Salivary and Gingival Crevicular Fluid Melatonin in Periodontal Health and Disease

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Background: Melatonin, with its antioxidant properties, plays a pertinent role in influencing the pathogenesis of periodontal disease. This study aims to detect the presence of melatonin in gingival crevicular fluid (GCF) and to assess the levels of salivary and GCF melatonin in periodontal disease and show the correlation between salivary and GCF melatonin.

Methods: Forty-five subjects, based on the gingival and Russell periodontal indexes, were grouped as 15 healthy subjects (group 1), 15 subjects with gingivitis (group 2), and 15 subjects with periodontitis (group 3). Saliva and GCF samples were collected from all subjects. Melatonin levels were assessed using an enzyme-linked immunosorbent assay. The paired-sample test was used to correlate between saliva and GCF.

Results: Melatonin was present in GCF (mean: 1.54 pg/ml) with significantly less concentration compared to that of saliva (mean: 2.17 pg/ml). Salivary and GCF melatonin levels were reduced to the lowest concentrations in patients with chronic periodontitis (salivary melatonin: 0.07 pg/ml; GCF melatonin: 0.06 pg/ml; $P<0.05$), which were inversely proportional to the clinical indices. There was no significant correlation between salivary and GCF melatonin levels ($P>0.05$).

Conclusions: Melatonin was expressed in GCF. Salivary and GCF melatonin levels varied from clinically healthy subjects (group 1) to subjects with periodontitis (group 3). Both salivary and GCF melatonin levels decreased in group 3 subjects compared to group 1 subjects, indicating that melatonin may have a protective role against periodontal disease, although further research is required to validate this hypothesis. *J Periodontol 2010;81:277-283.*

KEY WORDS

Enzyme-linked immunosorbent assay; gingival crevicular fluid; melatonin; periodontal disease; saliva.

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Periodontal disease is a chronic inflammatory condition. Damage to periodontal tissues results from a direct effect of the toxic products released by bacteria and from the action of the immune system stimulated by the bacterial infection. A notable feature of PD is the generation of free radicals, some derived from the bacteria themselves, and others as a consequence of the immune response.[1] The increased reactive oxygen and nitrogen species that occur in periodontal disease are responsible for the oxidative damage to the periodontal tissues.[2] Also, the increase in free radical generation coexists with a decrease in the antioxidant defense mechanisms. This imbalance between the pro-oxidant and antioxidant systems may lead to a further oxidative attack and a marked deterioration of the periodontal tissues.[3] Melatonin is an indoleamine synthesized and secreted mainly by the pineal gland. Chemically, it is N-acetyl-5-methoxy-tryptamine derived from the amino acid tryptophan. It is difficult to assign a single specific function to melatonin because it is involved in the regulation of many different physiologic and behavioral processes. Melatonin was shown to exert its effect on the oral cavity as an antioxidant and free radical scavenger, an immune modulator, and as a promoter of bone formation.[4]

The regulation of melatonin secretion is under neural control. Sympathetic

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innervations seem to play a major role via its release of noradrenalin. Normal plasma melatonin levels range between 14 to 60 pg/ml. Melatonin has its highest levels in plasma during nighttime, peaking between 12:00 am and 2:00 am and also between 2:00 am and 4:00 am, and is lowest during the day (between 12:00 pm and 2:00 pm).[4]

Melatonin diffuses passively into saliva from the bloodstream. Seventy percent of plasma melatonin is albumin bound,[5] and because only the free melatonin in plasma is thought to be present in saliva, salivary melatonin levels (normal range: 2 to 4 pg/ml) reflect the proportion of free-circulating melatonin.[6] Factors such as smoking, exposure to light, alcohol consumption, and increasing age decrease the levels of salivary melatonin, whereas gender does not influence the levels of melatonin.[7] The levels of salivary melatonin decrease as the severity of disease increases.[8] Considering the strong analogy that exists between saliva and gingival crevicular fluid (GCF) as potential sources of periodontal disease markers, the possible presence of melatonin in GCF and its role in periodontal disease were considered. Therefore, this study aims to evaluate the presence of melatonin in GCF and assess the levels of salivary and GCF melatonin in periodontal health and disease.

