ORIGINAL RESEARCH

Salivary, Plasma, and Gingival Levels of Melatonin and TNF-α in Nonsmokers and Current Smokers with and without Periodontal Disease

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Abstract

Aim: The aim of this study was to quantify the levels of gingival, salivary, and plasma melatonin and tumor necrosis factor-α (TNF-α) in healthy individuals and chronic generalized periodontitis patients with and without cigarette smoking habit and to investigate whether a relationship exists between melatonin and TNF-α levels in the samples.

Materials and methods: Blood of 5 mL, 5 mL of saliva, and gingival tissue samples were obtained from 30 periodontally healthy individuals without smoking habit (HP), 30 nonsmoking patients with chronic generalized periodontitis (CP), 30 periodontally healthy individuals with current smoking habit (SHP), and 30 current smoker patients with chronic generalized periodontitis (SCP). The levels of melatonin and TNF-α in the samples were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kit. The results obtained were statistically analyzed using SPSS statistical software (23.0 version).

Results: This study demonstrated the presence of melatonin and TNF-α in all the saliva, plasma, and gingival tissue samples. Gingival tissue melatonin levels were highest in the HP group and least in the SCP groups, while TNF-α levels were least in the HP group and highest in the SCP groups. No significant difference was observed between the groups with regard to salivary and plasma melatonin. An overall significant difference was also observed between the groups with regard to salivary TNF-α but not with regard to plasma TNF-α. Binary logistic regression analysis was carried out after dividing the study groups into current smokers and nonsmokers. Results revealed that a reduction in gingival melatonin and an increase in gingival TNF-α were associated with a transition from periodontal health to chronic generalized periodontitis in current smokers but not in nonsmokers.

Conclusion: This study sheds light on the anti-inflammatory actions of melatonin in the gingival tissues in states of periodontal health and disease in current smokers.

Clinical significance: Melatonin could be used as a supplement to boost anti-inflammatory mechanisms in periodontal therapy especially in cigarette smokers.

Keywords: Melatonin, Periodontitis, Smokers, Tumor necrosis factor-α.

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Introduction

Chronic periodontitis represents an inflammatory condition of microbial etiology that affects the tooth supporting apparatus characterized by connective tissue breakdown and alveolar bone resorption ultimately resulting in tooth loss. It is well-known that proinflammatory cytokines such as interleukin (IL)1β, TNF-α, and lipid mediators such as prostaglandin E2 are released by the host immune cells to battle the microbial challenge posed by the periopathogenic bacteria in the dental plaque biofilm.[1] In addition, reactive oxygen species (ROS) is released by the inflammatory and immune cells during the periodontal inflammation process. It is noteworthy that proinflammatory cytokines, lipid mediators, and the ROS molecules together mediate tissue destruction in periodontal disease.[1]

Tobacco smoking is one of the key predisposing risk factors for periodontal disease.[2] Smokers have been found to have more periodontal disease burden due to the deleterious components present in the tobacco smoke, which dysregulates the inflammatory process and causes aberrant production of proinflammatory cytokines.[3] Chronic inflammation and a jeopardized antioxidant oxidant balance are cardinal features that underlie the pathogenesis of chronic periodontitis in current smokers. It is at this juncture that TNF-α deserves mention as a cytokine-mediating tissue destruction and bone loss in periodontal disease and smoking. Tumor necrosis factor-α is a pleotropic cytokine produced by monocytes predominantly in response to bacterial challenge. Once released in the inflammatory microenvironment, it binds to its specific receptors and acts through the intracellular activation of the transcription factor nuclear factor (NF)-κB, which in turn switches on other proinflammatory cytokines and mediators to culminate in tissue destruction.[4]

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Stashenko et al. demonstrated elevated levels of TNF-α in sites of periodontal destruction in nonsmokers.[5] In a study of smokers, Boström et al. demonstrated higher levels of TNF-α from gingival crevice fluid in sites of periodontal destruction compared to healthy individuals.[6] Periodontal therapy has been found to reduce the levels of TNF-α in gingival crevice fluid,[7] demonstrating the fact that TNF-α could be a major inflammatory marker in periodontal pathology.

