the results of Experiment 3, aimed to examine the effect of melatonin on Cd-induced changes in the 24-h pattern of pituitary circadian clock gene expression and plasma PRL, LH, TSH and corticosterone levels. Significant time-related changes in clock gene expression in anterior pituitary were found in controls, *Clock* and *Bmal1* expression peaking during scotophase (acrophases at ZT 18:53 and 19:11, respectively) while that of *Per1* and *Per2*, and of *Cry1* and *Cry2*, at the beginning and the middle of photophase (ZT 02:10 and 02:16, and ZT 10:20 and 11:34, respectively, Table 5). As indicated by the significant interactions “Cd x time of day” in the factorial ANOVA, rats receiving CdCl₂ showed a suppression of daily rhythm in *Clock* expression and a significant disruption in daily rhythms of the remaining circadian genes ($p < 0.01$). The Cosinor analysis indicated that only the expression of *Bmal1*, *Per1* and *Cry2* fit a sinusoidal pattern in the anterior pituitary of CdCl₂-treated rats, showing acrophases at late scotophase (ZT 00:03), late photophase (ZT 11:44) and middle scotophase (ZT 19:33) (Table 5).

The co-administration of melatonin restored the sinusoidal pattern of circadian genes resembling controls only for *Clock* and *Bmal1* expression (acrophases at ZT 19:28 and 21:42, respectively). Maxima in pituitary expression of *Per1*, *Per2*, *Cry1* and *Cry2* shifted to the scotophase in Cd-melatonin-treated rats (acrophases at ZT 13:43, 13:58, 16:08 and 18:34, Table 5). As compared to controls, rats receiving melatonin alone showed significant changes in acrophases of *Per1* and *Per2* expression (from early to late photophase) and of *Cry1* expression (from late photophase to early scotophase).

The changes in the daily variations of PRL, LH, TSH and corticosterone are depicted in Fig. 6. In controls the four hormones tested exhibited significant 24-h variations with acrophases at ZT 15:40 (PRL), 19:06 (LH), 05:59 (TSH) and 08:22 (corticosterone). CdCl₂ treatment disrupted the 24-h rhythmicity of the 4 hormones examined as indicated by the significant interactions “Cd x time of day” in the factorial ANOVA ($p < 0.01$). The Cosinor analysis (Table 6) indicated that the acrophases of TSH and corticosterone rhythms were almost inverted in CdCl₂ rats from mid photophase to mid scotophase as compared to controls. The daily variations in plasma PRL and LH did not follow a sinusoidal pattern. The co-administration of melatonin was effective to restore rhythmicity of plasma PRL, LH and TSH with acrophases similar to those found in controls while in the case of corticosterone a phase delay on about 7 h was observed (from ZT 08:22 to 15:10, Table 6). For every hormone, the amplitude of the rhythm was significantly higher in Cd + melatonin-treated rats than in controls or Cd-treated rats ($p < 0.01$, Table 5). Rats receiving melatonin alone showed a significant delay in acrophase of plasma corticosterone rhythm (from ZT 08:22 to 15:10) and significant increases in amplitude of LH, TSH and corticosterone rhythms (Table 6).

Discussion

The questions posed in the Introduction may now be answered. A low dose of Cd has a demonstrable endocrine disruptor effect in rats as far as PRL synthesis and release, as shown by the suppression of 24-h variations in both parameters. CdCl₂ treatment also resulted in the disruption of 24-h rhythmicity of plasma LH, TSH and corticosterone. In the anterior pituitary, Cd increased significantly expression of PRL gene during the photophase, particularly at morning hours. Concomitantly, Cd augmented pituitary lipid peroxidation and expression of a number of pituitary redox enzyme genes, again at early photophase. Cd treatment had a significant disruptive effect on pituitary circadian clock gene expression making it feasible that genomic effects of the pollutant on cellular circadian clock mechanisms may underlie some or all of the above mentioned changes. The co-administration of melatonin counteracted the effect of Cd on 24-h rhythmicity of plasma PRL, LH, TSH and corticosterone levels. Melatonin was also effective to counteract the promoting effect of Cd on lipid peroxidation and expression of redox enzymes, when assessed at the time of maximal expression of pituitary effects (ZT 01:00), and to restore partly Cd-induced changes in pituitary circadian clock gene expression.

