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Melatonin levels in periodontal health and disease

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Background and Objective: The aim of this study was to measure melatonin levels in the gingival crevicular fluid and saliva of subjects with healthy periodontal tissues, plaque-induced gingival inflammation, chronic periodontitis and aggressive periodontitis.

Material and Methods: A total of 70 subjects were examined and assigned to four groups: healthy periodontium (10 subjects); plaque-induced gingival inflammation (20 subjects); chronic periodontitis (20 subjects); and aggressive periodontitis (20 subjects). Gingival crevicular fluid and saliva samples were collected from each subject and analyzed using ELISAs.

Results: The melatonin levels in both gingival crevicular fluid and saliva were lower in patients with chronic periodontitis (10.4 and 12.8 pg/mL, respectively) and aggressive periodontitis (8.4 and 8.8 pg/mL, respectively) than in patients with gingivitis (13.9 and 17.6 pg/mL, respectively) and in healthy subjects (16.6 and 22.9 pg/mL, respectively). The mean melatonin levels in both gingival crevicular fluid and saliva were statistically significantly higher in healthy patients compared with patients with chronic periodontitis and aggressive periodontitis; however, there was no significant difference in the plaque-induced gingival inflammation between the study groups.

Conclusions: The melatonin levels in gingival crevicular fluid and saliva are decreased in diseased periodontal tissues, especially periodontitis. The melatonin level was lowest in the aggressive periodontitis group.

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Periodontal diseases are versatile clinical entities with a complex, multifactorial etiology. The main initiating factor of the most prevalent forms of these diseases is dental plaque, which collaborates with a multitude of local and systemic risk factors, such as smoking, diabetes, putative pathogens and immune defects (1). Plaque-induced gingival inflammation (gingivitis) is the most common form of periodontal disease and may progress to periodontitis, which has several clinical forms. Of these forms, chronic and aggressive periodontitis share the clinical features of bone resorption and clinical attachment loss. The accepted definitions of the two conditions have changed regularly with increased understanding of the disease process (2). Aggressive periodontitis has a rapid rate of disease progression with the absence of large accumulations of dental plaque and calculus, and is seen in otherwise healthy individuals. This form of periodontitis usually affects younger people at or after puberty, and thus can be observed during the second and third decades of life (3).

Melatonin is a hormone secreted mainly by the pineal gland and to a lesser extent by the retina, lens, iris, ciliary body, lacrimal gland, skin and gut (4). It is released mainly during night and diffuses passively into saliva via the bloodstream, and can be reliably assayed (5). Although melatonin is present in food such as fruits, vegetables and wheat, the dietary source does not significantly contribute to the circulating levels of melatonin (6).

The daily variation persists in adulthood with peak serum levels occurring between midnight and 2 AM and is lowest during the day between noon and 2 PM (7). Eventually, in old age, this prominent night-time peak becomes markedly attenuated (8). Melatonin diffuses passively into saliva via the bloodstream, with the salivary melatonin concentration representing 24–33% of its plasma levels (5).

Melatonin exhibits a wide spectrum of activities, including antioxidant functions and protection of the mucosa against various irritants. Furthermore, it protects the oral cavity and the gastrointestinal tract from conditions such as stomatitis, esophagitis, gastritis and peptic ulcer (9). Melatonin appears to be an important modulator of the immune system as it enhances the natural and acquired immunity *in vivo*, and activates monocytes and neutrophils (10). Melatonin has an anti-inflammatory effect (11) and is chemotactic for cultured chick retinal pigment epithelial cells (12). It also stimulates type I collagen synthesis and promotes bone formation (13,14).

The relationship between periodontal status and melatonin levels is still unclear and inconclusive (15,16). The present study was conducted to measure melatonin levels in gingival crevicular fluid and saliva from subjects with and without periodontal disease. The null hypothesis assumes that there is no statistically significant difference among the study groups.