The human body devised several natural antioxidants and anti-inflammatory mechanisms to fight the deleterious effects of proinflammatory and pro-oxidant molecules like TNF-α. In this regard, melatonin is a key indoleamine molecule that has been found to play an important role in the pathobiology of periodontal disease. Melatonin is synthesized from the pineal gland and predominantly regulates circadian rhythm in the human body.[8] Apart from this, melatonin biosynthesis has been documented in the salivary glands,[9] retina,[10] ovaries,[11] gastrointestinal tract,[12] and cells like lymphocytes.[13] Melatonin, a potent antioxidant, is also known to have a strong immunomodulatory role.[14] Melatonin acts on the immune system predominantly by inhibiting NF-κB activation, which in turn inhibits the transcription and translation of inflammatory mediators such as IL-1β and TNF-α.[15] Numerous studies have demonstrated reduced levels of melatonin in saliva,[16] gingival crevice fluid,[17] and gingival samples[18] obtained from patients with chronic periodontitis in comparison with healthy subjects. So far, no study has investigated the melatonin levels in smokers with periodontal disease. The present study was hence performed to estimate the levels of melatonin and TNF-α in gingival tissue samples, saliva, and plasma of healthy individuals and chronic generalized periodontitis patients with and without cigarette smoking habit, so that the relationship between melatonin and TNF-α in periodontal pathogenesis was understood.

Materials and Methods

Study Plan and Recruitment of Volunteers

This planned case–control study was conducted after obtaining formal permission from the Institutional Ethics Committee of Sri Ramachandra University. A total number of 120 volunteers were chosen for the study after obtaining written informed consent. The study comprised four groups of 30 participants in each group, namely, HP, CP, SHP, and SCP. The inclusion criteria for HP group were nonsmokers with clinically healthy gingiva and absence of inflammatory signs and bleeding on probing. The inclusion criteria for CP group were nonsmoking patients diagnosed with chronic generalized periodontitis with clinical signs of inflammation in gingiva in a minimum of 10 teeth along with more than 30% of intraoral sites showing periodontal pockets with attachment loss and radiographic features depicting bone loss.[19] The inclusion criteria for SHP group were cigarette smokers with current smoking habit and who had smoked a minimum of hundred numbers of cigarette in their lifetime,[20] with clinically healthy gingiva and absence of inflammatory signs and bleeding on probing. The inclusion criteria for SCP group were 30 volunteers with cigarette smoking habit, who had the habit of smoking during the study, and had smoked a minimum of hundred numbers of cigarette in the past with an intraoral manifestation of chronic generalized periodontitis as assessed by definite parameters previously mentioned. The exclusion criteria for the HP and CP groups were cigarette smoking, while the general exclusion criteria for all groups were participants with a history of systemic disease and patients who were on one or more of the following drugs: antibiotics, pain relief medication, melatonin and vitamin supplements up to 6 months prior to the study. Patients who had undergone periodontal treatment and expectant and breastfeeding mothers were eliminated from the study.

Clinical Examination

After obtaining a detailed history, intraoral examination was done in the patients to assess the plaque index, which was measured by the presence or absence of supragingival plaque of around four surfaces in each tooth.[21] Gingival bleeding scores were calculated by measuring the percentage of sites demonstrating bleeding on instrumentation with a periodontal probe of around six sites in each tooth.[22] Probing depth and clinical attachment loss were assessed in six sites per tooth with a Michigan O probe with Williams markings by a single calibrated examiner who was calibrated by repetitive examination in five patients prior to the study. A κ value of 0.8 was the result of calibration which revealed good intraexaminer agreement.

