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A non-invasive simple method for measurement of urinary excretion of melatonin in undisturbed mice

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Abstract: Melatonin is a hormone involved in neuroendocrine responses; its plasma concentrations display a circadian pattern which is modified by stress. Studies for determining the effects of stressors on melatonin levels in laboratory animals present the difficulty that the procedures for blood sampling are by themselves potential stressors capable of influencing the levels of the hormone measured. A simple non-stressful method for measuring urinary excretion of melatonin has been consequently developed. The method is applicable to single undisturbed mice kept in conventional cages, and consists of urine collection on chromatographic paper followed by extraction and melatonin assay by radioimmunoassay. The use of this method with BD2F1 mice indicates nocturnal excretion of melatonin significantly higher than during the day; nighttime melatonin levels were shown to be suppressed by constant illumination. A significant increase in nocturnal melatonin excretion was caused by the application of rotational stress applied as a mild experimental stressor.

Laura Perissin,[1] Sonia Zorzet,[1] Valentina Rapozzi,[2] Tullio Giraldi[2]

[1]Institute of Pharmacology, University of Trieste, Trieste, Italy. [2]Department of Biomedical Sciences and Technologies, University of Udine, Udine, Italy.

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Address reprint requests to Laura Perissin, Istituto di Farmacologia, Università di Trieste, Via L. Giorgieri 7, 34100 Trieste, Italy.

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Introduction

Melatonin is an indole hormone secreted by pineal gland. In humans and animals this hormone shows a circadian rhythm with a nocturnal peak of its plasma levels [Reiter, 1986]. Melatonin appears to be involved in various biological functions [Reiter, 1991a], including the regulation of neuroendocrine responses [Maestroni et al., 1986; Reiter, 1991b]. Melatonin secretion appears to be altered in several pathological conditions related to neuroendocrine functions, and eventually to their modifications by stress [Maestroni et al., 1988]. Indeed, variations in plasma melatonin concentrations have been reported to occur in laboratory animals upon application of experimental stressors [Lynch and Deng, 1986; Lynch et al., 1973].

It is conceivable that melatonin secretion measured in these studies may have been affected by animal handling and blood sampling, since the procedure used to obtain blood specimens consisted of either decapitation or blood collection through the retrorbital plexus [Lynch and Deng, 1986; Maestroni et al., 1986]. That such procedures are obviously stressful is supported by a handling-induced increase in plasma corticosterone [Riley, 1981]. Likewise, the presence of a tumor reportedly increases blood corticosterone levels [LaBarba, 1970]. Also, an increase in metastasis in tumor bearing mice caused by an intraperitoneal injection of physiological saline reported by Giraldi et al. [1989] was possibly related to a rise in circulating stress-sensitive hormones.

A simple non-invasive and non-stressful method has been consequently developed for assaying melatonin in undisturbed mice individually maintained in conventional cages. The method is based on urine collection on chromatographic paper lining the bottom of the cage and subsequent melatonin recovery and assay by radioimmunoassay; it allows the measurement of urinary melatonin excretion in the range of 20–640 pg.

The results obtained using this assay to measure diurnal and nocturnal urinary melatonin excretion in BD2F1 mice, as a function of cycles of illumination and of application of an experimental stressor (spatial disorientation, rotational stress), are herein reported.

Materials and methods

The animals used were young BD2F1 female mice weighing 20–22 g; they were maintained 5 per cage in a protected housing with illumination (2500 lux in the cages) provided by Vitalite broad spectrum fluorescent lamps. The animals were kept for two weeks before experiments with a 12/12 light/dark (LD 12:12) cycle, in order to allow them to recover from the stress of shipment and to adapt to the housing and experimental conditions.

The stressor paradigm employed was a rotational stress (spatial disorientation); protected (low stress) housing and rotational stress application are described in detail elsewhere [Giraldi et al., 1989].