MATERIALS AND METHODS

Forty-five subjects (23 females and 22 males; age range: 20 to 45 years) were recruited for the study, which was conducted in February and March of 2008 at the Sri Dharmasthala Manjunatheshwara College of Dental Sciences and Hospital. Ethical clearance was obtained from this institution, and written informed consent was obtained from all subjects before starting the study. Three groups with 15 subjects each were designated as: group 1 (clinically healthy subjects), based on a gingival index[9] score of 2 and 3; group 2 (subjects with gingivitis), based on a Russell periodontal index[10] score of 6 and 8; and group 3 (subjects with chronic generalized periodontitis). Group 3 included subjects with moderate to severe chronic generalized periodontitis.[11]

Subject inclusion criteria were as follows:

1) Patients with a varying degree of periodontal disease (healthy, gingivitis, and chronic generalized periodontitis).
2) Good general health.
3) No invasive periodontal therapy in the past 6 months.

Subject exclusion criteria were as follows:

1) Systemic diseases like diabetes mellitus.
2) Neurologic disorders such as epilepsy and schizophrenia.
3) Pregnant subjects.
4) Smokers and alcoholics.
5) The presence of disease with possible effects on the immune system, e.g., chronic infection or cancer.
6) Treatment with any drug that might alter melatonin levels (e.g., diazepam).
7) The use of any antibiotics in the previous 6 months, and patients who had undergone non-invasive periodontal therapy (scaling and root planing).

A dental and medical history was compiled for all subjects with an oral examination, including caries assessments. The gingival index[9] and Russell periodontal index[10] scores were collected for each subject. The same investigator (RS) performed all data collection and examinations.

Determination of Salivary and GCF Melatonin

Collection of saliva. Participants were instructed to refrain from eating, drinking, and practicing oral hygiene habits after 12:00 am on the day of the saliva and GCF sample collection. Both test and control subjects reported to the hospital at 8:00 am. Samples were collected under dim light. Low-intensity light was used because a high-intensity light source diminishes the secretion of melatonin.[12,13] One milliliter of unstimulated saliva was collected using a collection device† (avoiding any possible contamination). The saliva samples were centrifuged at 3,000 × g for 20 minutes, and the clear supernatant was stored at – 20°C until an assay was performed.

Collection of GCF. Multiple test sites were dried and isolated with cotton rolls to prevent any contamination from saliva and blood. Prior to GCF sampling, supragingival calculus was removed using sterile curet. A standard volume of 5 μl was collected extracrevicularly using a calibrated, volumetric, microcapillary pipette measuring 1 to 5 μl with a plunger for 5 to 30 minutes. A pooled volume of GCF was collected for healthy subjects, whereas for subjects with gingivitis and periodontitis, site-specific scoring was followed. Samples were collected from sites exhibiting severe inflammation with a gingival index[9] score of 2 and 3 for group 2 and sites exhibiting a probing depth >5 mm for group 3. On visual examination, test sites that did not express any volume of GCF and micropipettes contaminated with blood and saliva were not included in the study. The GCF obtained was stored at – 20°C until the assay was performed.

Melatonin determination. Melatonin levels in saliva and GCF were measured using a competitive immunoassay‡ per the manufacturer's instructions. The competitive immunoassay uses a capture antibody (Ab) technique and the polyclonal Kennaway G280 antimelatonin Ab. A plate with eight-well strips was enough to test the samples.

† Buhlmann Saliva Collection Device, Buhlmann Laboratories, Schönenbuch, Switzerland.
‡ Buhlmann Direct Saliva Melatonin ELISA (EK-DSM), Buhlmann Laboratories.

After appropriate dilution, 200 μl of samples was used for the assay. Samples were pretreated per manufacturer's guidelines. During the first 3 hours of incubation, melatonin present in the pretreated saliva and GCF, controls, and ready-to-use standards, respectively, competed with the biotinylated melatonin for binding sites of this highly specific Ab.

After washing, the enzyme label, streptavidin conjugated to horseradish peroxidase, was added, which bound during a second 60-minute incubation step to melatonin-biotin-Ab complexes captured on the coated wells. The unbound enzyme label was removed by a second washing step, and a tetramethylbenzidine substrate was added to the wells. In a third 30-minute incubation step, a colored product was formed in inverse proportion to the amount of melatonin present in the sample. The color turned from blue to yellow after the addition of an acidic stop solution and was measured at 450 nm.