Sample Collection

All samples were collected at 9 am in the morning after a 12-hour overnight fasting. After 20 minutes of rest, 5 mL of saliva was obtained from the study subjects. In order to stimulate saliva production, the participants chewed a bit of paraffin wax for 7 minutes. Saliva produced during the first 2 minutes was discarded. After which the saliva was collected the following 5 minutes to avoid any possible contamination. The patients chewed the paraffin during the time of saliva collection. The saliva samples obtained were centrifuged at 3000 rpm, 4°C for 15 minutes following which the clear supernatant obtained was collected and frozen at −80°C until assays were performed.[23]

A 5-mL blood sample was collected from the subjects in vacutainer tubes and anticoagulated with EDTA. After centrifugation at 3000 rpm, 4°C for 15 minutes, the plasma was separated and stored at −20°C until the assays were performed.

In the HP and SHP groups, collection of gum tissue samples were done while the subjects reported for routine surgical crown lengthening. In CP and SCP groups, gingival samples were obtained prior to scaling and root planning. Local anesthesia was administered and a segment of the marginal and papillary gingiva was excised using a 15 number bard parker blade under aseptic protocol. The tissue sample was transferred to sterile Eppendorf tubes and stored at −80°C until the assays were performed.

All the samples were processed and the supernatant obtained was fractionated into two halves and used for melatonin and TNF-α quantification.

Sample Preparation Prior to Processing

The gingival tissue samples were thawed for tissue homogenization which was mechanically performed using a tissue homogenizer in cold phosphate buffered saline. Following complete homogenization, the supernatant obtained was extracted prior to the assay. Plasma samples were also extracted prior to the assay. Methanolic extraction of the tissue and plasma samples was done using the protocol mentioned in the ELISA kit. Saliva was used directly without the extraction procedure.

Measurement of Melatonin and TNF-α in the Samples

Saliva samples were analyzed for melatonin using commercially available human ELISA kit for salivary melatonin detection

(Catalogue no: RE54041; IBL laboratories, Hamburg, Germany). Gingival tissue and plasma samples were analyzed for melatonin using commercially available melatonin detection ELISA kit (Catalogue no: RE54021; IBL laboratories). Tumor necrosis factor-α was estimated in the gingival tissue, saliva, and plasma samples using commercially available Human TNF-α Mini ABTS ELISA Development kit (Catalogue no: 900-M25; Peprotech, Rocky Hill, New Jersey, USA). Manufacturer protocols were followed. After following the manufacturer's instructions, the readings of the ELISA procedure were subjected to spectrophotometric reading. A wavelength of 405 nm was fixed for melatonin and 405 nm with a correction of 650 nm was fixed for TNF-α. Upon completion of ELISA procedure, the results obtained were statistically analyzed using SPSS statistics software (23.0 version). One-way analysis of variance (ANOVA) was used for obtaining the overall significance between the groups and *post hoc* Tukey test was used for further statistical comparison. Binary logistic regression analysis was used to assess the relationship between melatonin and TNF-α. The level of significance was set at $p < 0.05$.

Results

A total of 120 subjects participated in the present study. The descriptive parameters of the study volunteers are given in Table 1.

Table 2 depicts the gingival, salivary, plasma melatonin, and TNF-α levels. Analysis showed the presence of both markers in all samples.

The one-way ANOVA demonstrated an overall difference in gingival tissue melatonin levels between the groups ($p < 0.05$), while no significant difference was observed in salivary and plasma melatonin levels (Table 2). Furthermore, *post hoc* Tukey honestly significant difference (HSD) test showed the highest levels of gingival tissue melatonin in HP group and the least amount of melatonin in the SCP group in comparison with the other groups. The results were statistically significant ($p < 0.05$) (Table 3).

One-way ANOVA showed an overall difference in gingival tissue ($p < 0.05$) and salivary TNF-α levels ($p < 0.05$) between the groups which was significant, while no significant difference was observed in plasma TNF-α levels (Table 2). With regard to the gingival tissue samples, *post hoc* Tukey HSD analysis revealed that TNF-α levels were lowest in the HP group, which is significant when compared with the other three groups ($p < 0.05$) and highest in the SCP group, which is statistically significant when compared with the HP group but not the CP and SHP groups ($p > 0.05$) (Table 4).

