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Melatonin expression in periodontal disease

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Background and Objective: It was the purpose of this study to examine the relationship between periodontal diseases and melatonin level.

Material and Methods: Forty-six patients with periodontal disease, together with 26 age- and gender-matched healthy controls, were included. Periodontal status was assessed using the Community Periodontal Index. Plasma and salivary melatonin levels were determined using specific commercial radioimmunoassays, whereas lymphocyte subpopulations (e.g. CD3, CD4, CD8, C19 and natural killer cells) were analyzed using flow cytometry.

Results: Patients with periodontal disease had significantly ($p < 0.001$) lower plasma (9.46 ± 3.18 pg/mL) and saliva (2.55 ± 0.99 pg/mL) melatonin levels than healthy control patients (14.33 ± 4.05 and 4.22 ± 0.87 pg/mL, respectively). A biphasic relationhip was observed between plasma melatonin levels and Community Periodontal Indices. The plasma melatonin level was reduced in patients with a lower Community Periodontal Index value (1 or 2) and increased in patients with a higher Community Periodontal Index value (3 or 4). Salivary melatonin parallels the changes of plasma melatonin. The higher the Community Periodontal Index, the older the patient and the higher the total lymphocyte counts. CD4 concentrations also increased as the disease worsened.

Conclusion: The results obtained from this study suggest that melatonin could act as a protective function in fighting periodontal infection. However, further studies in this area are encouraged.

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Melatonin, or *N*-acetyl-5-methoxytryptamine, is a hormone synthesized and secreted mainly in the pineal gland. It is released during the night via postsynaptic activation of the β-adrenergic receptors, thereby earning it the name of *nocturnal messenger* (1,2). The pineal gland cells (or pinealocytes) respond to changes in the light/darkness cycle, causing their metabolic activity to synchronize with the 24-h day period (a phenomenon known as a circadian rhythm), mediated by the hypothalamic suprachiasmic nucleus. Peak melatonin secretion occurs in healthy subjects between 24:00 h and 02:00 h, and the lowest secretion is observed between 12:00 h and 14:00 h. The production of melatonin declines after the age of 40–45 years, with a continuing reduction in its levels with increasing age (3,4).

Currently, melatonin is not regarded as a hormone in the classical sense, but rather as a cell protector, because it is not synthesized in a single organ and does not exert effects upon a specific target organ (5). By far, most of the melatonin released into the bloodstream (95%) is metabolized and conjugated in the liver, whereas in the central nervous system it is rapidly oxidized to *N*-acetyl-5-methoxykynuramine – a substance representing 15% of the total urinary metabolites of the hormone (6).

Melatonin diffuses passively into saliva via the bloodstream, and salivary melatonin can be reliably assayed. The ratio between plasma and salivary melatonin in a (24-h) cycle varies from 0.24 to 0.33, which means that the salivary melatonin concentration is equivalent to ≈24–33% of the plasma levels (7–9). Approximately 70% of plasma melatonin is bound to plasma albumin, with no appreciable amounts of such protein-bound melatonin being

found in the saliva. Thus, salivary melatonin represents the portion of circulating melatonin not bound to proteins (i.e. the free fraction of the hormone). Melatonin modulates immune responses, protects cells via anti-inflammatory effects (acting as an antioxidant and free radical scavenger), stimulates type I collagen synthesis and promotes bone formation (10–27).

An important consideration in periodontal disease is the generation of free radicals (28), some of which derive from the oral bacteria themselves, others originating from the induced immune response (29–30). It has been suggested that an increase in both reactive oxygen and nitrogen species during periodontal disease is responsible for the oxidative damage to periodontal tissues (31). The increase in free radical production co-exists with a decrease in antioxidant defense. The imbalance between the pro-oxidant and antioxidant systems may lead to further oxidative attack and to substantial deterioration of the periodontal tissues (32,33).

However, the relationship between periodontal diseases and melatonin level remains unknown. Hence, the present study was conducted to examine the relationship between periodontal disease and melatonin levels, as well as to assess the lymphocyte subpopulations (e.g. CD3, CD4, CD8, CD19 and natural killer) and their relationship to melatonin.

Material and methods

A total of 72 subjects were included in the study. Informed consent was obtained from all patients before participation in the study, which was approved by the University Ethics Committee and performed in accordance with the Code of Ethics of the World Medical Association according to the Declaration of Helsinki. The subjects were divided into two groups: control (26 healthy subjects, 15 women and 11 men, 47.2 ± 9.1 years); and test (46 age- and gender-matched patients with periodontal disease).

