Japanese Journal of Physiology, **50**, 149–153, 2000

The Effect of Melatonin on Liver Superoxide Dismutase Activity, Serum Nitrate and Thyroid Hormone Levels

Güler ÖZTÜRK, Şule COŞKUN, Deniz ERBAŞ, and Enver HASANOĞLU*

Department of Physiology and *Department of Pediatric Nephrology,
Faculty of Medicine, University of Gazi, Ankara, Turkey

Abstract: Melatonin is a main neurohormone of the pineal gland. The effects of melatonin on the level of serum thyroid-stimulating hormone (TSH), thyroxine (T_4), triiodothyronine (T_3), nitrate, melatonin and liver superoxide dismutase (SOD) activity were examined in rats. Melatonin was injected at the dose of 10 mg/kg for 7 days, 2 h before turning the lights off. Rats were decapitated at 10:00 a.m. and 02:00 a.m., which are the times of the lowest and highest serum melatonin levels, respectively. Blood and tissue samples were collected. Decreased TSH, T_3, T_4 and nitrate levels were determined in the melatonin-injected and nighttime groups. Melatonin levels showed a diurnal rhythm. SOD activity increased in the melatonin-treated group. The results demonstrate that increased SOD activity, and reduced serum TSH, T_3, T_4 and nitrate levels correlated with the serum melatonin levels. [Japanese Journal of Physiology, 50, 149–153, 2000]

Key words: melatonin, nitrate, superoxide dismutase, thyroxine, triiodothyronine.

Melatonin is a neurohormone produced during the night by the pineal gland. Its secretion is regulated by circadian and seasonal variations in day length, transmitted via visual projections to the suprachiasmatic nucleus, which functions as a circadian clock in mammals [1].

Melatonin is now considered an important antioxidant [2]. It is a free-radical scavenger and general antioxidant. It possibly works via electron donation to directly detoxify free radicals such as the highly toxic hydroxyl radical [3]. Melatonin has also been reported to alter the activities of enzymes improving the total antioxidative defense capacity of the organism, i.e., nitric oxide synthase, SOD [4]. It is also implicated in the regulation of blood flow because pinealectomy produces hypertension and exogenous melatonin reverses this phenomenon [5]. Thyroid hormones also increase the blood flow in many tissues [6].

Many types of mammalian cells produce nitric oxide (NO), and it plays a major role in the regulation of blood pressure. NO functions as a vasodilator and is synthesized by the isoforms of nitric oxide synthases (NOS) [7]. Suppression of melatonin secretion by NO has been reported [8]. The neural isoform of NOS is present in the pineal gland and its function appears to be under photoneural regulation [9].

The lowering effect of melatonin and thyroid hormones on serum cholesterol has also been documented showing a parallel effect on lipid metabolism [10].

The aim of this study was to investigate the correlation between diurnal variations of melatonin and thyroid hormones, antioxidant status, and nitrate production in plasma.

MATERIAL AND METHODS

Melatonin (*N*-acetyl-methoxy-tryptamine) was purchased from Sigma and dissolved in absolute ethanol, and later in phosphate-buffered saline (PBS) with a final ethanol concentration of less than 1%.

Animals. A total of 24 male Sprague-Dawley rats, age 2 months, were divided into control and melatonin-treated groups. Animals were maintained at 20±2°C and under 12:12-h light : dark cycle (light from 6:00 a.m. to 6:00 p.m.). After 1 week of acclimation, animals were randomly divided into four groups of six rats each and fed standard pellet food and tap

Received on June 9, 1999: accepted on December 27, 1999
Correspondence should be addressed to: Deniz Erbaş, Department of Physiology, Faculty of Medicine, University of Gazi, 06500, Beşevler, Ankara, Turkey. Tel: +90–312–212–9010, Fax: +90–312–213–4338, E-mail: erbasd@tr-net.net.tr

water *ad libitum*. The rats received 7 daily subcutaneous (s.c) injections of melatonin (10 mg/kg) or PBS as the control 2 h before turning the lights off, and were decapitated under ether anesthesia (under ethical consideration) at 10:00 a.m. and 02:00 a.m.