In the present study, and with the aim of mimicking human exposure, we gave Cd in the drinking water for 1 month at a low concentration (5 ppm). A single dose of Cd, rather

than a dose-response design, was chosen because of the experimental design and the number of animals employed. The dose of Cd was selected by the BAS normalization method [21] to resemble the acceptable limit put forth by WHO (1 µg/day for a lifespan of 60 yr) [22]. It mimicked exposure level in moderately to heavily polluted areas or under occupational exposure conditions [2].

The effect of low doses of Cd on plasma PRL levels had been reported previously [9], but the mechanisms by which Cd alters PRL secretion remained unsettled. In vitro, Cd causes oxidative damage of lactotrophs and inhibits PRL release by increasing radical oxygen species (ROS) in a mitochondrial-dependent way and by inducing apoptosis [15,16,28]. These in vitro effects of Cd on pituitary cells were prevented by antioxidants.

In the present study Cd treatment augmented significantly lipid peroxidation presumably by augmenting nitric oxide (NO), which is also a relevant modulator of pituitary hormone release [29,30]. The expression of both NOS isoforms, NOS-1 and NOS-2 increased in Cd-treated rats, particularly during the photophase. Indeed, Cd affects NO production in different tissues [31,32] and several hypotheses have been entertained about the role of NO in Cd toxicity ranging from mediation of toxicity [33] to non-effects [34] or protective effects [35,36].

Since ROS generation is a continuous and physiological phenomenon, the cells possess efficient antioxidant systems that protect them from oxidative damage (for reviews, see [37,38]). HO has an important role in controlling the redox state of the cell by functioning as a rate-limiting enzyme in the heme degradation process which results in the formation of equimolar amounts of two potent antioxidants, i.e. carbon monoxide and biliverdin [37]. In the present study, and concomitant with the disrupting effect that Cd has on PRL synthesis during the photophase, an increase in HO-1 and HO-2 expression was observed. The results suggest that an overexpression of mRNA for HO-1 and HO-2 may be counteracting the oxidative damage caused by excess NO.

The detoxification of ROS in cells involves the cooperative action of intracellular antioxidant enzymes like Cu/Zn SOD, Mn SOD and catalase [39]. In addition, GPx and GRd help to maintain adequate levels of reduced glutathione, a major antioxidant defense of the cells. The changes in redox enzyme mRNA expression brought about by CdCl₂ treatment in the present study can be interpreted in terms of a compensatory increase caused by the augmented oxidative load. Since ROS play a role in cellular signaling processes, including transcription factors activities such NF-κB and AP-1, the increase of free radicals caused by Cd would allow regulation of gene transcription via modulation of redox-sensitive transcription factors [39,40].

Melatonin, the major secretory product of the pineal gland, participates in diverse physiological functions signaling not only the length of the night (the chronobiotic effect, [17,18]) but also enhancing ROS scavenging, the immune response and cytoprotection [19]. At least two previous observations supported a protective role of melatonin on Cd-induced pituitary changes in rats. Poliandri et al. demonstrated that in the anterior pituitary, melatonin administration prevented Cd-induced increases in lipid peroxidation and in mRNA levels for NOS-1, NOS-2 and HO-1 [20]. Miler et al. reported, in a study

aimed to assess whether Cd-induced oxidative stress in pituitary gland was reversed by removing the pollutant source, that in rats exposed to 5 ppm Cd the augmented expression of pituitary HO-1 was prevented by the concomitant administration of melatonin [6]. In both studies, the dose of melatonin used was similar to that employed herein.

When assessed at early morning, i.e., at the time interval at which Cd treatment showed maximal effects, melatonin treatment was effective to suppress the effect of Cd on PRL synthesis and circulating levels. Melatonin was also effective to counteract the promoting effect of Cd on lipid peroxidation and the concomitant expression of NOS-1, NOS-2, Cu/Zn SOD, Mn SOD and catalase. Moreover, a significant effect of melatonin in decreasing PRL synthesis and release and in augmenting HO-2, catalase, GRd, MT-1 and MT-3 expression was found. Since the binding of Cd to MTs facilitates the tissue deposit of the metal, any toxic effect of Cd could be modified by compounds able to modulate the synthesis of MT proteins [41]. It should be noted that the effect of melatonin depicted in Figures 5 and 6 could be due, rather than to actual blunting, to a phase-shifting effect of the methoxyindole. Further studies are needed to clarify this point.

In the present study the effect of melatonin on expression of antioxidant enzymes given by Cd was generally inhibitory except for GPx. Thus the beneficial effect of melatonin to counteract the augmented lipid peroxidation induced by CdCl₂ appears to be more related to the decrease in expression of NOS-1 and NOS-2 and to the augmentation of MT-1 and MT-3, since most of Cd in the body is bound to small, cysteine-rich, MTs [42]. Further studies are needed to verify whether the actual levels of MTs are also increased by melatonin.