A similar trend was observed for salivary TNF-α levels, i.e., the least amounts present in the HP group and the highest amounts present in the SCP group. The results were statistically significant during intergroup comparison of HP group with CP and SCP groups ($p < 0.05$), not significant with SHP group ($p > 0.05$). In the SCP group, the values were significantly high when in comparison with the HP and SHP groups ($p < 0.05$), but not significant when compared with the CP group ($p > 0.05$) (Table 5).

A binary logistic regression analysis was performed after dividing the study participants into nonsmokers and smokers. This was done to assess the relationship between changes in melatonin and TNF-α levels in the gingiva, saliva, and plasma samples in the transition from periodontal health to chronic generalized periodontitis. A logistic regression model was constructed and a dichotomous score of "0" was assigned for periodontal health and a value of "1" was assigned for chronic generalized periodontitis. The β values, standard error, Wald values, and *p* values that emerged are presented in Table 6 for nonsmokers (HP and CP groups) and Table 7 for current smokers (SHP and SCP groups). The negativity or positivity of the β value was used to interpret the relationship

Table 1: Demographic details of the study participants

Group	*Total number of participants*	*Gender M/F*	*Mean age (years)*	*Mean plaque index*	*Mean bleeding on probing scores (%)*	*Mean attachment loss (mm)*	*Mean smoking scores (pack years)*
HP	30	15/15	45.47 ± 1.36	13.03 ± 0.78	3.4 ± 0.27	0.00	0.00
CP	30	15/15	41.50 ± 1.19	88.47 ± 1.61	79.73 ± 2.06	1.74 ± 0.07	0.00
SHP	30	30/0	43.17 ± 1.37	12.13 ± 0.77	2.63 ± 0.22	0.00	1.18 ± 0.08
SCP	30	30/0	42.63 ± 1.34	86.13 ± 1.74	3.27 ± 0.31	3.45 ± 0.14	1.419 ± 0.12
p value		$p < 0.001$	0.194	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

HP denotes periodontally healthy individuals without smoking habit, CP denotes nonsmoking patients with chronic generalized periodontitis, SHP denotes periodontally healthy individuals with current smoking habit, SCP denotes current smoker patients with chronic generalized periodontitis ($n = 30$ in each group)

Table 2: Mean melatonin and TNF-α levels in the gingival tissue, saliva and plasma samples of the study participants

	HP	*CP*	*SHP*	*SCP*	*F test ratio df:3*	*p value*
Mean gingival tissue melatonin levels (pg/mL)	234.61 ± 3.70	172.49 ± 10.62	183.81 ± 14.18	131.47 ± 10.49	16.458	$p < 0.001$
Mean salivary melatonin levels (pg/mL)	0.77 ± 0.038	0.55 ± 0.13	0.71 ± 0.14	0.57 ± 0.08	0.226	0.878
Mean plasma melatonin levels (pg/mL)	24.4 ± 8.7	21.74 ± 5.6	11.62 ± 1.05	10.50 ± 3.01	1.949	0.126
Mean gingival tissue TNF-α levels (pg/mL)	20.72 ± 5.45	381.5 ± 79.58	335.70 ± 83.68	509.9 ± 106.69	6.953	$p < 0.001$
Mean salivary TNF-α levels (pg/mL)	20.63 ± 3.67	106.17 ± 5.49	48.21 ± 9.65	144.07 ± 17.20	28.727	$p < 0.001$
Mean plasma TNF-α levels (pg/mL)	109.47 ± 17.12	172.60 ± 30.89	161.57 ± 49.00	223.97 ± 23.56	2.098	0.104

HP denotes periodontally healthy individuals without smoking habit, CP denotes nonsmokers with chronic generalized periodontitis, SHP denotes periodontally healthy individuals with current smoking habit, SCP denotes current smoker patients with chronic generalized periodontitis