Material and methods

This study was approved by the Ethics Committee of the Riyadh Colleges of Dentistry and Pharmacy, Riyadh, Saudi Arabia. The exact procedures were explained to all patients who provided signed, informed consent before participation. At the initial examination, detailed medical and dental histories were obtained for each subject and this was followed by a complete periodontal examination. Subjects were excluded if they were smokers, pregnant, lactating, or had uncontrolled systemic disorders or intellectual disability. Patients on medications that could alter melatonin levels (e.g. antidepressants and antipsychotics) were also excluded. Patients who had periodontal treatment in the last 12 mo were not included in the study. Following history-taking and extra-oral and intra-oral examinations, thorough periodontal examinations were performed by the same examiner. Subjects were classified into the following four groups based on the diagnosis of the periodontal condition.

Periodontally healthy subjects

Subjects of this group were periodontally healthy; as confirmed by the gingival index of Loe and Silness (17), patients showed no attachment loss and recession with a sulcus depth not exceeding 3 mm. For both saliva and gingival crevicular fluid, sampling time was fixed to around 8.30 PM for all groups.

Plaque-induced gingival inflammation

Patients of this group had severe generalized plaque-induced gingival inflammation. Using the gingival index of Loe and Silness (17), patients had severe gingival inflammation, as defined by marked redness, hypertrophy, and tendency for spontaneous bleeding and ulceration. At least 18 teeth were present in each patient (excluding third molars), of which 12 teeth were posterior. Patients in this group had no evidence of attachment loss and had a clear diagnosis of plaque-induced gingival inflammation.

Chronic periodontitis

All patients of this group received a thorough periodontal examination. This included assessments of: clinical attachment level; probing depth; hygiene index; gingival index; bleeding on probing; tooth mobility; furcation probing; and gingival recession. Only patients with generalized severe chronic periodontitis were included. Extent was determined based upon the percentage of affected sites according to Armitage (18). Only patients with a generalized involvement (> 30% of sites involved) were included in this group. The group included patients with at least 18 remaining teeth (excluding third molars) of which at least 12 were molars/premolars. Severity of chronic periodontitis was determined on the basis of the amount of clinical attachment loss ($\geq$ 5 mm) (18). For collection of gingival crevicular fluid samples, four sites were randomly selected to obtain a representative sample for each patient. Owing to the natural random distribution of the disease, samples were not taken from specific areas.

Aggressive periodontitis

Patients of this group fulfilled the following criteria.

1 Apart from periodontitis, patients were clinically healthy.
2 Rapid rate of disease progression, as indicated by the age of the patient and the level of periodontal destruction.
3 Inconsistency between the amount of local deposits and the extent of tissue destruction.
4 Possible family history.

Full charting and sample collection for patients of this group were performed as previously described for the chronic periodontitis group. Agreement on the clinical diagnosis was made by two clinicians. At the end of data collection, a total of 70 subjects were included in the study. Detailed distribution of the age and gender distribution, and of periodontal clinical parameters, are shown in Table 1.

Sample collection

For saliva collection, participants were instructed not to eat, drink, chew gum or brush teeth for at least 30 min before sampling. The mouth was rinsed thoroughly with cold water 5 min before sample collection. To stimulate salivary flow, patients were asked to chew a piece of paraffin wax for 7 min (19). Saliva produced in the first 2 min was discarded, and only

Table 1. Gender, age and periodontal clinical data

	Gender/age distribution				Periodontal clinical data			
	Male	Female	Age range (years)	Age (years)	Tooth mobility	Periodontal pocket (mm)	Attachment loss (mm)	Gingival index
Group I (healthy periodontium)	7	3	20–44	28.3 ± 6.7	0	0	0	0
Group II (gingivitis)	18	2	18–40	29.9 ± 7.0	0	0	0	3
Group III (chronic periodontitis)	16	4	40–55	47.2 ± 4.6	2	5.7 ± 0.5	7.2 ± 0.2	2
Group IV (aggressive periodontitis)	15	5	28–40	34.7 ± 5.4	3	5.4 ± 0.5	8.0 ± 0.2	3

