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MELATONIN IN HUMAN VOLUNTEERS: FOCUS ON NASAL LAVAGE LEVEL AND ITS FREE RADICAL SCAVENGING PROPERTIES WITH POSSIBILITY TO APPLY IN FOOD RESEARCH – A SHORT REPORT

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Key words: blood plasma, nasal lavage fluid, melatonin, SOD activity, peroxyl radical trapping activity

The present paper is the first to report the results of the work carried out on daytime melatonin levels in paired plasma and nasal lavage fluid (NAL) samples obtained from healthy adult subjects. Lavage fluid was collected from 25 volunteers using a modified Foley catheter. Blood plasma was collected using normal procedure. The immunoreactive melatonin was present in all plasma samples and in 80% of the investigated NAL samples. Blood plasma melatonin level ranged from 0.98 pg/mL to 34.97 pg/mL however the level found in NAL was lower and ranged from 0.46 to 2.42 pg/mL There was no correlation between paired plasma and nasal lavage melatonin concentration (r=0.02). Moreover, the superoxide dismutase activity (SOD) in NAL samples was determined and then the correlation coefficient between the melatonin concentration and EC-SOD activity was r= 0.18. The peroxyl radical trapping activity of 1 μmol/L melatonin was equivalent to 3 μmol/L Trolox. It has been concluded that nasal lavage fluid melatonin has limited utility in the field of melatonin research.

INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine), the principal hormone of the pineal gland of vertebrates, is implicated in several physiological processes controlled by photoperiod like diurnal rhythm of locomotor activity as well as seasonal rhythm of reproduction (Figure 1). There is also an increasing number of evidence that melatonin modulates the function of the immune system and growth of cancer [Maestroni *et al.,* 1986; Blask *et al.,* 1992]. Next to the neurohormonal chronobiotic effects, melatonin is a very potent free radicals scavenger [Tan *et al.,* 1993; Reiter, 1997]. It detoxifies a variety of free radicals and reactive oxygen intermediates including the hydroxyl radical, peroxynitrite anion, singlet oxygen and nitric oxide. Additionally, it reportedly stimulates several antioxidative enzymes including glutathione peroxidase, glutathione reductase, glucoso-6-phosphate dehydrogenase and superoxide dismutase; conversely, it inhibits a prooxidative enzyme, nitric oxide synthase [Reiter *et al.,* 1999].

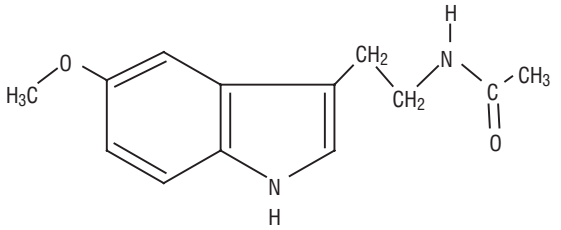

FIGURE 1. Chemical structure of melatonin (N-acetyl-5-methoxytryptamine).

The circulating melatonin concentration increases during nighttime in response to the melatonin production in the pineal gland. The pineal melatonin production declines progressively with age such that in elderly humans the levels of melatonin available to the organism are a fraction of that of young individuals [Reiter, 1994]. Beside the pineal gland, melatonin is also synthesized in some other tissues including the gastrointestinal tract, where this indole is involved in local regulatory mechanisms and may also act as antioxidant [Chow *et al.,* 1996; Bubenik *et al.,* 1999]. The total quantity of melatonin in the gastrointestinal tract is about 400 times higher than in the pineal [Huether *et al.,* 1992], but hepatic barrier prevents its large release to peripheral blood. However, there is an increasing number of evidence showing that melatonin from gastrointestinal tract is, at least in some situations, a significant contributor to the peripheral melatonin concentrations [Bubenik *et al.,* 1996].

It has been demonstrated that edible plant tissues contain melatonin [Dubbels *et al.,* 1995; Zieliński *et al.,* 2001a, b] and their consumption increases the circulating blood melatonin level in vertebrates [Hattori *et al.,* 1995]. Moreover, the presence of radioimmunoassayable melatonin in medici-

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nal plants as well as in cereal-based food products has been shown [Murch *et al.,* 1997; Zieliński *et al.,* 2001a, b]. For this reason, taking into account simple and non-invasive method of biological fluid sample collection, question which was addressed in this study was to find out whether human nasal lavage fluid can be used in melatonin research in food sciences.