Table 3: Comparison of gingival tissue melatonin levels between the study groups

					95% confidence interval	
Dependent variable	*Group*	*Group*	*Mean difference ± SE*	*p value*	*Lower bound*	*Upper bound*
Gingival tissue melatonin	HP	CP	62.11 ± 14.799	0.000*	23.54	100.69
		SHP	50.80 ± 14.79	0.005*	12.22	89.37
		SCP	103.14 ± 14.79	0.000*	64.56	141.71
	CP	HP	−62.11 ± 14.79	0.000*	−100.69	−23.54
		SHP	−11.31 ± 14.79	0.870	−49.89	27.25
		SCP	41.02 ± 14.79	0.032*	2.44	79.59
	SHP	HP	50.80 ± 14.79	0.005*	−89.37	−12.22
		CP	11.31 ± 14.79	0.870	−27.25	49.89
		SCP	52.33 ± 14.79	0.003*	13.76	90.91
	SCP	HP	−103.14 ± 14.79	0.000*	−141.71	−64.56
		CP	−41.02 ± 14.79	0.032*	−79.59	−2.44
		SHP	−52.33 ± 14.79	0.003*	−90.91	−13.76

HP denotes periodontally healthy individuals without smoking habit, CP denotes nonsmoking patients with chronic generalized periodontitis, SHP denotes periodontally healthy individuals with current smoking habit, SCP denotes current smoker patients with chronic generalized periodontitis ($n = 30$ in each group), SE denotes standard error
*Significant

Table 4: Comparison of gingival TNF-α levels between the study groups

					95% confidence interval	
Dependent variable	*Group*	*Group*	*Mean difference ± SE*	*p value*	*Lower bound*	*Upper bound*
Gingival tissue TNF-α	HP	CP	−0.36 ± 0.11	0.008*	−0.65	−0.07
		SHP	−0.31 ± 0.11	0.028*	−0.60	−0.02
		SCP	−0.48 ± 0.11	0.000*	−0.77	−0.19
	CP	HP	0.36 ± 0.11	0.008*	0.07	0.65
		SHP	0.04 ± 0.11	0.976	−0.24	0.33
		SCP	0.12 ± 0.11	0.661	−0.41	0.16
	SHP	HP	0.31 ± 0.11	0.028*	0.02	0.60
		CP	−0.04 ± 0.11	0.976	−0.33	0.24
		SCP	−0.17 ± 0.11	0.406	−0.46	0.11
	SCP	HP	0.48 ± 0.11	0.000*	0.19	0.77
		CP	0.12 ± 0.11	0.661	−0.16	0.41
		SHP	0.12 ± 0.11	0.406	−0.11	0.46

HP denotes periodontally healthy individuals without smoking habit, CP denotes nonsmoking patients with chronic generalized periodontitis, SHP denotes periodontally healthy individuals with current smoking habit, SCP denotes current smoker patients with chronic generalized periodontitis ($n = 30$ in each group), SE denotes standard error
*Significant

Table 5: Comparison of salivary TNF-α levels between the study groups

					95% confidence interval	
Dependent variable	*Group*	*Group*	*Mean difference ± SE*	*p value*	*Lower bound*	*Upper bound*
Salivary TNF-α	HP	CP	−0.08 ± 0.01	0.000*	−0.12	−0.04
		SHP	−0.02 ± 0.01	0.245	−0.06	0.01
		SCP	−0.12 ± 0.01	0.000*	−0.16	−0.08
	CP	HP	0.08 ± 0.01	0.000*	0.04	0.12
		SHP	0.05 ± 0.01	0.001*	0.01	0.09
		SCP	0.03 ± 0.01	0.054	−0.07	0.00
	SHP	HP	0.02 ± 0.01	0.245	−0.01	0.06
		CP	−0.05 ± 0.01	0.001*	−0.09	−0.01
		SCP	−0.09 ± 0.01	0.000*	−0.13	−0.05
	SCP	HP	0.12 ± 0.01	0.000*	0.08	0.16
		CP	0.037 ± 0.01	0.054	−0.00	0.07
		SHP	0.09 ± 0.01	0.000*	0.05	0.13