A dental and medical history was compiled for all patients, following World Health Organization criteria (34). The inclusion criteria for patients with periodontal disease were: age 18–65 years; and evidence of periodontal disease (e.g. bone loss, pocket depth). Exclusion criteria included the presence of other concomitant systemic disorders (such as epilepsy and schizophrenia) and diseases capable of affecting the immune system, such as chronic infectious and neoplastic processes (35,36). Patients receiving pharmacological treatment that could alter melatonin levels were excluded from the study (37,38).

Data were assessed by a single-masked examiner. The intra-examiner reliability was calculated to be 84%. Periodontal status was evaluated using the Community Periodontal Index (34). The Community Periodontal Index, currently recommended by the World Health Organization, consists of dividing the oral cavity into six sextants, with tooth indexing in each. Teeth index are 17/16 for the first sextant, 11 for the second, 26/27 for the third, 36/37 for the fourth, 31 for the fifth and 47/46 for the sixth. Teeth were examined using a probe with two marks located at 3.5 and 5.5 mm. The Community Periodontal Index scores used for recording periodontal status were as follows: 0 = healthy periodontium; 1 = moderate bleeding; 2 = presence of supra- or subgingival dental calculus; 3 = periodontal pocket measuring 4–5 mm; and 4 = periodontal pocket 6 mm or more in depth.

Determination of plasma melatonin

Both test and control patients reported to the laboratory at 08:00 h and were seated for 30 min before sampling. Blood samples (20 mL) were collected from the antecubital vein and centrifuged at 3000 *g* for 10 min, followed by separation of the plasma fraction, which was then frozen (−20°C) until assay (39). Plasma melatonin was determined using a commercial radioimmunoassay (DVD Biochemie GmbH, Marburg, Germany) (40,41), and a quality control was performed yielding intra- and interassay coefficients of variation of 10.5% and 5.9%, respectively. The recovery of added melatonin was 86.3%, with an assay sensitivity of 4.45 pg/mL.

Determination of salivary melatonin

Saliva was obtained after chewing a piece of paraffin. Saliva produced during the first 2 min was discarded. Saliva (5–10 mL) was then collected during the following 5 min, avoiding any possible contamination. The saliva samples were centrifuged at 3000 *g* for 20 min, and the clear supernatant was frozen at −20°C. Melatonin levels in saliva were measured by radioimmunoassay (IBL GmbH, Hamburg, Germany) (42), and a quality control was performed yielding intra- and interassay coefficients of variation of 11.8% and 6.3%, respectively. The recovery of added melatonin was 82.5%, with an assay sensitivity of 2.09 pg/mL.

Determination of lymphocyte subpopulations

A flow cytometer (Becton Dickinson FACS Vantage; Becton Dickinson, Franklin Lakes, NJ, USA) was used to quantify the lymphocyte subpopulations.

Determination of $CD4^+$ and $CD8^+$ lymphocytes

CD4 and CD8 are markers for T lymphocytes. Ortho Trio CD4/CD8/CD3 monoclonal antibodies were used to identify these subpopulations. These antibodies are supplied as a mixture of three mouse monoclonal antibodies: CD4 conjugated to fluorescein isothiocyanate; CD8 conjugated to phycoerythrin; and CD3 conjugated to the tandem cyano-5-phycoerythrin.

Determination of $CD16^+$ and $CD19^+$ lymphocytes

CD16 and CD19 are markers for B lymphocytes. We used Ortho Trio CD16/CD19/CD3 monoclonal antibodies, which are supplied as a mixture of three mouse monoclonal antibodies (CD16 conjugated to fluorescein isothiocyanate, CD19 conjugated to phycoerythrin and CD3 conjugated to the tandem cyano-5-phycoerythrin), to identify these subpopulations.

Determination of natural killer cells

The clone HP 2/1 (conjugated to fluorescein isothiocyanate) was used to identify natural killer cells. The reagent from Immunotech-Coulter Co. (Marseille, France) (cat. no: 1404) was used in the test.

Statistical analysis

Quantitative variables were expressed as mean ± standard deviation, whereas absolute and relative frequencies were calculated for qualitative variables. The Mann–Whitney *U*-test was used to correlate quantitative with qualitative variables, and the Student's *t*-test was applied to compare the means of quantitative variables. The Spearman correlation coefficient was used to correlate quantitative and qualitative variables. The behavior of the lymphocyte subpopulations (expressed as percentages) was investigated using the Welch test. The relationship between a qualitative variable with more than two modalities (Community Periodontal Index) and the quantitative variables was examined by the Kruskal–Wallis test and/or one-way analysis of variance.