The present paper is the first to report the results of the work carried out on the presence of daytime melatonin in nasal lavage fluid samples obtained from healthy volunteers. Moreover, the extra-cellular superoxide dismutase activity (EC-SOD) in NAL samples was determined and then the correlation coefficient between the melatonin concentration and EC-SOD activity was calculated. Moreover, the peroxyl radical trapping capacity of melatonin was also investigated under *in vitro* test system.

MATERIALS AND METHODS

Chemicals. ^{125}I-melatonin (spc. act. 2200 Ci/mmol/L) was purchased from Du Pont NEN; rabbit antiserum R/R/19540-16876 from Stockgrand Ltd, University of Surry Guilford UK. The antisera used were sufficiently specific for clinical application without pre-assay sample preparation. Sodium chloride and toluene were from POCh-Poland; gelatin from Merck-Germany; N-acetyl-5-methoxytryptamine (cold melatonin), phosphate buffered saline pH 7.4 (PBS – 0.01 mol/L sodium and potassium phosphate buffer, containing 0.0027 mol/L potassium chloride and 0.137 mol/L sodium chloride), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and all other reagents were from Sigma Co. Superoxide Dismutase kit (Cat No SD 125) was obtained from Randox Laboratories Ltd (United Kingdom). 2,2'-Azobis(2-amidopropane) hydrochloride (ABAP) was purchased from Wako Pure Chemical Industries, LTD., Osaka, Japan.

Subjects and lavage. The experiment was carried out at King's College London. Consenting subjects over 18 years of age were examined by a physician and excluded from the study if they had gross anatomical nasal deformity; a history of allergy or asthma; an upper respiratory tract infection in the previous six weeks; suffered from cardiovascular, respiratory, endocrine or any other condition considered cause for exclusion, are currently taking medication, are smokers or passive smokers, or work in an environment where occupational exposure to dust or fumes may occur. The nasal cavity of all subjects was lavaged according to the procedure described by Housley *et al.* [1995]. Recovered nasal lavage fluids were sieved through 150 μmesh, centrifuged at 300 g for 10 min at 10°C and the supernatant was removed for storage at -70°C. Blood plasma was collected using normal procedure. All plasma and nasal lavage fluid samples were treated with deferoxamine mesylate and 2,6-di-tert-butyl-4-methyl phenol (BHT) (final concentration of 20 μmol/L) to stabilise liable antioxidant constituents. The samples were collected during the day.

Melatonin radioimmunoassay. The melatonin immunoreactivity was assayed by a slightly modified direct method of Fraser *et al.* [1983]. Briefly, 200 μL of antiserum R/R/19540-16876, diluted 1:150 000 in assay buffer (tricine 0.1 mol/L, sodium chloride 9 g/L, gelatine 1 g/L), were added to 100 μL of plasma or 400 μL of NAL (or set of standards containing 0, 0.5, 1, 2.5, 5, 10, 25, 50, 100 pg of cold melatonin in 100 μL of plasma endogenous melatonin depleted by C18 SPE method or in 400 μL of PBS). The mixture was incubated at room temperature for 30 min. Then, 100 μL of ^{125}I-melatonin, diluted directly before using in the assay buffer to approximately 10 000 cpm/100 μL was added. After 18 h of incubation at 4°C, antibody-bound melatonin was separated from the free fraction by incubation with 250 μL or 500 μL of dextran-coated charcoal (1.5 g Norit A and 60 mg dextran in 100 mL of the assay buffer) for 15 min at 4°C, for plasma and NAL samples, respectively. After centrifugation at 3000 *g* and 4°C for 20 min, the radioactivity of supernatant was measured using a toluene-based scintillation cocktail and Beckman counter LS 6500. The toluene based scintilation fluid contained 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyl-2-oxazolyl) benzene (POPOP). Samples were assayed in duplicates. For each sample the non-specific binding was determined (in triplicate) by the addition of tricine buffer instead of antibody solution. The assay was validated by running the samples containing different amounts of exogenous melatonin (in triplicates). Melatonin concentration was calculated using a four-parameter logistic curve (Immuno Fit EIA/RIA ver. 3.0a, Beckman).