Determination of melatonin in serum. The serum was estimated by ELISA (RE 540 21 IBL-Hamburg). Extraction columns were placed into glass tubes, 2×1 ml of methanol (undiluted) was added, the solution centrifuged at 200×*g* for 1 min, and then 2×1 ml of bidistilled water was added. After column preparation, 0.5 ml of standards, controls and samples were added to the columns and centrifuged at 200×*g* for 1 min. The columns were then washed with 2×1 ml of 10% methanol in bidistilled water (v/v) and centrifuged at 500×*g* for 1 min. Next, the columns were placed into clean glass tubes and 1 ml methanol was added followed by centrifugation at 200×*g* for 1 min. The columns were removed from tubes, the methanol was evaporated by nitrogen, and samples were reconstituted in 0.15 ml of bidistilled water. Fifty microliters of each extracted standard, control and sample were pipetted into the appropriate wells. Fifty microliters of Melatonin-Biotine and 50 µl antiserum were added into each well and the plate was shaken carefully. The plate was sealed with an adhesive foil and incubated for 18 h at +4°C. Each well was then washed with assay buffer and 150 µl of enzyme conjugate was pipetted into the wells followed by incubation of the plate for 120 min at room temperature.

Each well was washed three times with assay buffer, and 200 µl of substrate solution was pipetted into each well. The plate was then incubated at room temperature for 1 h. Fifty microliters of stop solution was then pipetted into each well and the plate was read at an optical density of 405 nm with a microtiter plate reader. The lowest detectable level of the assay was 5 pg/ml.

Determination of thyroid hormones and thyroid-stimulating hormone. Hormones were measured by radioimmunoassay kits (Bio Bak, USA). The lowest detection limit of the assay was 0.011 µIU/ml for TSH, 0.2 ng/ml for T_3 and 0.5 µg/dl for T_4.

Determination of the liver SOD activity. SOD activity was estimated by the spectrophotometric method (SOD-525, Bioxytech, France). Tissues were blotted on paper and weighted after washing in ice-cold 0.25 M sucrose. Tissues were then homogenized in ice-cold 0.25 M sucrose. Homogenates were centrifuged at 3,000×*g* for 10 min at +4°C and supernatant was collected. Four-hundred microliters of ice-cold extraction reagent [ethanol–chloroform, 62.5 : 37.5 (v/v)] was added to 250 µl of the supernatant, and the solution was centrifuged at 3,000×*g* for 10 min at +4°C. The aqueous upper layer was then collected. Subsequently 40 µl samples were taken from each supernatant (40 µl water for the negative control), and 900 µl diluting buffer at 37°C was added [50 mM 2-amino-2-methyl-1,3-propanediol/HCI, pH= 8.8, 0.11 mM diethylenetriaminepentaacetic acid (DTPA) as the chelating agent (Bioxytech)]. Thirty microliters of 1,4,6-trimethyl-2-vinylpropyl-trifluoromethano-sulphonate [R2 (Bioxytech)] was then added to separate the mercaptans (RSH groups), followed by energetic stirring to a vortex for 3–4 s and incubation in a thermal bath at 37°C (for 1 min). Thirty microliters of 5,6,6*a*,11*b*-tetrahydro-3,9,10-trihydroxybenzo-fluorene [R1 (Bioxytech)], the chromophore which will be self oxidized as a function of the SOD concentration, was then added, followed by stirring to a vortex (4–5 s). The samples were transferred to a quartz tube and the SOD activity was measured for absorbance at 525 nm (for 1 min). The experimental ratio V_s/V_c was obtained by reference tables which relate it to the units of activity of the enzyme: $V_s/V_c=1+(\mathrm{SOD})/a(\mathrm{SOD})+b$; V_s=percentage of sample with SOD, V_c=mean of at least four controls, $a=0.073$, $b=0.93$. Hence: $(\mathrm{SOD})=0.93\ (V_s/V_c-1)/1.073-0.073(V_s/V_c)$ [11].

The resulting value is multiplied by the dilution factor and expressed as units of activity of per milliliter. The detection limit of the assay was 0.2 U/ml.

Nitrate measurement. The amount of total nitrate in the test samples was determined by modification of the procedure described by Bramen and Hendrix [12] using the purge system of Sievers (Model 280 NOA). A saturated solution of VCI_3 in 1 M HCI was prepared and filtered before use. Five milliliters of this reagent was added to a purge vessel and it purged with nitrogen for 5 to 10 min before use. The purge vessel was equipped with a cold-water condenser and a water jacket to permit heating of the reagent to 95°C using a circulating water bath. The HCI vapors were removed by a gas babbler containing 15 ml of 1 M NaOH. The gas flow rate into the chemiluminescence detector was controlled using a needle valve.