For urine collection, the mice were housed individually in conventional cages (21 × 27 × 14 cm), containing a stainless steel grid. Urine was collected on chromatographic paper (Whatman 3MM) placed below the stainless steel grid. The paper was subsequently removed and cut in pieces of 1 cm^2, from which melatonin was extracted twice with 10 ml 0.07 M phosphate buffer pH 5.5. The extract was purified by a single chromatographic step by absorption on a C-18 column (SEP-PAK Water Associates, Millipore) followed by elution with 1 ml chloroform. The solvent was evaporated under a gentle stream of nitrogen, and the melatonin present in the dry residue was assayed by means of a ^{125}I radioimmunoassay (Clone-System Diagnostics, Nuclear Medica, Padova, Italy).

20–640 pg melatonin in 0.5 ml 0.07 M phosphate buffer pH 5.5 were applied on filter paper in order to determine linearity and recovery; appropriate blanks on filter paper were used. The assay was linear in the range examined, and recovery was constant and equal to 94.5 ± 1.2%. No interference was observed by feces or food debris.

Results and discussion

The procedure of blood sampling for subsequent measurement of hormone concentrations may influence the level of the hormone determined because of the stress caused by handling and sampling procedures. This possibility is more serious in the case of repeated sampling, and appears to apply for melatonin whose levels have been reported to change upon coping with stressful stimuli [Lynch and Deng, 1986]. A simple method has been thus developed which permits repetitive urine collection for subsequent melatonin assay in undisturbed mice individually kept in conventional cages.

The use of metabolic cages is avoided with this method. This is important since such studies usually require caging of several mice (because of their small size) in an individual cage for urine collection; hence, the ensuing measurement is an average of these animals. Moreover, the adaptation to the new environment constituted by the metabolic cage also is avoided.

TABLE 1. Urinary excretion of melatonin in BD2F1 mice as a function of cycle of illumination and application of rotational stress

Rotational stress	Light/dark cycle	Urinary melatonin excretion (pg)	
		Light hours	Dark hours
–	12/12	<20	107 ± 16[a]
+	12/12	<20	599 ± 86[a]
–	24/0	<20	<20

Urine from 10 mice was individually collected separately for 12 hr starting at 0800 (light hours) or at 2000 (dark hours). Lights were on from 0800 until 2000 (12/12), or were continuously on (24/0) as indicated. Rotational stress or continuous illumination were applied for 24 hr, during which 2 samples of urine were collected for 12 hr intervals starting at either 0800 or 2000 and subsequently assayed. Each value is the mean ± S.E. Means with the same letter are significantly different, t-test for grouped data, $P < 0.01$ [Tallarida and Murray, 1987].

The present method allows the individual collection of urine produced at chosen intervals (12 hr in the present investigation) on chromatographic paper lining the bottom of the cage, and its subsequent assay by radioimmunoassay (Table 1). When tested with melatonin standards applied to the paper, the measurement is proved to be specific and linear in the range of 20–640 pg (Fig. 1).

When total urinary melatonin excretion is measured using this method, a significantly higher amount (107 pg) is found during dark hours (2000–0800), in comparison with light hours (<20 pg, 0800–2000). Upon constant illumination, the melatonin urinary excretion during night hours was significantly reduced to the levels measured during the day (<20 pg); this is a consequence of the 'functional pinealectomy' produced by light exposure at night [Wurtman et al., 1963]. The application of an experimental stressors (rotational stress) caused a significant increase to 599 pg, in total melatonin excretion during dark hours.

In conclusion, the results presented indicate that the simple method described allows the measurement of melatonin excretion in single unrestrained mice, avoiding the potentially stress-inducing procedures of blood sampling. In general, these data on urinary excretion of melatonin are in agreement with those obtained by blood sampling in mice, showing a nocturnal increase in melatonin levels [Maestroni et al., 1986] and in pineal activity of hydroxyindole-O-methyltransferase in rats maintained in constant light [Wurtman et al, 1963]. The