Total protein concentration. The method utilised for the determination of total protein concentrations in nasal lavage fluid samples was based upon a modification of the Lowry method [Lowry *et al.,* 1951] proposed by Smith *et al*. [1985]. The assay is based upon the observation that when incubated with the cupric ion in an alkaline environment, proteins will reduce Cu (II) to Cu (I) in a concentration-dependent manner. In this assay bicinchoninic acid (BCA), a highly specific chromogenic reagent for Cu(I) was substituted for the Folin-Ciocalteau Reagent used in the Lowry assay. BCA reacts with Cu (I) to form a stable purple complex which has an absorption maximum at 562 nm, the intensity of which is directly proportional to protein concentration over a broad concentration range. A series of standards: 0.0–1.0 mg/mL, were prepared from the stock bovine serum albumin solution (BSA, fraction V) using the phosphate buffer as a diluting agent. Exactly 50-μL aliquots for each the standards and nasal lavage fluid samples were added to 1.4 mL of the BCA solution (50 volumes of BCA to 1 volume of 4% $CuSO_4 \times 5\,H_2O$ solution, prepared immediately prior to use). Following incubation at 37°C for 30 min, the absorbance of the standards/samples was read at 562 nm using a spectrophotometer (UV-160 1PC with CPS-Controller, Shimadzu, Japan). The final protein concentration was calculated by reading the absorbance of the samples against the standard curve and was expressed as mg/mL.

Determination of extra-cellular superoxide dismutase (EC-SOD) concentrations in the nasal lavage fluid. EC-SOD concentrations were quantified in nasal lavage fluid samples using a superoxide dismutase kit (RANSOD). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-

nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The superoxide dismutase scavenging activity of the investigated samples was then measured by the degree of inhibition of this reaction at 505 nm by using UV/VIS spectrophotometer. The superoxide dismutase with an activity of 5.4 U/mL was used as a standard. The percent of reaction inhibition was plotted against $\log_{10}$ of different SOD activities (SOD/mL) giving a standard curve and then SOD activity of the sample was calculated on SOD unit/mL of the investigated fluids. The results were finally expressed in SOD unit/mg of protein. Assays were performed at 37°C using a recording spectrophotometer (UV-160 1PC with CPS-Controller, Shimadzu, Japan). The test has required 50 μL of sample, with a read time of 3 min.

Measurement of the peroxyl radical-trapping capacity of melatonin. The assay was carried out according to Bartosz *et al*. [1998]. Sodium phosphate buffer (0.1 mol/L, pH 7.0) was pre-heated to 37°C and added to a cuvette so as to obtain 3 mL of the final reaction volume. Then 90 μL of 5 mmol/L ABTS solution and 80 μL of melatonin solution or Trolox solution were added followed by 300 μL of 200 mmol/L 2,2'-azobis(2-amidopropane) hydrochloride (ABAP) solution. The melatonin solution was prepared in methanol in the range of 1–5 μmol.L. The cuvettes were placed in a thermostated recording spectrophotometer (UV-160 1PC with CPS-Controller, Shimadzu, Japan) adjusted to 37°C inside the cuvettes and absorbance was measured at 414 nm. The results represent mean values of three determinations. The final results were expressed with reference to a known amount of Trolox, a water-soluble vitamin E analogue.

RESULTS AND DISCUSSION

The immunoreactive melatonin was present in all plasma samples and in 80% of the investigated NAL samples. Blood plasma melatonin level ranged from 0.98 to 34.97 pg/mL however the level found in NAL was lower and ranged from 0.46 to 2.42 pg/mL (Table 1). There was no correlation between the melatonin level found in plasma nad NAL samples(r=0.02). That observation was made indicating that those subjects who had low melatonin concentration in plasma had no detected immunoreactive melatonin in NAL samples (subjects No. 2, 14, 15, 19 and 24, respectively).

The extra-cellular superoxide dismutase (EC-SOD) concentrations in the nasal lavage fluids ranged from 0.055 to 0.24 U/mL (Table 2). The correlation coefficients between

TABLE 1. Concentration of immunoreactive melatonin in paired plasma and nasal lavage fluid (NAL) samples of twenty five healthy subjects.