Results

Table 1 shows the comparison between periodontal disease patients and healthy individuals. The patients with periodontal disease had significantly lower plasma and salivary melatonin concentrations than the healthy subjects ($p < 0.001$). However, the salivary/plasma melatonin ratio was similar in both groups. A significant, negative correlation was found between plasma melatonin and age in the control group ($r = -0.672$, $p < 0.001$). In healthy individuals, the older the patient, the higher the plasma melatonin concentration. However, this correlation was not noted in the periodontal disease patients ($r = 0.02$, not significant). When the percentage of lymphocyte subpopulations was examined, no difference was noted between test and control subjects, except for CD3. Periodontal disease patients showed a significantly higher percentage of $CD3^+$ lymphocytes than the control group ($p < 0.05$).

Application of the Kruskal–Wallis test yielded a *p*-value of < 0.001, reflecting the age-dependent increase in the Community Periodontal Index (Table 2).

A biphasic relationship was observed between plasma melatonin and the Community Periodontal Index. The plasma melatonin level was decreased in patients with a Community Periodontal Index of 1–2 (the youngest patients), and increased to peak values in patients with a Community Periodontal Index of 4. Salivary melatonin was shown to parallel the changes of plasma melatonin (Fig. 1).

Patients with a Community Periodontal Index score of 4 showed the highest T-cell counts (Fig. 2). On evaluating $CD4^+$ cell behaviour and the Community Periodontal Index ($F = 0.77$; not significant), it was found that when the Community Periodontal Index was ≥3, the $CD4^+$ lymphocyte count increased. Moreover, in patients with a Community Periodontal Index of 4, the $CD8^+$

Table 1. Comparison of the study variables between the controls and patients with periodontal disease

Variable	Controls ($n = 26$)	Periodontitis patients ($n = 46$)
Age	47.2 ± 9.1	52.07 ± 9.3
PM	14.33 ± 4.05	9.46 ± 3.18**
SM	4.22 ± 0.87	2.55 ± 0.99**
SM/PM ratio	0.30 ± 0.06	0.31 ± 0.07
$CD3^+$	73 ± 11.2	78.39 ± 7.89***
$CD19^+$	9.5 ± 3.0	10.31 ± 4.43
$CD4^+$	44.5 ± 13.7	46.56 ± 9.17
$CD8^+$	29.5 ± 7.8	28.29 ± 9.57
NK^+	10.5 ± 8.2	11.91 ± 6.35

Data are given as mean ± SD. Age is expressed in years, melatonin in pg/mL, and the lymphocyte subpopulations in percentages. Values in parenthesis indicate the number of cases.
NK, natural killer; PM, plasma melatonin; SM, salivary melatonin.
**$p < 0.001$ vs. control.
***$p < 0.05$ on applying the Welch test.

Table 2. Mean patient age according to the Community Periodontal Index (CPI) score

CPI	*n*	Age (years)
1	9	39.9 ± 10.6
2	11	50.2 ± 8.6
3	17	56.6 ± 10.3
4	9	61.6 ± 7.8

Data are expressed as the mean ± SD; *n* = number of cases.

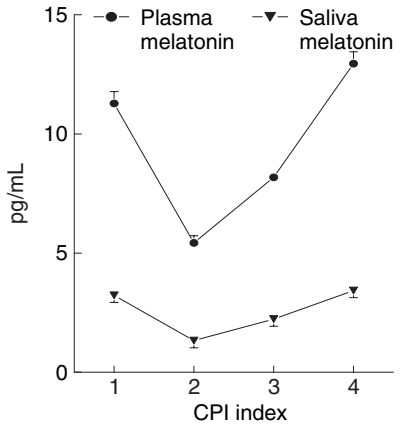

Fig. 1. Plasma and saliva melatonin levels in patients with periodontal disease.

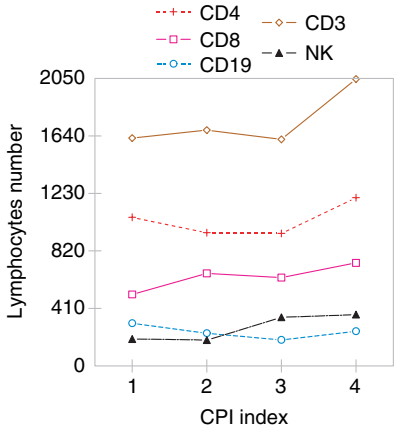

Fig. 2. Lymphocyte subpopulations among periodontal patients in relation to the Community Periodontal Index (CPI). NK, natural killer.