In the present study significant time-related changes in circadian clock gene expression were found in the anterior pituitary of control rats. The peaks of pituitary *Clock* and *Bmal1* expression were in antiphase with those of *Per1* and *Per2* expression. *Per1* and *Per2* peaked at the beginning of the light phase while *Clock* and *Bmal1* peaked during scotophase. In the case of *Cry1* and *Cry2* their maximal expression took place in the middle of photophase, with a phase delay of 4-8 h as compared to *Per1* or *Per2*. Rats receiving CdCl₂ exhibited a disruption in expression of every pituitary clock gene tested as well as suppression of rhythmicity of *Clock* and *Bmal1* expression. In addition to the daily variations of PRL, those of LH, TSH and corticosterone became disrupted by Cd.

The co-administration of melatonin restored rhythmicity in *Clock* and *Bmal1* expression but shifted the maxima in *Per1*, *Cry1* and *Cry2* expression to the scotophase. Melatonin was also effective to restore rhythmicity of plasma PRL, LH and TSH with acrophases similar to those found in controls while in the case of corticosterone a phase delay of about 7 h was observed. In addition, melatonin has a significant effect by itself, i.e., for every hormone the amplitude of the rhythm was significantly higher in melatonin-treated rats. Collectively, these observations are compatible with the chronobiotic role of melatonin proposed by others [17,18]. However, it is important to realize that findings from laboratory rats cannot be directly extrapolated to humans, since in a nocturnally active species like the rat high circulating melatonin is associated with neuronal and

locomotor activity, the opposite to what is seen in a diurnally active species like the human.

One important limitation of the present descriptive study is that gene expression needs to be completed with Western blotting analysis of the proteins assessed in order to obtain a better understanding of Cd-melatonin interactions on pituitary redox enzymes and circadian clock genes. It is also necessary to establish whether the changes in amplitude and timing of 24-h rhythms in gene expression are due to effects exerted on the hypothalamic suprachiasmatic nuclei or at the level of some of their output(s). Previous results from this Laboratory indicated that Cd administration disrupted the 24-h rhythmicity and overall expression of redox enzyme and clock genes in rat medial hypothalamus, an effect partly counteracted by melatonin administration [43,44]. As reported herein on PRL, LH, and TSH and corticosterone daily rhythms, the chronic exposure of rats to similar low doses of Cd affected the circadian variation of pituitary hormone release [11,45,46]. Collectively this information would allow predicting the occurrence of changes in behavioral and other physiological rhythms after Cd. However, there is no recent information on this point, this important subject remaining unanswered.

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Disclosure statement

The authors declared no conflict of interest.

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Table 1. Sequence of the primers used for real-time PCR

Gene	Primers		Product Size (bp)
β - Actin	Forward	ctctcttcagccttccttc	99
	Backward	ggctctttacggatgtcaacg	
PRL	Forward	ttcttggggaagtgtggtc	86
	Backward	tcatcagcaggaggagtgtc	
NOS-1	Forward	atcggcgctccgtgactactg	92
	Backward	tcctcatgtccaaatccatcttcttg	
NOS-2	Forward	tggcctccctctggaaaga	93
	Backward	gggtggccatgatggtcacat	
HO-1	Forward	tgctcgcatgaacactctg	123
	Backward	tcctctgtcagcagtgcc	
HO-2	Forward	agcaaagttggccttacaa	84
	Backward	gtttgtgctgccctcacttc	
Cu/Zn SOD	Forward	gggtgtccacgagaaacaag	98
	Backward	caatcacaccacaagccaag	
Mn SOD	Forward	aaggagcaaggtcgcttaca	94
	Backward	acacatcaatccccagcagt	
Catalase	Forward	gaatggctatggctcacaca	100
	Backward	caagttttgatgccctgggt	
GPx1	Forward	tgcaatcagttcggacatc	120
	Backward	cacctcgacttctcaaaca	
GRd	Forward	atcaaggagaagcgggatg	96
	Backward	gcgtagccgtggatgactt	
MT-1	Forward	gttgctccagattcaccaga	105
	Backward	gcatttgcagttcttgag	
MT-3	Forward	ctgctcggacaaatgcaaa	96
	Backward	ttggcacacttctcacatcc	
Clock	Forward	tgccagctcatgagaagatg	98
	Backward	catcgctggctgtgtaatg	
Bmal1	Forward	ccgtggaccaaggaagtaga	102
	Backward	ctgtgagctgtgggaagggtt	
Per1	Forward	ggctccggtacttctctttc	106
	Backward	aataggggagtggtcaaagg	
Per2	Forward	acacctcatgagccagacat	99
	Backward	cttgactcttgccactggt	
Cry1	Forward	cagttgcctgtttcctgacc	91
	Backward	cagtcggcgtcaagcagt	
Cry2	Forward	attgagcggatgaagcagat	103
	Backward	ccacagggtgactgaggtct	