Subject	Immunoreactive melatonin concentration (pg/mL)	
	plasma	nasal lavage fluid
1	5.33 ± 0.77	1.57 ± 0.24
2	4.20 ± 0.90	ND
3	14.07 ± 1.53	1.43 ± 0.29
4	8.51 ± 0.16	0.55 ± 0.08
5	3.80 ± 1.13	0.61 ± 0.16
6	4.97 ± 0.27	0.46 ± 0.21
7	21.62 ± 6.36	1.61 ± 0.34
8	26.45 ± 2.16	1.06 ± 0.32
9	21.88 ± 4.21	1.84 ± 0.28
10	16.29 ± 8.66	2.42 ± 0.44
11	3.90 ± 1.30	0.58 ± 0.09
12	2.93 ± 1.40	1.95 ± 0.51
13	5.56 ± 1.11	0.73 ± 0.12
14	6.60 ± 3.14	ND
15	2.43 ± 1.00	ND
16	0.98 ± 0.35	0.66 ± 0.12
17	31.57 ± 5.13	0.86 ± 0.30
18	34.97 ± 9.53	0.87 ± 0.19
19	1.29 ± 0.66	ND
20	11.15 ± 1.89	0.72 ± 0.35
21	3.12 ± 0.42	2.27 ± 0.34
22	19.98 ± 1.79	0.71 ± 0.35
23	4.01 ± 1.89	1.40 ± 0.21
24	7.00 ± 0.78	ND
25	13.66 ± 1.70	1.32 ± 0.24

ND, not detected

TABLE 2. Concentration of protein and extra-cellular superoxide dismutase (EC-SOD) in the nasal lavage fluids (NAL) samples of twenty five healthy subjects.

Subject	Protein (mg/mL)	EC-SOD (U/mL)	EC-SOD (U/mg protein)
1	0.96	0.15	0.16
2	0.54	0.24	0.45
3	0.82	0.15	0.19
4	0.76	0.15	0.19
5	0.55	0.11	0.20
6	0.44	0.08	0.17
7	0.65	0.09	0.14
8	0.59	0.09	0.15
9	1.04	0.09	0.09
10	1.29	0.09	0.07
11	1.29	0.13	0.10
12	3.42	0.11	0.03
13	0.40	0.06	0.14
14	0.16	0.08	0.48
15	0.59	0.13	0.22
16	0.82	0.08	0.10
17	0.83	0.11	0.14
18	0.68	0.08	0.12
19	0.54	0.11	0.19
20	0.64	0.06	0.10
21	1.36	0.13	0.09
22	0.70	0.09	0.13
23	1.81	0.08	0.04
24	0.83	0.07	0.09
25	0.61	0.10	0.16

melatonin concentration *vs*. EC-SOD (U/mL) and protein in the nasal lavage samples were 0.18 and 0.53, respectively (the data for subjects No. 2, 14, 15, 19 and 24 was excluded from the calculation).

The peroxyl radical-trapping activity of melatonin is shown in Figure 2. Interesting was to find out that inhibition of the peroxyl radicals derived from thermal decomposition of ABAP was observed at the reaction time 15 min and that melatonin at all levels tested inhibited the peroxyl radical reaction. Judging from the Figure 2, the efficiency of 1 μmol/L melatonin was equivalent to 3 μmol/L trolox. This finding supports previous observations that melatonin possesses remarkable antioxidant properties *in vivo*, and its activity *in vitro* suggests that it acts primarily as a trap for free radicals with an $OH^{\bullet}$ structure [Tan *et al*., 1993; Reiter, 1997; Reiter *et al*., 1999]. Melatonin, which is both hydrophilic and hydrophobic [Shida *et al.,* 1994], can enter every cell and all subcellular compartments. In view of melatonin's ability to cross all morphological barriers it is potentially an important component of the antioxidative defense system of the organism. The data provided in this study indicate that in aqueous environment melatonin appears to differ in behavior towards peroxyl radical trapping than other antioxidants. Considering the synergistic interactions of melatonin with antioxidants such as Trolox, glutathione, ascorbate in aqueous system [Poeggler *et al.,* 1995] and α-tocopherol in a lipid bilayer [Livrea *et al.,* 1997], it may be concluded that melatonin may play a role in the protection against radical mediated cellular damage in the nasal cavity. Melatonin is also considered to be a natural oncostatic agent for animals and humans [Hill *et al.,* 1988]. Melatonin is known to be rapidly taken up from the gastrointestinal tract when it is administered orally. A dose of synthetic melatonin of 1.0 to 1.5 μg/kg is sufficient to raise the daytime blood melatonin levels to high values normally seen in humans at night [Vakkurio *et al.,* 1985]. It could be suspected that food-derived melatonin may play an important local role in the gastrointestinal tract, where this indoleamine is involved in the regulation of food consumption, motility and tone of musculature, physiology of mucosa and absorption of water and electrolytes [Chow *et al.,* 1996]. It has been demonstrated that melatonin prevents formation of gastric ulceration in the rat [Khan *et al.,* 1990] and the domestic pig [Ayles *et al.,* 1996] as well as reduces severity of dextran-induced colitis in the mice [Pentney & Bubenik, 1995].