HP denotes periodontally healthy individuals without smoking habit, CP denotes nonsmoking patients with chronic generalized periodontitis, SHP denotes periodontally healthy individuals with current smoking habit, SCP denotes current smoker patients with chronic generalized periodontitis. 30 participants in each group, SE implies standard error
*Significant

Table 6: Relationship between melatonin and TNF-α levels in the samples with periodontal status in nonsmokers

Variables measured	*B*	*SE*	*W*	*df*	*p value*
Gingival tissue melatonin	−0.463	81.418	0.000	1	0.995
Salivary melatonin	3.693	1808.727	0.000	1	0.998
Plasma melatonin	0.066	34.346	0.000	1	0.998
Gingival tissue TNF-α	0.293	39.725	0.000	1	0.994
Salivary TNF-α	0.972	207.964	0.000	1	0.996
Plasma TNF-α	−0.061	14.765	0.000	1	0.997
Constant	20.885	23740.391	0.000	1	0.999

B denotes β value, SE denotes standard error, W denotes the Wald values, df denotes degree of freedom.

Table 7: Relationship between melatonin and TNF-α levels in the samples with periodontal status in current smokers

Variables measured	*B*	*SE*	*W*	*df*	*p value*
Gingival tissue melatonin	−0.020	0.009	4.578	1	0.032*
Salivary melatonin	−0.710	0.698	1.034	1	0.309
Plasma melatonin	0.005	0.056	0.008	1	0.929
Gingival tissue TNF-α	0.003	0.001	6.808	1	0.009*
Salivary TNF-α	0.034	0.010	12.245	1	0.000*
Plasma TNF-α	−0.003	0.002	2.069	1	0.150
Constant	−0.042	1.927	0.000	1	0.983

B denotes β value, SE denotes standard error, W denotes the Wald values, df denotes degree of freedom
*Significant

of melatonin and TNF-α with periodontal disease progression. This trend was considered significant if the associated *p* value was less than 0.05.

When this analysis was conducted for the nonsmokers (HP and CP groups), no relationship could be reported between gingival, salivary, and plasma melatonin and TNF-α and periodontal disease progression (Table 6). However when this analysis was performed in the smokers (SHP and SCP groups), a statistically significant negative β value was observed for gingival melatonin (β value: −0.020) and a statistically significant positive β value was observed for gingival TNF-α (β value: 0.003) and salivary TNF-α (β value: 0.034), showing that a decrease in gingival melatonin and an increase in gingival and salivary TNF-α were associated with a transformation from periodontal health to chronic generalized periodontitis (Table 7).

It could hence be inferred that melatonin levels were high in healthy gingival tissues and reduced in periodontitis and smoking. The vice versa situation operated in relation to TNF-α in saliva and gingival tissues which was found to be lowered in healthy individuals and increased in periodontitis and smoking. Further the binary logistic regression analysis revealed that melatonin levels dip and TNF-α levels increase as there is a transition from periodontal health to disease.

Discussion

This present study was performed to assess the levels of melatonin and TNF-α in status of periodontal health and disease to understand the role and interrelationship between the two molecules in periodontal pathology. The study included current smokers, as smoking is significantly associated with destructive periodontal disease. In epidemiological studies, current smokers are observed to have more periodontal disease burden than nonsmokers.[24,25] Nicotine, the principal component of tobacco, plays a major role in modulating the pathogenesis of periodontal disease. Smoking induces oxidative stress in human tissues and causes dysregulation in the immune response by reducing neutrophil chemotaxis and phagocytosis[26] and impairing oxidative burst.[27] Since both the immune response and redox status are jeopardized by smoking, this condition was researched as it would be worthwhile to assess melatonin which is believed to have both anti-inflammatory and anti-oxidant roles in the human body.