Table 2. Cosinor analysis of the effect of cadmium on 24-h pattern in adenohypophyseal PRL gene expression and plasma PRL levels in rats. Data from Fig. 1.

	Mesor	Amplitude	Acrophase (ZT)	R ² , probability
<u>Control</u>				
PRL relative gene expression	0.96 ± 0.06	0.17 ± 0.03	09:36 ± 01:13	0.61, p<0.02
Plasma PRL levels (ng/mL)	9.06 ± 1.11	3.36 ± 0.56	14:23 ± 02:18	0.72, p< 0.01
<u>Cadmium</u>				
PRL relative gene expression	1.19 ± 0.06*	-	-	0.25, N.S.
Plasma PRL levels (ng/mL)	8.63 ± 0.98	-	-	0.11, N.S.

Shown are the means ± SEM. * p< 0.05 vs. control, Student's t test. R² values and their probability are also shown. Mean values at each time series in Fig. 1 (n= 6) was used to calculate the acrophase and amplitude of rhythms. To calculate the mesor and R² statistical validity the total number of individual values was considered (n= 43-46). For further statistical analysis see text.

Table 3. Cosinor analysis of the effect of Cd on 24-hour changes in adenohipophysial expression of mRNA for NOS-1, HO-1, GPx, NOS-2, HO-2 and GRd in rats.

	Mesor (relative gene expression)	Amplitude (relative gene expression)	Acrophase (ZT)	R², probability
<u>Control</u>				
NOS-1	1.04 ± 0.20	0.17 ± 0.02	03:05 ± 02:12	0.81, p< 0.001
HO-1	0.97 ± 0.10	0.28 ± 0.05	07:44 ± 01:10	0.86, p< 0.001
GPx	1.43 ± 0.09	0.23 ± 0.04	15:15 ± 02:16	0.32, p< 0.05
NOS-2	0.91 ± 0.12	0.47 ± 0.06	04:23 ± 01:34	0.62, p< 0.02
HO-2	1.10 ± 0.07	-	-	0.18, N.S.
GRd	1.26 ± 0.12	0.27 ± 0.03	11:06 ± 01:05	0.84, p< 0.001
<u>Cadmium</u>				
NOS-1	1.46 ± 0.20**	0.47 ± 0.10**	05:40 ± 02:43	0.79, p< 0.01
HO-1	0.95 ± 0.11	0.21 ± 0.05	01:19 ± 02:34*	0.52, p< 0.02
GPx	1.16 ± 0.10**	0.26 ± 0.03	19:32 ± 02:56	0.77, p< 0.01
NOS-2	0.95 ± 0.08	0.96 ± 0.07**	03:50 ± 02:14	0.63, p< 0.01
HO-2	1.34 ± 0.08*	-	-	0.11, N.S.
GRd	1.31 ± 0.11	0.31 ± 0.05	02:11 ± 01:37*	0.72, p< 0.01

Shown are the means ± SEM. * p< 0.05, ** p< 0.01 vs. control, Student's t test. R² values and their probability are also shown. Mean values at each time series in Fig. 2 (n= 6) was used to calculate the acrophase and amplitude of rhythms. To calculate the mesor and R² statistical validity the total number of individual values was considered (n= 42-48). For further statistical analysis see text.

Table 4. Cosinor analysis of the effect of Cd on 24-hour changes in expression of mRNA for Cu/Zn SOD, Mn SOD, catalase, MT-1 and MT-3 and in lipid peroxidation in rat adenohypophysis.