T-cell count was increased ($F = 0.50$; not significant). The lowest $CD8^+$ count was observed in subjects with a Community Periodontal Index score of 1 ($F = 0.43$; not significant) (Fig. 2).

The patients with a Community Periodontal Index score of 1 presented the highest number of B lymphocytes (Fig. 2). The number of B lymphocytes decreased at a Community Periodontal Index score of 2 (age inflexion point) to a minimum at a Community Periodontal Index score of 3. Nevertheless, a rise was subsequently recorded at a Community Periodontal Index score of 4 ($F = 0.77$; not significant). Regarding the natural killer cells ($F = 1.46$; not significant), the counts increased with increasing Community Periodontal Index score.

An r of -0.305 ($p < 0.05$) was found between the percentage of $CD4^+$ T cells and plasma melatonin concentration. Similar results were obtained on relating $CD4^+$ percentage to salivary melatonin, because the salivary concentrations of the hormone parallel those found in plasma ($r = -0.255$; $p < 0.01$).

Discussion

The importance of melatonin in relation to the immune system has been established, and melatonin is presently being used as coadjutant therapy in patients with certain tumors because it effectively reinforces the host immune defenses (26,43,44). In the present study, melatonin levels in healthy subjects were significantly lower in older individuals. This situation could be attributed to the known decrease in the production of melatonin as individuals begin to age (i.e. at around 40–45 years old). However, the older patients preserved their response capacity to Community Periodontal Index scores of 3 and 4, attaining melatonin concentrations, in plasma (Community Periodontal Index 3, 8.5 pg/mL; Community Periodontal Index 4, 13.5 pg/mL) and in saliva (Community Periodontal Index 3, 2.5 pg/mL; Community Periodontal Index 4, 3.8 pg/mL), that were lower than in healthy individuals but sufficient to maintain periodontal stability (Tables 1 and 2). In earlier studies (22,23), this increase in salivary and plasma melatonin resulted in stimulation of the $CD4^+$ T cells, which possess membrane and nuclear receptors for the hormone (20,21). This would stimulate the other immune cell populations via cytokine secretion (e.g. $CD3^+$, $CD19^+$, $CD4^+$ and $CD8^+$ cells with natural killer cell stimulation) – thereby facilitating the host reaction to an existing oral infection. This is in agreement with our data that indicated the higher the Community Periodontal Index index score, the greater the total lymphocyte counts ($F = 0.73$; not significant). Furthermore, our data also showed that when oral health is deficient and infectious foci develop (e.g. at a Community Periodontal Index score of 3), the $CD4^+$ lymphocyte count expanded – in close similarity to the melatonin concentration curve.

Data from our study showed that periodontal disease patients with lower $CD4^+$ counts tend to exhibit higher melatonin levels. Considering that the immune cell populations increase as melatonin concentrations rise in situations of increasing Community Periodontal Index scores, the question arises as to what causes melatonin to increase at a given moment and stimulate the host immune system. In this context, melatonin possesses two functions of great interest to dental professionals: (i) its capacity to scavenge free radicals, thereby exerting antioxidative action (clearly surpassing all known antioxidants such as vitamin C, E and coenzyme Q) (45–49); and (ii) the cell-protective effect exerted by melatonin in situations of inflammation (13,28), stimulating bone regeneration by favoring collagen type I production (18) and modulating osteoblast and osteoclast activity (16,19).

On examining the effects of melatonin upon the immune system, it may be considered that abundant reactive oxygen species are produced in situations of periodontal disease, characterized by an increase in peroxidation products generated by the infiltrating polymorphonuclear cell population (31), this, in turn, leading to an increase in melatonin levels. From our perspective, this not only would stimulate the immune system through the plasma fraction of the hormone, but would also afford local protection though the salivary melatonin fraction (50). As a result, the bone and cell population affected by the periodontal process would be protected from the reactive oxygen species generated by the existing inflammatory process, and bone repair would also be stimulated. Such effects could open new perspectives for the treatment of oral inflammatory processes (50,51). In conclusion, the results obtained from this study suggest that melatonin could have a protective function in fighting periodontal infection. However, future studies in this area are encouraged to validate the initial results reported here.

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