Sample and standards were injected into the purge vessel to react with the VCI_3/HCI reagent, which converted nitrate and nitrite and s-nitroso compounds to NO. The NO produced was detected by ozone-induced chemiluminescence in the chemiluminescence detector. A standard curve was constructed using various concentrations of $NaNO_3$ (10–100 µM). The detection limit of the assay was nanomolar to millimolar

Table 1. Effect of melatonin on liver SOD, serum nitrate and thyroid hormones levels (mean±SD).

	Control group		Melatonin-treated group	
	10:00 a.m. (n=6)	02:00 a.m (n=6)	10:00 a.m. (n=6)	02:00 a.m. (n=6)
Melatonin (pg/ml)	10.28 ± 1.81^{a}	74.05 ± 13.22^{b}	56.42 ± 7.17^{c}	122.2 ± 36.2^{d}
Nitrate (μM)	3.18 ± 1.60^{e}	1.29 ± 0.43^{f}	2.70 ± 1.56^{g}	1.12 ± 0.37^{h}
SOD (U/ml)	10.85 ± 3.23^{i}	21.96 ± 10.40^{j}	27.37 ± 6.59^{k}	39.41 ± 12.12^{l}
T_3 (ng/ml)	0.74 ± 0.05^{m}	0.52 ± 0.06^{n}	0.65 ± 0.06^{o}	0.50 ± 0.07^{p}
T_4 (mg/dl)	5.12 ± 0.13^{r}	3.60 ± 0.53^{s}	4.81 ± 0.47^{t}	3.11 ± 0.11^{u}
TSH (μIU/ml)	2.01 ± 0.53^{v}	0.62 ± 0.30^{w}	1.26 ± 0.11^{x}	0.24 ± 0.09^{y}

a–b, a–c, a–d, c–d, i–k, i–l, m–n, m–p, r–s, r–u, t–u, v–w, v–y, w–x, x–y $p<0.001$. e–f, e–h, v–x, n–o, o–p $p<0.01$. c–d, j–l, s–t, w–y $p<0.02$. b–d, f–g, g–h, i–j, k–l, m–o, s–u $p<0.05$.

Table 2. Groups and levels (Group factor has two levels: control and melatonin treated; and time factor has two levels: 10:00 a.m. and 02:00 a.m.).

	n	Control group X±SD	Melatonin-treated group X±SD	10:00 a.m. X±SD	02:00 a.m. X±SD
Melatonin	12	42.16 ± 34.49^{a}	89.20 ± 42.44^{b}	33.28 ± 24.5^{c}	98.09 ± 9.9^{d}
Nitrate	12	2.23 ± 1.47^{e}	1.91 ± 1.36^{f}	2.94 ± 1.50^{g}	1.21 ± 0.39^{h}
SOD	12	16.39 ± 9.35^{i}	33.39 ± 11.22^{j}	19.08 ± 9.90^{k}	30.70 ± 14.00^{l}
T_3	12	0.63 ± 0.12^{m}	0.57 ± 0.10^{n}	0.69 ± 0.07^{o}	0.51 ± 0.07^{p}
T_4	12	4.36 ± 0.87^{r}	3.96 ± 0.94^{s}	4.96 ± 0.3^{t}	3.36 ± 0.44^{u}
TSH	12	1.32 ± 0.83^{v}	0.75 ± 0.54^{w}	1.63 ± 0.53^{x}	0.43 ± 0.29^{z}

a–b, c–b, i–j, o–p, t–u, x–z $p<0.001$. v–w $p<0.01$. g–h, k–l, m–n $p<0.05$.

and assay sensitivity was ~1 pM.

Statistical analysis. All data are expressed as the mean±standard deviation (SD). A two-way factorial analysis of variance (ANOVA) test was applied. There were two levels of group factor: control and melatonin-treated. Two time factors were also involved: 02:00 a.m. and 10:00 a.m. (Table 2).

RESULTS

Nighttime serum melatonin levels were found to be significantly higher than those of daytime levels in control ($p<0.001$) and melatonin-treated groups ($p<0.02$) (Tables 1 and 2).

The SOD activity in the liver was increased at nighttime in control and melatonin-treated groups ($p<0.001$) (Tables 1 and 2).

Serum nitrate levels were decreased in the melatonin-treated and nighttime groups. This was a singificant difference between daytime and nighttime levels in the control and melatonin-injected groups ($p<0.01$ and $p<0.05$ respectively) (Tables 1 and 2).

T_3, T_4 and TSH levels during nighttime were found to be significantly lower than those during daytime for the control and melatonin-injected groups ($p<0.001$). There were also significant difference between the levels of the control and melatonin-treated groups during in daytime and nighttime ($p<0.05$) (Tables 1 and 2).