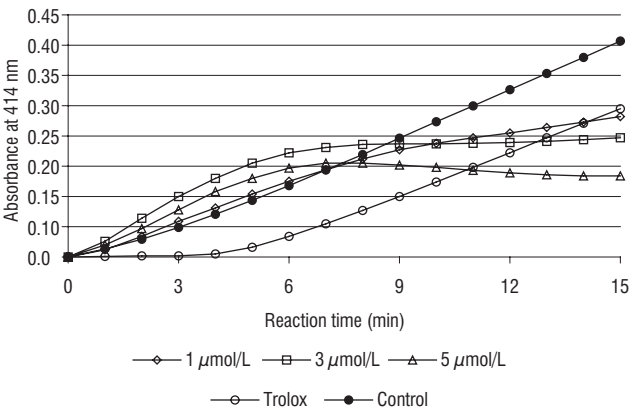

FIGURE 2. Time course of absorbance increase during oxidation of ABTS by ABAP-derived free radicals in the presence of different concentrations of melatonin and 3 μmol/L of Trolox solution (n = 6).

In summary, this is the first report that attempted to measure melatonin in nasal fluid and to compare the levels with those in the blood. There was no correlation between melatonin level found in plasma nad NAL samples. Moreover, the extra-cellular superoxide dismutase (EC-SOD) was found in the nasal lavage fluids (NAL) samples of twenty five healthy subjects. The correlation coefficient between melatonin concentration *vs*. ES-SOD (U/mg protein) in the nasal lavage samples was r=-0.55 and the respective correlation curve of melatonin levels and the EC-SOD activity is illustrated in Figure 3.

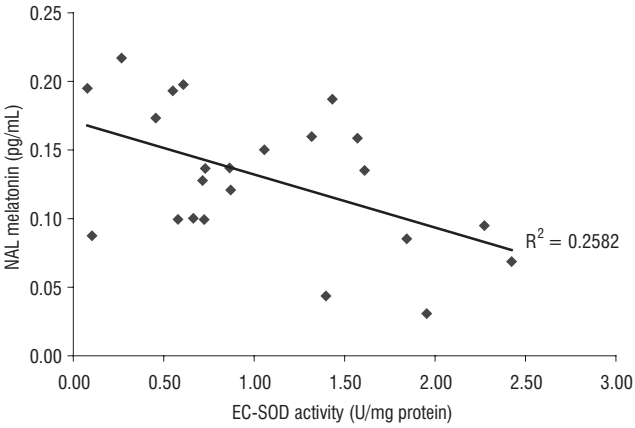

FIGURE 3. A correlation curve of melatonin levels and the EC-SOD activity in the nasal lavage fluid samples.

CONCLUSION

It has been concluded that nasal lavage fluid melatonin has limited utility in the field of melatonin research.

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POZIOM MELATONINY W PŁYNIE UZYSKANYM Z PŁUKANIA KOMORY NOSOWEJ ORAZ JEGO AKTYWNOŚĆ PRZECIWUTLENIAJĄCA – KRÓTKI KOMUNIKAT

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W pracy po raz pierwszy przedstawiono zawartość melatoniny w osoczu krwi i płynie uzyskanym z płukania komory nosowej zdrowych dorosłych 25 ochotników. Próby do badań pobierano w dzień według przyjętych procedur. Obecność melatoniny stwierdzono we wszystkich próbach osocza oraz w 20 próbach płynu uzyskanego z płukania komory nosowej.

Poziom melatoniny w osoczu był w przedziale od 0,98 pg/mL do 34,97 pg/mL (tab. 1) natomiast w uzyskanym płynie zmieniał się od 0,46 do 2,42 pg/mL (tab. 2). Nie stwierdzono korelacji pomiędzy zawartością melatoniny w osoczu i płynie z płukania komory nosowej od indywidualnych ochotników (r=0,02). Zbadano także aktywność dysmutazy ponadtlenkowej (SOD) w płynie i stwierdzono jej słabą korelację z zawartością w nim melatoniny (r= 0,18) (rys. 3). Prześledzono ponadto zdolność melatoniny do wymiatania rodników nadtlenkowych. Wykazano, że zdolność 1 μmola melatoniny do wymiatania rodników nadtlenkowych była równoważna tej wykazywanej przez 3 μmole Troloksu (rys. 2). Uznano, że płyn uzyskiwany z płukania komory nosowej zdrowych ochotników nie stanowi ważnego materiału biologicznego w badaniach nad występowaniem i właściwościami melatoniny.