Values are given as mean ± SD, except for tooth mobility and gingival index which are given as the mode.

saliva generated in the remaining 5 min was collected. Samples were centrifuged at 2795 *g* for 20 min and the supernatant was collected and frozen at -20°C until required for analysis (20). To obtain gingival crevicular fluid, multiple test sites were randomly selected from the four quadrants. Test sites were dried and isolated with cotton rolls to prevent any contamination with saliva or blood. Before gingival crevicular fluid sampling, supragingival calculus was removed using suitable instrumentation. A standard volume of 5 μL of gingival crevicular fluid was collected extra-crevicularly over a 5 to 10 min time period using a volumetric, microcapillary pipette with a capacity of 1–5 μL. A pooled volume of gingival crevicular fluid was collected from healthy subjects, whereas for subjects with gingivitis and periodontitis, random sampling from four sites was performed. On visual examination, test sites that did not express any gingival crevicular fluid or yielded samples mixed with blood or saliva were excluded. The gingival crevicular fluid samples were stored at −20°C until evaluated. Collection of samples was performed by the same examiner.

Melatonin measurement

The melatonin levels in saliva and gingival crevicular fluid were measured using a competitive immunoassay [DRG® Melatonin Saliva, Marburg, Germany (Non-extraction) (SLV-4779); 1]. Direct Saliva Melatonin ELISA (DRG Instrumentals GmbH, Marburg, Germany) is a competitive immunoassay using a capture antibody technique. The assay follows the basic principle of competitive ELISA whereby there is competition between a biotinylated and a nonbiotinylated antigen for a fixed number of antibody-binding sites. The amount of biotinylated antigen bound to the antibody is inversely proportional to the concentration of analytes of the sample. When the system was in equilibrium, the free biotinylated antigen was removed by washing and the antibody bound to biotinylated antigen was determined using streptavidin peroxidase as the marker and tetramethylbenzidine (TMB) as the substrate. Values of > 50 μL pg/mL (Standard F) were diluted with Standard A into the linear range of the standard curve (e.g. by dilution 1:10 – example: 50 μL of saliva + 450 μL of Standard A). Dilution was made in glass tubes. The results obtained were multiplied by the dilution factor to obtain corrected results. Values lower than 0 pg/mL were not repeated.

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Statistical analysis

Data were collected and analyzed using Statistical Package for the Social Sciences, version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). First, a two-way analysis of variance (ANOVA) was performed to determine the interaction and the differences among the groups using the two sampling media (saliva and gingival crevicular fluid). ANOVA was followed by Scheffé's test to analyze the means of multiple paired comparisons that was deemed necessary after using ANOVA. An alpha level of 0.05 was used to indicate the statistical significance ($p \leq 0.05$).

Results

Melatonin was detected in all samples tested, and the level of melatonin varied among samples and groups. The levels of melatonin in saliva and gingival crevicular fluid showed a progressive decrease from healthy subjects to subjects with aggressive periodontitis. The highest concentration of melatonin was detected in the saliva and gingival crevicular fluid samples of the control group using the two sample sources. The concentration of melatonin in gingival crevicular fluid was 16.6 ± 4.2 pg/mL (mean ± standard error) while in saliva, the melatonin concentration was 22.9 ± 4.5 pg/mL. The lowest mean concentration of melatonin was detected in the aggressive periodontitis group (8.5 ± 0.9 pg/mL in gingival crevicular fluid and 8.9 ± 1.0 pg/mL in saliva). The concentrations of melatonin in both saliva and gingival crevicular fluid of all groups are shown in Table 2 and Fig. 1.