Statistical Analyses

The collected data were entered in a spreadsheet application and statistical analyses were performed using a statistical program.§ One-way analysis of variance (ANOVA) was used to test the significant difference among the groups. The Scheffe test was used to compare each group separately. To determine the correlation between the clinical indices and the levels of melatonin, parametric Pearson correlation analysis and tests of significance were used. Statistical significance was established at $P<0.05$. The paired-sample test was used to compare salivary and GCF melatonin.

RESULTS

All samples in each group showed the presence of melatonin. The highest concentration was in group 1 for saliva and GCF (saliva melatonin: 2.17 pg/ml; GCF melatonin: 1.54 pg/ml), and the lowest mean melatonin was in group 3 (saliva melatonin: 0.07 pg/μl; GCF melatonin: 0.06 pg/ml). The mean concentration for all groups for saliva and GCF is shown in Table 1. One-way ANOVA was used to test the significant difference among the groups, and a significance difference in the mean concentration of melatonin was observed in saliva and GCF (Table 2).

Multiple comparisons using the Scheffe test were carried out to compare the concentration of melatonin for all groups for saliva and GCF and to determine which pair or pairs were significant at a 5% confidence interval (Table 3). A statistically significant difference was obtained in salivary melatonin levels in all three groups, but when GCF melatonin levels were compared between groups 2 and 3, no significant difference was present ($P>0.05$).

Table 1.

Melatonin Levels (pg/ml) in Saliva and GCF in Study Subjects

Groups	n	Mean	SD
Salivary samples			
Healthy (group 1)	15	2.17	0.43
Gingivitis (group 2)	15	0.67	0.73
Periodontitis (group 3)	15	0.07	0.098
GCF samples			
Healthy (group 1)	15	1.54	0.46
Gingivitis (group 2)	15	0.25	0.14
Periodontitis (group 3)	15	0.06	0.12

Table 2.

One-Way ANOVA to Test the Significant Differences in Concentrations Among the Study Groups for Saliva and GCF

Melatonin	Sum of Squares	Mean Square	Significance
Saliva	34.90	17.45	0.00
GCF	19.34	9.67	0.00

The results suggest that the levels of salivary and GCF melatonin decreased from healthy subjects to subjects with periodontitis.

A comparison between the levels of melatonin and the indices was done using the Pearson correlation coefficient (Tables 4 and 5). There was strong positive correlation between the concentration of melatonin and clinical indices in saliva and GCF, showing that the levels of both salivary and GCF melatonin decreased as the values of the indices increased.

Furthermore, the paired-sample test was used to compare saliva and GCF (Table 6), and there was no statistically significant correlation between saliva and GCF ($P>0.05$).

DISCUSSION

Melatonin, a hormone secreted by the pineal gland has recently received considerable attention because of its antioxidant, antiaging, and anti-inflammatory properties in the medical and dental fields. The presence of melatonin in saliva and serum was recognized.[4-8] However, the presence of melatonin in GCF was not assessed. Studies by Eley and Cox[14] and Page[15] showed that the components of periodontal inflammation and tissue degradation increased in GCF in accordance with the severity of disease and,

§ SPSS statistical package (PC version 7.0), SPSS, Chicago, IL.

Table 3.

Scheffe Test for Comparing Among the Study Groups for Saliva and GCF

Dependent Variable	(I) Group	(J) Group	Mean Difference (I–J)	Significance
Saliva melatonin	1	2	1.498*	0.000
		3	2.093*	0.000
	2	1	–1.498*	0.000
		3	0.595*	0.007
	3	1	–2.093*	0.000
		2	–0.595*	0.007
GCF melatonin	1	2	1.2840*	0.000
		3	1.4771*	0.000
	2	1	–1.2840*	0.000
		3	0.1931	0.201
	3	1	–1.4771*	0.000
		2	–0.1931	0.201

The letters (I) and (J) are used for statistical convenience to show the comparison between the groups.
* The mean difference was significant at the <0.05 level.

Table 4.

Correlation Between the Gingival Index (GI) and Salivary and GCF Melatonin in All Groups

Groups	GI	Saliva Melatonin	GCF Melatonin
Salivary melatonin			
Pearson correlation	–0.929*	1.000	0.826*
Significance (two-tailed)	0.00	0.00	0.000
N	45	45	45
GCF melatonin			
Pearson correlation	–0.850*	0.826*	1.000
Significance (two-tailed)	0.00	0.00	0.00
N	45	45	45

* Correlation was significant at the 0.05 level.

therefore, were correlated with the disease severity and disease activity. The presence and activity of melatonin in saliva were proven;[5] however, the presence of melatonin in GCF was not explored. Therefore, this study aims to evaluate the presence of melatonin in GCF, its level in periodontal health and disease, and to compare it with salivary melatonin.