	Mesor (relative gene expression)	Amplitude (relative gene expression)	Acrophase (ZT)	R², probability
<u>Control</u>				
Cu/Zn SOD	1.27 ± 0.11	0.24 ± 0.03	13:18 ± 01:12	0.85, p< 0.001
Mn SOD	1.42 ± 0.14	0.33 ± 0.07	13:13 ± 02:45	0.83, p< 0.001
Catalase	1.31 ± 0.15	0.31 ± 0.04	08:43 ± 02:43	0.71, p< 0.01
MT-1	1.07 ± 0.11	-	-	0.16, N.S.
MT-3	0.96 ± 0.08	0.16 ± 0.03	04:22 ± 01:58	0.57, p< 0.02
Lipid peroxidation (%)	91 ± 7	-	-	0.28, N.S.
<u>Cadmium</u>				
Cu/Zn SOD	1.57 ± 0.10*	0.39 ± 0.04**	22:56 ± 01:17*	0.64, p< 0.01
Mn SOD	1.58 ± 0.31	0.26 ± 0.07	23:26 ± 02:32*	0.81, p< 0.001
Catalase	1.40 ± 0.32	0.27 ± 0.07	03:33 ± 02:56	0.69, p< 0.01
MT-1	0.99 ± 0.02	0.33 ± 0.08	05:54 ± 02:12	0.98, p< 0.001
MT-3	1.04 ± 0.22	0.21 ± 0.06	05:57 ± 01:54	0.84, p< 0.001
Lipid peroxidation (%)	113 ± 7*	-	-	0.21, N.S.

Shown are the means ± SEM. * p< 0.05, ** p< 0.01 vs. control, Student's t test. R² values and their probability are also shown. Mean values at each time series in Fig. 3 (n= 6) was used to calculate the acrophase and amplitude of rhythms. To calculate the mesor and R² statistical validity the total number of individual values was considered (n= 42-47). For further statistical analysis see text.

Table 6. Cosinor analysis of the effect of melatonin on Cd-induced changes in 24-h pattern of circulating PRL, LH, TSH and corticosterone levels.

	Mesor	Amplitude	Acrophase (ZT)	R ² , probability
<u>Control</u>				
PRL	9.53 ± 1.11	3.30 ± 0.41	15:40 ± 02:45	0.87, p< 0.001
LH	409 ± 37	163 ± 13	19:06 ± 02:17	0.96, p< 0.001
TSH	2.35 ± 0.32	0.50 ± 0.04	05:59 ± 01:45	0.43, p< 0.03
Corticosterone	490 ± 43 ^a	121 ± 13	08:22 ± 02:02 ^a	0.83, p< 0.001
<u>Cadmium</u>				
PRL	8.27 ± 0.91	-	-	0.07, N.S.
LH	615 ± 44 ^a	-	-	0.21, N.S.
TSH	2.79 ± 0.32	0.34 ± 0.01	16:56 ± 01:59 ^a	0.54, p< 0.03
Corticosterone	337 ± 45	65 ± 8	16:10 ± 01:19	0.64, p< 0.02
<u>Cadmium + melatonin</u>				
PRL	7.33 ± 1.13	6.70 ± 0.87 ^a	14:45 ± 01:40	0.91, p< 0.001
LH	393 ± 47	208 ± 23	18:15 ± 01:57	0.86, p< 0.001
TSH	3.16 ± 0.32	1.11 ± 0.23 ^b	03:52 ± 01:45	0.50, p< 0.03
Corticosterone	278 ± 32	234 ± 15	15:10 ± 01:23	0.88, p< 0.001
<u>Melatonin</u>				
PRL	4.23 ± 0.51 ^a	2.72 ± 0.16	17:08 ± 02:15	0.84, p< 0.001
LH	349 ± 42	213 ± 32	18:52 ± 02:58	0.83, p< 0.001
TSH	2.28 ± 0.42	1.31 ± 0.21 ^b	04:30 ± 02:54	0.56, p< 0.03
Corticosterone	309 ± 34	187 ± 12 ^b	15:02 ± 02:05 *	0.72, p< 0.01

Mesor and amplitude values are expressed as ng/mL (TSH, testosterone, corticosterone) or pg/mL (LH). Shown are the means ± SEM. Superscripts denote significant differences in a one way ANOVA and post-hoc Bonferroni's tests as follows: ^a p< 0.01 vs. the remaining groups; ^b p< 0.01 vs. control and cadmium groups. R² values and their probability are also shown. Mean values at each time series in Fig. 7 (n= 6) was used to calculate the acrophase and amplitude of rhythms. To calculate the mesor and R² statistical validity the total number of individual values was considered (n= 44-46). For further statistical analysis see text.