Two-way ANOVA was used to determine the effect of media (saliva and gingival crevicular fluid) and subjects (groups) on melatonin levels as a response (dependent) variable. Two-way ANOVA revealed no interaction between media and disease ($p = 0.764$), as indicated in Table 3. There was no significant difference between the media analyzed ($p = 0.099$), as shown

Table 2. Melatonin levels in saliva and gingival crevicular fluid samples from each study group

	Aggressive periodontitis		Chronic periodontitis		Gingivitis		Healthy periodontium	
	Gingival crevicular fluid	Saliva	Gingival crevicular fluid	Saliva	Gingival crevicular fluid	Saliva	Gingival crevicular fluid	Saliva
Mean	8.5	8.9	10.4	12.82	13.9	17.6	16.6	22.9
Standard error	0.9	1.0	1.1	1.9	1.8	3.6	4.2	4.5
Sample size (*n*)	20	20	20	20	20	20	10	10

All values are given as pg/mL.

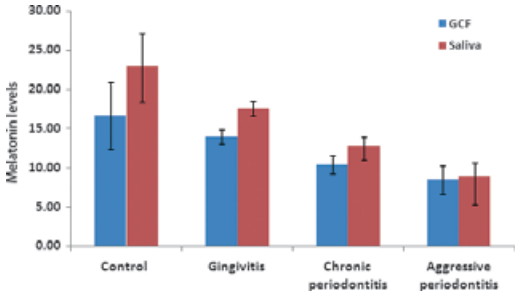

Fig. 1. Melatonin levels in saliva and gingival crevicular fluid samples. Data are presented as mean ± standard error.

Table 3. Two-way analysis of variance (ANOVA), performed to test differences in the concentrations of melatonin in saliva and gingival crevicular fluid among the study groups

Source	Type III sum of squares	df	Mean square	F	Significance
Corrected model	33.525	7	4.789	3.617	0.001
Intercept	1569.333	1	1569.333	1.185E3	0.000
Media	3.654	1	3.654	2.760	0.099
Disease	29.255	3	9.752	7.365	0.000
Media × disease	1.531	3	0.510	0.385	0.764
Error	174.772	132	1.324		
Total	1834.368	140			
Corrected total	208.297	139			

df, degree of freedom.

in Fig. 2. However, upon testing the effect of subject level (groups) on melatonin level, a statistically significant difference was detected ($p = 0.000$), as shown in Table 3.

Using Scheffé's multiple comparisons, significant differences were found upon comparing the healthy periodontium group with the chronic and aggressive periodontitis groups. A statistically significant difference was also found between the gingivitis group and the aggressive periodontitis group. The remaining paired comparisons revealed no statistical difference, as shown in Table 4.

Discussion

Periodontal diseases result from the effect of bacterial products, which participate significantly in the disease process, on the host response. It has been suggested that certain biologic mediators contribute significantly to the protection or destruction of periodontal tissues. The well-known destructive factors include the bone-resorbing mediators, specifically, interleukin-1, prostaglandin E_2 and tumor necrosis factor alpha (21). Detection and evaluation of local or systemic mediators, of a destructive and/or protective nature, on the periodontium are routinely performed using oral fluids specifically, saliva and gingival crevicular fluid. These fluids are valuable for use in the diagnosis of periodontal diseases as well as for detecting systemic problems. Significant data and evidence have accumulated from the use of gingival crevicular fluid and saliva for the diagnosis of periodontal diseases, as well as from the evaluation of periodontal tissues in health and disease (22,23).

In the present investigation, gingival crevicular fluid and saliva were collected to assess melatonin levels and any possible variability of this hormone in periodontal health and disease. Samples were taken during evening (8–9 PM) to ensure that the sampling time would not be affected by the diurnal cycle of melatonin, which peaks between midnight and 2 AM (7). Within the limitations of the current study, although the salivary levels of melatonin were higher than the gingival crevicular fluid levels of melatonin, the difference between both sampling methods was found to be statistically insignificant, confirming the findings of other investigators (15). Therefore, the remaining part of this section will focus on the level of melatonin, regardless of its sampling method.