DISCUSSION

The action of melatonin is highly pleiotropic. It involves: firstly, direct effects via spesific binding sites in various peripheral tissues and cells of vertebrates, including immunomodulation; secondly, systemic influence on the cytoskeleton and nitric oxide formation, mediated by calmodulin; and thirdly, antioxidative protection [13].

In vitro studies have demonstrated that melatonin is a scavenger of oxyradicals and peroxynitrite, and an inhibitor of NO [14]. Melatonin can inhibit NO production by reducing iNOS expression in leucocytes and inhibit catalytic activity of NOS in rat cerebellum and hypothalamus by a calmodulin- mediated mechanism [15–17]. Recent studies indicated that NO is involved in ischemic brain damage. In addition to the inhibition of neuronal NOS activity, the cytoprotective effect of melatonin during ischemia may be due to its ability to directly scavenge free radicals [18]. It is suggested that the inhibitory effect of melatonin on NO production may be responsible for the protective effect of the hormone on neuronal structures [18]. NO and

cGMP rhythms in the frontal cortex of the chick are inversely related to the melatonin rhythm. When melatonin levels increase (at night or after melatonin injection) NO and cGMP levels decrease [18, 19]. In our study, serum nitrate levels decreased inversely related to the serum melatonin levels, and this is in agreement with previous studies [14, 18].

Thyroid hormones increase the basal rate of oxygen consumption in all tissues except the brain, gonads and spleen and dismutase levels, resulting in increased superoxide anion free radical formation. This may contribute to the deleterious effects of chronic hyperthyroidism and also decrease cholesterol levels [20]. Total light deprivation reduces serum T_4. This effect is prevented by pinealectomy. In contrast, pinealectomy induced thyroid hypertrophy, an effect reversed by melatonin [21, 22]. Chronic night treatment with melatonin in the drinking water for mice significantly lowers night levels of T_3 and T_4 in peripheral blood [22]. In this study, it has been found that melatonin injection decreased the serum T_3, T_4, and TSH levels. This is in conjunction with other studies except for the results of TSH. As is expected, TSH should be increased after serum T_3 and T_4 levels decline, and this effect may be the result of direct melatonin effect on the hypothalamic plane.

SOD is a metalloenzyme which catalyses the dismutation of $O_2^- \cdot$ into O_2 and H_2O_2. It is important in the antioxidative defense mechanism [3]. NOS is also included in free radical generation in the absence of L-arginine [23]. Melatonin, with its hepatic metabolite, 6-hydroxymelatonin sulfate, is a free radical scavenger. Rodriguez and co-workers have shown that SOD activity in ring dove leucocytes decreased in the presence of melatonin [3]. On the other hand, Antolin and co-workers reported that both Mn-SOD and Cu,Zn-SOD increased after treatment with melatonin in the harderian gland, and they suggested that melatonin may induce mRNA increments for SOD [24]. This is not the first report about melatonin on SOD activity, but we know that this is the first report showing the effect of diurnal variations of melatonin on liver SOD activity. In this study, increased SOD activity in the rat liver were determined. The antioxidant effect of melatonin may be the cause of increased SOD activity. Our findings were parallel with those of Genç and co-workers, who shown increased SOD activity in the liver after melatonin administration [25].

In conclusion, the present results demonstrated that melatonin administration raises the serum melatonin level, reduces the serum nitrate and thyroid hormone levels and, as an antioxidant, increases the antioxidant capacity of tissues.

This work was supported by the Turkish Prime Ministry State Planning Organization and Gazi University Research Center.