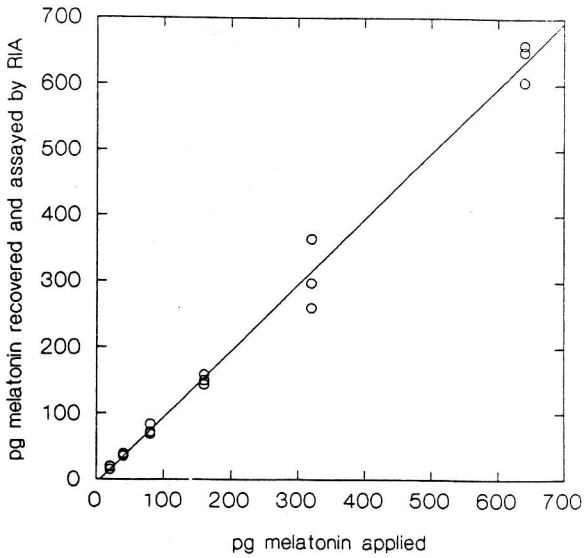

Fig. 1. Assay and recovery of melatonin from chromatographic paper. Melatonin, dissolved in 0.5 ml of 0.07 M phosphate buffer pH 5.5, was applied on filter paper which was allowed to dry. Melatonin was subsequently extracted and assayed as described in the experimental section. Recovery was 94.5 ± 1.2%, and no interference was observed by feces or food debris resulting from presence of a mouse in the cage.

possibility that the stress resulting from experimental procedures, and particularly handling and blood sampling, may affect the measurement of melatonin levels, is supported by the finding that the application of rotational stress (spatial disorientation) as a mild experimental stressor. This procedure was found to significantly increase the nocturnal excretion of melatonin. Also, these results support previous reports showing that melatonin is a stress sensitive hormone [Lynch and Deng, 1986]. They also indicate the validity of the method proposed for avoiding the stress of experimental procedures during melatonin assay, and appear to suggest the possibility of extending the use of urine collection on chromatographic paper followed by extraction and assay for measurement of other stress sensitive hormones in single undisturbed mice. Finally, although the methods described was used to determine the day-night excretion of melatonin, presumably it would have been equally useful for the collection and assay of melatonin metabolites, e.g., 6-hydroxymelatoninsulfate.

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Literature cited

GIRALDI, T., L. PERISSIN, S. ZORZET, P. PICCINI, V. RAPOZZI (1989) Effects of stress on tumor growth and metastasis in mice bearing Lewis lung carcinoma. Eur. J. Cancer Clin. Oncol. 25:1583–1588.

LABARBA, R.C. (1970) Experiential and environmental factors in cancer. Psychosom. Med. 32:259–276.

LYNCH, H.J., J.P. ENG, R.J. WURTMAN (1973) Control of pineal indole biosynthesis by changes in sympathetic tone caused by factors other than environmental lighting. Proc. Natl. Acad. Sci. USA 7:1704–1707.

LYNCH, H.J., M.H. DENG (1986) Pineal responses to stress. J. Neural Transm. 21:461–473.

MAESTRONI, G.J.M., A. CONTI, W. PIERPAOLI (1986) Role of the pineal gland in immunity. Circadian synthesis and release of melatonin modulates the antibody response and antagonizes the immunosuppressive effect of corticosterone. J. Neuroimmunol. 13:19–30.

MAESTRONI, G.J.M., A. CONTI, W. PIERPAOLI (1988) Role of the pineal gland in immunity. III. Melatonin antagonizes the immunosuppressive effect of acute stress via an opiatergic mechanism. Immunology 63:465–469.

REITER, R.J. (1986) Normal pattern of melatonin levels in the pineal gland and body fluids of humans and experimental animals. J. Neural Transm. 21:35–54.

REITER, R.J. (1991a) Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocrine Rev. 12:151–180.

REITER, R.J. (1991b) Pineal gland. Interface between the photoperiodic environment and endocrine system. Trends Endocrinol. Metabol. 2:13–19.

RILEY, V. (1981) Psychoneuroendocrine influences on immunocompetence and neoplasia. Science 212:1100–1109.

TALLARIDA, R.J., R.B. MURRAY (eds.) (1987) Manual of Pharmacologic Calculation with Computer Program. Springer, New York, pp. 131–134.

WURTMAN, R.J., J. AXELROD, L.S. PHILLIPS (1963) Melatonin synthesis in the pineal gland: control by light. Science 142:1071–1073.