In the current study, it was interesting to find that higher melatonin levels were related to health or

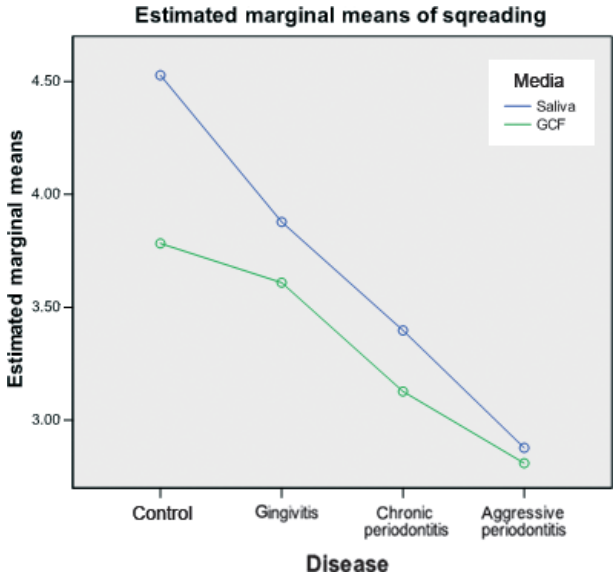

Fig. 2. Interaction between the media (saliva and gingival crevicular fluid) and the melatonin level in the different study groups.

Table 4. p values of Scheffé's test for multiple paired comparisons

	Healthy periodontium	Gingivitis	Chronic periodontitis
Aggressive periodontitis	0.001*	0.008*	0.452
Chronic periodontitis	0.050*	0.326	
Gingivitis	0.636		

*Significance at $p \leq 0.05$.

inflammation (gingivitis), but not to periodontal destruction (periodontitis). This was confirmed by the detection of higher levels of melatonin in samples taken from the healthy and gingivitis subjects. On the other hand, lower melatonin levels were found in both aggressive and chronic periodontitis groups compared with healthy and gingivitis groups. Although the melatonin level was lower in the periodontitis groups compared with healthy subjects and the plaque-induced gingival inflammation group, the results revealed no statistically significant difference between the chronic and aggressive forms of periodontitis. This result confirms an earlier report, which revealed that melatonin levels varied from clinically healthy subjects to those with advanced periodontal destruction (15). However, this is the first study to investigate melatonin levels in aggressive periodontitis.

Factors that increase the host susceptibility to periodontal diseases include familial aggregation, single nucleotide polymorphisms, defective neutrophil functions or primed neutrophils, antibodies to bacteria, smoking, stress and herpesvirus infections (24). Therefore, periodontal diseases are multifactorial in etiology. The overall finding of higher levels of melatonin in health may indicate a protective role of this hormone. In addition, lack of melatonin in periodontitis may emphasize the role of remotely produced molecules in the pathogenesis of periodontitis and may add to the overall picture and the currently familiar paradigm of periodontal pathogenesis.

In the study of periodontal disease pathogenesis, some biologic mediators of the host response are protective to periodontal tissues, as in other parts of the body. Antioxidants are protective elements that reduce the quantity of free radicals that are destructive in nature. Many of these free radicals originate from periodontopathic bacteria (25). It has been suggested that an increase in both reactive oxygen and nitrogen during periodontal disease is responsible for the oxidative damage to periodontal tissues (26). The increase in free radical production coexists with a decrease in antioxidant defence. The imbalance between the pro-oxidant and antioxidant systems may lead to further oxidative attack and substantial deterioration of the periodontal tissues (27). Therefore, a factor with an antioxidant effect would counteract the adverse effects of the periodontopathic bacteria, at least indirectly. The exact mechanism through which melatonin may affect the periodontium has not been thoroughly investigated. The well-known effects of melatonin are its antioxidant, anti-aging, anti-inflammatory and antimicrobial actions in the medical and dental fields (28). The increased reactive oxygen and nitrogen products that are present in advanced periodontal disease are responsible for the oxidative damage to the periodontal tissues (27). Melatonin may reduce this oxidative stress and hence participate in tissue protection. It was proposed that the increased generation of free radicals coexists with a decrease in the antioxidant defence mechanisms (29). Therefore, the decrease of melatonin levels in periodontitis might be the result of by-products or mediators that could interfere with the melatonin levels. A decrease of the melatonin level as an antioxidant in advanced periodontal diseases may cause an imbalance between the pro-oxidant and antioxidant systems, which may lead to a further oxidative attack and a marked deterioration of the periodontal tissues (29).