One of the significant molecules in the human body that has both an antioxidant and anti-inflammatory role is "melatonin". Numerous studies described the orchestrated pathways by which melatonin exerts a strong antioxidant activity.[28] But its role as a potent anti-inflammatory molecule is being currently researched especially with reference to TNF-α. The present study was performed to assess the anti-inflammatory role of melatonin in the pathogenesis of periodontal disease in individuals with and without cigarette smoking habit. To achieve the said objective, melatonin and TNF-α levels were measured in plasma, saliva, and gingival tissue samples. Tumor necrosis factor-α was chosen as the inflammatory marker to be assessed in this study due to the participation of this molecule in periodontal disease pathogenesis. Tumor necrosis factor-α is a multifaceted cytokine that is proven to be elevated in periodontal disease vs health.[21] Moreover, this cytokine has a systemic impact as it has been found to play a significant role in the pathobiology of type 2 diabetes mellitus,[29] atherosclerosis,[30] and adverse pregnancy outcomes.[31] It could hence be hypothesized that periodontal disease could be a major risk factor for the abovementioned systemic conditions through the elevation of a significant cytokine such as TNF-α.

The ELISA technique was used to measure melatonin and TNF-α levels in the plasma, saliva, and gingival tissue samples of the study participants. There was an overall significant difference in gingival tissue levels of melatonin between the four groups studied ($p < 0.05$). However, no difference was observed in the plasma and salivary levels of melatonin ($p > 0.05$). In this regard, the study contradicts previous studies that have demonstrated significantly lower levels of plasma and salivary melatonin in periodontally diseased individuals

in comparison with healthy volunteers.[16,17] With reference to gingival tissue melatonin, the highest levels were observed in the HP group ($p < 0.05$) and the lowest levels were observed in the SCP group ($p < 0.05$). With regard to the CP group, gingival melatonin levels were significantly lower than the HP group ($p < 0.05$). These observations are justifiable as the HP group participants were nonsmokers with clinically healthy gingiva free of inflammation. Several endogenous antioxidant molecules like coenzyme Q and vitamin E are found in the healthy gingival tissues, maintaining the antioxidant balance.[32] In a similar way, melatonin could also serve the same role as an endogenous gingival antioxidant. With regard to the finding of lowered gingival melatonin in the CP group, it can be justified as this group was comprised of chronic generalized periodontitis patients. Several studies have demonstrated elevated levels of biomarkers of oxidative damage in the saliva, gingival crevicular fluid, and plasma samples of patients with chronic periodontitis.[33–35] To mitigate and scavenge free radicals and ROS, the human body has evolved numerous antioxidant mechanisms. One of the most potent antioxidants is melatonin and its metabolic by-products. It has to be re-emphasized at this point that melatonin can protect cells against oxidative stress more efficiently than other antioxidants under *in vivo* conditions. Melatonin metabolites such as cyclic-3-hydroxymelatonin, N1-acetyl-5-methoxykynuramine (AMK), and N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) are more potent than melatonin in scavenging cell lethal ROS such as the hydroxyl radical.[36] It is hence understandable that gingival melatonin in periodontal disease is lowered as it would be converted into its metabolites to scavenge the free radicals and ROS generated. The justification for lowest melatonin levels in the SCP group could be based on the fact that this group comprised smokers with chronic periodontitis. Both cigarette smoking and periodontal disease are associated with increased oxidative stress. Melatonin levels could be extremely depleted in these states as explained by the abovementioned mechanisms.