The GCF from healthy subjects was collected to assess whether melatonin was secreted and to correlate it with periodontal disease. Supragingival calculus was removed, and the test sites were dried and isolated to prevent any contamination from products of supragingival calculus, saliva, and blood. Contamination with saliva and blood alters the levels of GCF. Salivary samples were collected to correlate the levels of melatonin in GCF. Stimulating salivary secretions by using paraffin and citric acid altered the levels of melatonin.[5] Hence, unstimulated saliva was collected.

This study shows the presence of melatonin in GCF in a concentration ~60% less than that of serum melatonin (14 to 60 pg/ml).[4] This amount cannot be standardized due to the relatively small sample size (15 subjects). Therefore, additional studies are needed to standardize the concentration of melatonin in GCF. However, when the levels of melatonin in GCF were analyzed, there was a reduction in melatonin levels from healthy subjects (group 1) to subjects with periodontitis (group 3). The levels of salivary and GCF melatonin were compared to clinical indices that included the gingival index[9] and Russell periodontal index.[10] The Russell periodontal index[10] was used to categorize patients as a periodontitis group, but it does not indicate disease severity. The other limitations of the Russell periodontal index[10] are that it does not grade clinical attachment loss, it scores gingivitis and periodontitis on the same weighted scale, it does not elaborate disease distribution because it compresses all the groups into one group, and it does not provide information regarding disease progression.[16] Because the present study may be the first of its kind, cutoff parameters need to be validated in future studies. This may be one limitation of the present study. Results of this study demonstrate the presence of melatonin in GCF in a concentration of 1.54 pg/ml, which was ~30% less than that of saliva (i.e., 2.17 pg/ml) (Table 1). This could be attributed to the fact that salivary melatonin was 24% to 30% less than that of plasma (plasma melatonin: 14 to 60 pg/ml), and therefore, GCF melatonin was ~60% less than that of plasma melatonin,[4] and both salivary and GCF melatonin levels were reduced in subjects with gingivitis and periodontitis compared to healthy subjects.

The Kennaway G280 antimelatonin Ab incorporating the enzyme-linked immunosorbent assay was used in this study, which, to our knowledge, was a new procedure and provided an assay of circulating melatonin.[17] This required a minimum sample of 200 μl, and thus, we chose to collect 5 μl pooled GCF from the deepest sites using microcapillary pipettes, which was then diluted with 195 μl phosphate buffered saline to get a standardized volume of 200 μl. Forty-five patients (23 females and 22 males; age range: 20 to 45 years) were recruited, as evidence suggested that melatonin secretion and concentration varies considerably according to age. Infants <3 months of age secrete very little melatonin.[18] The peak nocturnal concentrations are highest between the ages of 1 to 3 years, after which they decline gradually. In normal

young adults, the average daytime and peak nighttime values for serum melatonin are 10 and 60 pg/ml, respectively.[18] In older adults, issues such as hyposalivation limit the feasibility and validity of salivary melatonin.[8] Light has two effects on melatonin: day–night cycles modify the rhythm of its secretion, and brief pulses of light of sufficient intensity and duration abruptly suppress its production. In normal subjects, exposure to light inhibits melatonin in a dose-dependant manner. The threshold is 200 to 400 lux (equivalent to ordinary fluorescent light), and maximal inhibition occurs after exposure to intense light (≥600 lux).[12,13] Hence, the samples were collected under dim light. Gender does not influence the secretion and regulation of melatonin.[19]

The results revealed the presence of melatonin in GCF, and the mean melatonin level reduced from groups 1 to 3 in saliva and GCF. These values ran inversely proportional to the values of clinical indices (i.e., the more severe the inflammation, the higher the index score and the lower the melatonin level) (Tables 4 and 5). This supports the antioxidant and anti-inflammatory effects of melatonin.[4]

When compared to GCF melatonin using the paired-sample test, salivary melatonin levels exhibited a clinically significant reduction in GCF and salivary melatonin, but statistically, there was no significant correlation between saliva and GCF (Table 6).