Figure 1

Effect of cadmium on 24-h pattern in adenohypophysial PRL gene expression and plasma PRL levels in rats. The rats received CdCl_2 (8 $\mu\text{g}/\text{ml}$, 5 ppm Cd) in drinking water or drinking water alone for 1 month (control). Groups of 6-8 rats were euthanized by decapitation at 6 different time intervals throughout a 24 h cycle. mRNA levels encoding the PRL gene and plasma PRL levels were measured as described in the text. Shown are the means $\pm$ SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples and the means $\pm$ SEM of plasma PRL levels. Asterisks denote significant differences (* $p < 0.05$, ** $p < 0.02$) as compared to control in Student's t tests performed at every time interval. The analysis of data by Cosinor is summarized in Table 2. For further statistical analysis, see text.

Figure 2

Effect of cadmium on 24-h pattern in adenohipophysial expression of mRNA for NOS-1, NOS-2, HO-1, HO-2, Gpx and GRd in rats. For experimental details see legend to Fig. 1. mRNA levels encoding the enzymes were measured as described in the text. Shown are the means $\pm$ SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. Asterisks denote significant differences (* $p < 0.05$, ** $p < 0.02$) as compared to control in Student's t tests performed at every time interval. The analysis of data by Cosinor is summarized in Table 3. For further statistical analysis, see text.

Figure 3

Effect of cadmium on 24-h pattern in expression of mRNA for Cu/Zn SOD, Mn SOD, catalase, MT-1 and MT-3, and lipid peroxidation in rat adenohypophysis. For experimental details see legend to Fig. 1. Shown are the means $\pm$ SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. Lipid peroxidation was assessed by the thiobarbituric acid reactive substances procedure as described in Methods. Asterisks denote significant differences (* $p < 0.05$, ** $p < 0.02$) as compared to control in Student's *t* tests performed at every time interval. The analysis of data by Cosinor is summarized in Table 4. For further statistical analysis, see text.

Figure 4

Effect of melatonin on Cd-induced in adenohypophysial PRL gene expression and plasma PRL levels in rats. Rats were divided into 4 groups and treated for 1 month as follows: (i) CdCl_2 (8 $\mu\text{g}/\text{ml}$ drinking water); (ii) CdCl_2 (8 μg) plus melatonin (3 μg) per ml drinking water; (iii) melatonin (3 μg) per ml drinking water; (iv) drinking water alone (control). Groups of 6-8 rats were euthanized by decapitation at ZT 1. mRNA levels encoding the PRL gene and plasma PRL levels were measured as described in the text. Shown are the means $\pm$ SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples and the means $\pm$ SEM of plasma PRL levels. ^a $p < 0.01$ vs. the remaining means, ^b $p < 0.01$ vs. control and Cd administered animals, one way ANOVA, Bonferroni's tests. For further statistical analysis, see text.

Figure 5

Effect of melatonin on Cd-induced changes in expression of mRNA for NOS-1, NOS-2, HO-1, HO-2, Cu/Zn SOD, Mn SOD, catalase, Gpx, GRd, MT-1 and MT-3 and in lipid peroxidation in rat adenohypophysis. The experimental details are given in the legend to Fig. 4. Shown are the means $\pm$ SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. Lipid peroxidation was measured by the TBARS assay as described in Methods. ^a $p < 0.01$ vs. the remaining means, ^b $p < 0.01$ vs. control and Cd administered animals, ^c $p < 0.01$ vs. Cd and Cd + melatonin groups, ^d $p < 0.01$ vs. control and melatonin groups, one way ANOVA, Bonferroni's tests. For further statistical analysis, see text.

Figure 6

Effect of melatonin on Cd-induced changes in 24-h pattern in adenohypophysial expression of mRNA of *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1* and *Cry2*. The rats were divided into 4 groups and treated for 1 month as follows: (i) CdCl_2 (8 $\mu\text{g}/\text{ml}$ drinking water); (ii) CdCl_2 (8 μg) plus melatonin (3 μg) per ml drinking water; (iii) melatonin (3 μg) per ml drinking water; (iv) drinking water alone (control). Groups of 6-8 rats were euthanized by decapitation at 6 different time intervals throughout a 24 h cycle. Shown are the means $\pm$ SEM of mRNA determination as measured by triplicate real-time PCR analyses of RNA samples. The analysis of data by Cosinor is summarized in Table 5. For further statistical analysis, see text.

Figure 7

Effect of melatonin on Cd-induced changes in 24-h pattern of circulating PRL, LH, TSH and corticosterone. The experimental details are given in the legend to Fig. 6. Shown are the means $\pm$ SEM. The analysis of data by Cosinor is summarized in Table 6. For further statistical analysis, see text.