With reference to TNF-α, an overall significance between the groups was observed for gingival tissue samples ($p < 0.05$) and saliva ($p < 0.05$) but not plasma ($p > 0.05$). The gingival tissue and salivary levels of TNF-α were lowest in the HP group and highest in the SCP group. These values were statistically significant ($p < 0.05$). With regard to the CP group, the gingival and salivary levels of TNF-α were significantly higher than the HP group ($p < 0.05$). The abovementioned findings can be justified as the HP group participants had clinically healthy gingiva which is histologically not free of inflammation and has low numbers of infiltrating inflammatory cells which produce small amounts of TNF-α. The CP group represented nonsmoking patients with chronic generalized periodontitis. A significant proinflammatory cytokine that plays a pivotal role in the pathogenesis of periodontal disease is TNF-α. The findings of elevated TNF-α in the gingival tissue samples of the CP group in comparison with the HP group are in accordance with the findings of Stashenko et al. who observed elevated levels of TNF-α in gingival tissue samples of patients with periodontal destruction.[5] In the SCP group, the patients were smokers with chronic periodontitis. Smoking has been known to cause a hyperinflammatory status. Immune dysregulation in smoking results in aberrant production of cytokines, which could be responsible for elevated TNF-α levels in smokers with periodontal disease. Hence, the SCP group plausibly demonstrated the highest levels of gingival and salivary TNF-α.

To analyze the relationship between melatonin and TNF-α in the samples, a binary logistic regression analysis was performed after grouping the subjects into current smokers and nonsmokers. It was found that a decrease in gingival melatonin and an increase in gingival TNF-α was associated with a transition from periodontal health to chronic generalized periodontitis in current smokers but not in nonsmokers.

This study to our knowledge is the first to measure and correlate the melatonin and TNF-α levels in plasma, saliva, and gingival tissues of nonsmokers and current smokers in the states of periodontal health and disease. Elevated melatonin levels in gingival tissues were associated with low TNF-α levels and vice versa in current smokers with chronic periodontitis. This connection sheds light on the inverse relationship between melatonin and TNF-α and also reveals an anti-inflammatory role played by melatonin in periodontal homeostasis. Mounting evidence suggests that inflammation can suppress melatonin production in the pineal gland. This finding forms the basis of a bidirectional relationship between the pineal glands and the immune system. An *in vitro* study demonstrated suppressed melatonin production following the administration of TNF-α to cultured pineal gland cells.[37] Similarly, a clinical study found suppression of nocturnal melatonin in mothers with mastitis, which was highly correlated with increased TNF-α production.[38] With regard to the anti-inflammatory role played by melatonin, an *in vitro* study observed that melatonin was found to inhibit LPS-stimulated TNF-α, IL-1β, IL-6, IL-8, and IL-10 production in Raw264.7 cells through suppression of NF-κB activation.[15] In another study, melatonin was also found to suppress the production of LPS-activated IL-6 and NO in *Prevotella intermedia* by causing inhibition of NF-κB signaling and suppression of STAT-1 signaling.[39] In syncytial virus-infected Raw264.7 cells, melatonin decreased TLR3-mediated TNF-α and inducible nitric acid synthase expression through the inhibition of NF-κB activation.[40] Hence, melatonin could function as an anti-TNF-α compound in periodontal homeostasis also. The results of the present study demonstrates the anti-inflammatory role played by melatonin in periodontal homeostasis. The lacunae in the present investigation include the fact that in the study design, only males constituted the smoker group (SHP and SCP), whereas the nonsmoker group comprised both males and females (HP and CP). This is justifiable as in our country smoking habits are not prevalent with the female gender. Since all the study participants fell between the age ranges of 41–45 years, it can be hypothesized that the influence of gonadal hormones on periodontal disease pathogenesis and melatonin production would not be very significant.

Conclusion

Melatonin has been evolving in periodontal research as a potent perioceutics agent. Several animal and human clinical studies have been performed using melatonin as a host modulatory agent. However, no study so far has been done on smokers with chronic periodontitis. Based on the findings of the present study future clinical studies need to be performed in smokers with periodontal disease to exploit the anti-inflammatory potential of melatonin.

Clinical Significance

Melatonin could be used as a supplement to boost anti-inflammatory mechanisms in periodontal therapy.

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