The protective role of melatonin on periodontal tissues might be explained to some extent by its antimicrobial

action against *Porphyromonas gingivalis*, *Streptococcus mutans* and *Prevotella intermedia* (15). Melatonin appears to be an important modulator of the immune system as it enhances the natural and acquired immunity *in vivo* and activates monocytes and neutrophils (10), in addition to its anti-inflammatory effect (11). Melatonin may modulate periodontal destruction by interfering with prostaglandin E_2, thereby inhibiting the differentiation of osteoclasts (30). Furthermore, melatonin can modulate some of the proteins that regulate the bone-resorption process in periodontal disease and interact with other biologic agents, such as calcitonin (31). Melatonin may act at the level of the osteoclast lacuna because of its antioxidant properties and its ability to neutralize reactive species, where it inhibits bone resorption (32). It stimulates osteogenic differentiation in bone marrow stem cells, induces the proliferation and differentiation of osteoblasts and increases gene expression of type I collagen, osteopontin, bone sialoprotein and osteocalcin (33,34).

The clinical use of melatonin as an immunotherapeutic agent seems promising as the application of melatonin in dental sockets after tooth extraction and placement of endosseous dental implants in beagle dogs resulted in a greater amount of new-bone formation in contact with the implant in primary and secondary immunodeficiencies (35). Within the results of the current study, further investigations into the effect of melatonin on immune functions and bone metabolism in the periodontium are recommended. Cutando *et al.* (7), found that salivary melatonin levels vary according to the degree of periodontal disease. As the degree of periodontal disease increased, the salivary melatonin level decreased, indicating that melatonin may act to protect the body from external bacterial insults. Therefore, melatonin could be potentially valuable in the treatment of periodontal diseases.

The present investigation revealed statistically reduced levels of melatonin in subjects with periodontitis as compared with healthy subjects and those with gingivitis. Reduction of the melatonin level may contribute to depression and insomnia, which are usually associated with aggressive periodontitis. This is the first study to evaluate the levels of melatonin in subjects with aggressive periodontitis. In another study, it was concluded that the systemic manifestations of fatigue, depressive mood, loss of appetite and weight loss were strongly associated with aggressive periodontitis (36). Therefore, the reduced level of melatonin indicates a negative psychologic effect, which may explain the presence of aggressive and severe periodontitis in individuals suffering from stress (37). The overall result of reduction of melatonin and increased stress may contribute to periodontal destruction, which might have an opposite effect to cortisol. Another explanation of the positive effects of melatonin on periodontal structures is its antagonist effects on matrix metalloproteinases, which are key elements of periodontal destruction (38,39). Further investigations are required to elaborate on the possible protective effect of melatonin on specific mediators involved in periodontal destruction.

The findings of the current study, although preliminary, may encourage further research on the use of melatonin as an adjunctive diagnostic marker of periodontal disease. Melatonin may be added to the list of gingival crevicular fluid and saliva components currently used to differentiate between periodontitis, which is characterized by attachment loss, and other periodontal conditions without loss of attachment (gingivitis).

Conclusions

The following points can be concluded from this study.

1 The levels of melatonin in saliva and gingival crevicular fluid decrease in periodontal disease, especially periodontitis.
2 The decrease in melatonin levels in diseased periodontal tissues might be related to the absence of its protective role or to degradation by inflammatory mediators of periodontal destruction. Further research, particularly on patients taking melatonin as a medication, is recommended.

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