Although the collection of GCF using microcapillary pipettes was used by various authors, namely Krasse and Eagelberg,[20] Mann,[21] and Kaslick et al.,[22] Cimasoni reported it to be a deceiving method with regard to the collection of a sample in normal subjects.[23] This technique appears to be ideal because it provides an undiluted sample of native GCF with a volume that can be accurately assessed; however, it is difficult to collect an adequate volume of GCF in a short period. Another complication of this technique is the difficulty of removing the complete sample from the tubing.[24] Therefore, we used a microcapillary pipette with a plunger. However, it was shown that an average of 10 to 40 μl GCF can be collected in subjects with a gingival and plaque index score of 3.[23] Considering the difficulties in the methods of collection and the quantity of GCF, the assessment of salivary melatonin could be considered a better marker of periodontal disease.

Melatonin modulates the development of periodontal disease by acting on prostaglandin E_2, thereby inhibiting the differentiation of osteoclasts induced by cell-to-cell

Table 5.

Correlation Between the Russell Periodontal Index (RI) and Salivary and GCF Melatonin

Groups	RI	Saliva Melatonin	GCF Melatonin
Salivary melatonin			
Pearson correlation	–0.689*	1.000	0.826*
Significance (two-tailed)	0.00	0.00	0.00
N	45	45	45
GCF melatonin			
Pearson correlation	–0.591*	0.826*	1.000
Significance (two-tailed)	0.00	0.00	0.00
N	45	45	45

* Correlation was significant at the 0.05 level.

Table 6.

Paired-Sample Test Between Salivary and GCF Melatonin Levels Among Study Groups

	Paired Differences							
				95% Confidence Interval of the Difference				
Groups	Mean	SD	SE of the Mean	Lower	Upper	*t*	df	Significance (two-tailed)
Pair 1								
Healthy (saliva) Healthy (GCF)	0.63 000	0.46 2	0.12 871	0.38 92	0.88 08	5.33	14.0	0.000
Pair 2								
Gingivitis (saliva) Gingivitis (GCF)	0.414 000	0.788 6	0.20 388	–0.02 23	0.85 23	2.04	14.0	0.06
Pair 3								
Periodontitis (saliva) Periodontitis (GCF)	0.011 800	0.5 5	0.037 734	–0.07 32	0.093 32	0.31	14.0	0.76

t = test for equality of means; df = degrees of freedom.

contact between osteoblasts and osteoclasts.[7] Moreover, melatonin can modulate all proteins that regulate the resorption process in periodontal disease and interact with other biologic agents like calcitonin, therefore inhibiting bone resorption or promoting bone-marrow cell differentiation.[7]

Although melatonin is secreted in a very minimal concentration compared to some of the other biochemical markers of periodontal disease, taking its anti-inflammatory, bone-forming, and antioxidant properties[5] into account, it may still be considered a marker of periodontal disease. We tested the antimicrobial property of melatonin tablets (30 mg), and the results showed that the isolated strains of *Porphyromonas gingivalis*, *Streptococcus mutans*, and *Prevotella intermedia* were sensitive at 4, 2, and 2 μg, respectively, opening up the possibility of using melatonin as an antibacterial agent locally (unpublished data).

Considering the various pharmacologic and physiologic actions of melatonin, melatonin may be considered one of the markers of periodontal disease. However, more studies are needed to support this hypothesis.

CONCLUSIONS

The results of the present study show the presence of melatonin in GCF. It can be hypothesized that there is a substantial reduction in salivary and GCF melatonin concentration as the severity of periodontal disease increases. Thus, GCF melatonin could be useful for monitoring the severity of periodontal disease. However, further longitudinal studies with a larger sample size are required to confirm the concentration of melatonin in GCF and its variation as the severity of disease increases.

Within the limitations of this study, the following conclusions could be drawn:

1) Melatonin was expressed in GCF and may be considered one of the markers of periodontal disease.

2) GCF melatonin within this sample size was ~1.54 pg/ml, which was 60% less than that of serum.

3) Further research is required to standardize the amount of GCF melatonin.

4) Levels of salivary and GCF melatonin decreased in the gingivitis and periodontitis groups compared to the healthy group, showing the protective nature of melatonin, which is mainly credited to its anti-inflammatory properties.

5) Although there was no significant correlation between salivary and GCF melatonin, GCF may still act as a source of melatonin to study the activity of periodontal disease. Further research is warranted in this field.

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