REFERENCES

1. Delegrange P and Guardiola-Lemaitre B: Melatonin, its receptors, and relationships with biological rhythm disorders. Clin Neuropharmacol 20: 482–510, 1997
2. Cuzzocrea S, Zingarelli B, Gilad E, Hake P, Salzman AL, and Szabo C: Protective effect of melatonin in carrageenan-induced models of local inflammation: Relationship to its inhibitory effect on nitric oxide production and its peroxynitrite scavenging activity. J Pineal Res 23: 106–116, 1997
3. Rodriguez AB, Nogales G, Ortega E, and Barriga C: Melatonin controls superoxide anion levels: Modulation of superoxide dismutase activity in ring dove heterophils. J Pineal Res 24: 9–14, 1998
4. Reiter RJ: Pharmacological actions of melatonin in oxygen radical pathophysiology. Life Sci 60: 2255–2271, 1997
5. Reiter RJ: Functional aspects of the pineal hormone melatonin in combating cell and tissue damage induced by free radicals. Eur J Endocrinol 134: 412–420, 1996
6. Guyton A and Hall JL: Text Book of Medical Physiology, 9th Ed, Saunders Company, Philadelphia, p. 950, 1996
7. Archer S, Hample V, Mckenzie Z, Nelson D, Huang J, Shultz PJ, and Weir EK: Role of endothelial-derived nitric oxide in normal and hypertensive pulmonary vasculature. Semin respir Crit Care Med 15: 179–189, 1994
8. Maronde E, Middendorff R, Mayer B, and Olcese J: The effect of NO donors in bovine and rat pineal cells. Stimulation of cGMP and cGMP-independent inhibition of melatonin synthesis. J Neuroendocr 7: 207–214, 1995
9. Schaad NC, Vanecek J, and Schulz PE: Photoneural regulation of rat pineal NOS. J Neurochem 62: 2496–2499, 1994
10. Esguifino A, Agrasal C, Valezquez E, Villanua MA, and Cardinali DP: Effect of melatonin on serum cholesterol and phospholipid levels, and on prolactin, thyroid-stimulating hormone and thyroid hormone levels, in hyperprolactinemic rats. Life Sci 61: 1051–1058, 1997
11. Nebot C, Moutet M, Huet P, Xu J-Z, Yadan J-C, and Chaundiere J: Spectrophotometric assay of superoxide dismutase activity based on the activated autooxidation of a tetracyclic catechol. Anal Bioch 214: 442–451, 1993
12. Bramen RS and Hendrix SA: Nanogram nitrite and nitrate determination in enviromental and biological materials by vanadium (III) reduction with chemiluminescence detection. Anal Chem 61: 2715–2718, 1989
13. Hardeland R: New actions of melatonin and their relevance to biometeorology. Int J Biometeorol 41: 47–57, 1997
14. Cuzzocrea S, Zingarelli B, Costantino G, and Caputi AP: Protective effect of melatonin in a non-septic shock model induced by zymosan in the rat. J Pineal Res 25: 24–33, 1998

15. Pozo D, Reiter RJ, Calvo JR, and Guerrero JM: Physiological concentrations of melatonin inhibit nitric oxide synthase in rat cerebellum. Life Sci 55: 455–460, 1994
16. Pozo D, Reiter RJ, Calvo JR, and Guerrero JM: Inhibition of cerebellar nitric oxide synthase and cyclic GMP production by melatonin via complex formation with calmodulin. J Cell Biochem 65: 430–442, 1997
17. Bettahi I, Pozo D, Osuna C, Reiter RJ, Acuna-Castroviejo D, and Guerrero JM: Melatonin reduced nitric oxide synthase activity in rat hypothalamus. J Pineal Res 20: 205–210, 1997
18. Guerrero JM, Reiter RJ, Ortiz GG, Pablos MI, Sewerynek E, and Chuang J-I: Melatonin prevents increases in neural nitric oxide and cylic GMP production after transient brain ischemia and reperfusion in the Mongolian gerbil (*Meriones unguiculatus*). J Pineal Res 23: 24–31, 1997
19. Guerrero JM, Pablos MI, Ortiz GG, MI, Agapito MT, and Reiter RJ: Nocturnal decreases in nitric oxide and cyclic GMP contents in the chick brain and their prevention by light. Neurochem Int 29: 417–421, 1996
20. Greenspan FC and Baxter JD: Basic & Clinical Endocrinology, 4th ed, p 182, 1994
21. Houssay AB and Pazo JH: Role of pituitary in the thyroid hypertrophy of pinealectomized rats. Experientia 24: 813–814, 1968
22. Pierpoli W, Dall'ara A, Pedrinis E, and Regelson W: The pineal control of aging. The effects of melatonin and pineal grafting on the survival of older mice. Ann NY Acad Sci 621: 291–393, 1993
23. Davies MG, Fulton GJ, and Hagen PO : Clinical biology of nitric oxide. Brit J Surg 82(12): 1598–1610, 1995
24. Antolin I, Rodriguez C, Sainz RM, Mayo JC, Uria H, Kotler ML, Rodriguez-Colunga MJ, Toliva D, and Menendez-Pelaez A: Neurohormone melatonin prevents cell damage: effect on gene expression for antioxidant enzymes. FASEB J 10: 882–890, 1996
25. Genç S, Gürdol F, Öner IY, and Onaran I: The effect of melatonin administration on ethanol-induced lipid peroxidation in rats. Pharmacol Res 37: 37–40, 1998