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BIOCHEMICAL ESTIMATION OF MELATONIN LEVELS IN THE SALIVA OF SMOKERS AND NON SMOKERS SUFFERING FROM CHRONIC PERIODONTITIS

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ABSTRACT:

Background: Melatonin, a hormone secreted by the pineal gland possesses anti-oxidant, free-radical scavenging and immune-enhancing properties. Periodontal disease is an inflammatory disease coursing with an increase in free radical production. Cigarette smoke is also a significant source of oxidative stress.

Aim: The aim of this study was to estimate the salivary melatonin levels of smokers and non smokers in patients suffering from chronic generalised periodontitis.

Materials and methods: A total of sixty patients were included in this study. They were divided into three groups, Forty patients with generalised chronic periodontitis, 20 of which were smokers (group C) and 20 non smokers (group B), also 20 patients who were clinically healthy and non smokers (group A) were included in the study as a control group. Salivary melatonin levels were assayed using a commercially available ELISA kit. (IBL, Germany)

Results: Melatonin was detected in all three groups. There was a statistically significant difference between all three groups with the mean being 4.097422 for group A, 2.450833 for group B and 1.237223 for group C.

Conclusion: Salivary melatonin levels reduced in patients with periodontal disease and reduced even further in patients with periodontal disease who smoke. This suggests melatonin may act as a potent antioxidant in the oral cavity.

Keywords: Melatonin, Smokers, Chronic Periodontitis, Antioxidant, Saliva, Reactive oxygen species.

INTRODUCTION:

Although cigarette smoking is a widely recognized health hazard and a major cause of mortality, people continue to consume cigarettes on a regular basis. Approximately one third of the world's population, 15 years or older smokes cigarettes daily. [1]

Cigarette smoke (CS) is a complex mixture of over 4700 identified constituents and four hundred of them have been proven to be carcinogens (Maurizio Battino *et al.,* 2007). Enormous numbers of free radicals

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or ROS are produced during cigarette smoking (Fatma Fidan *et al.,* 2006). Free radicals in the particulate (tar) of cigarette smoke appear to be a relatively stable semiquinone (Freischlag *et al.,* 1999) and those in the gas phase that contains more than 1014 low-molecular weight compounds are short-lived carbon, oxygen-centered organic radicals and a high concentration of nitrogen oxides per puff (Pryor, 1997;Fatma Fidan *et al.,* 2006).4 Smoking is also known to cause depletion of systemic endogenous antioxidant capacity, resulting in increased pro-oxidant burden. [2]

Periodontal disease is an inflammatory disease initiated and perpetuated by a small group of predominantly gram negative, anaerobic or micro-aerophilic bacteria that colonise the sub gingival area. Recently, the role of reactive oxygen species (ROS) has been established in the pathogenesis of periodontitis. [3] some of which derive from the oral bacteria themselves, others originating from the induced immune response. 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. 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. [4]

Melatonin is an indoleamine produced in various parts of the body, mainly in the pineal gland. It 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. [4]

About 24–33% of the plasma melatonin appears in the saliva, where it is easily measured by ELISA. Salivary melatonin measurement provides a readily accessible means of obtaining data on the melatonin excretion via this route. [5]

Hence, The aim of this study was to estimate the salivary melatonin levels of smokers and non smokers with chronic generalized periodontitis. This study was undertaken with the following objectives-

- To evaluate the effect of cigarette smoking on the levels of melatonin.
- To evaluate the relationship between melatonin levels in smokers with and without chronic periodontitis and to compare it with the levels in healthy non smokers.
- To evaluate the efficacy of salivary melatonin levels as diagnostic marker.

MATERIAL AND METHODS:

A total of 60 patients with age ranging from 30-50 years (mean 37.43±5.49) yrs of both sexes, visiting outpatient department of AJ Institute of Dental Sciences, Mangalore participated in this

study. Ethical clearance for the study was obtained from the AJ Institute of Medical Sciences Ethical committee prior to the commencement of the study. A written informed consent was obtained from the subjects who participated in the study.

The patients were questioned regarding their smoking status. Patients who had undergone any periodontal, surgical or non surgical therapy for the past 6 months, patients with a history of underlying systemic or neurological diseases, or diseases known to affect the immune system, pregnant and lactating women and those using hormonal contraceptives and patients on medications known to affect melatonin levels were excluded from this study. Clinical examination was carried out by a single examiner. Individuals with clinically healthy periodontium (Bleeding on probing negative, gingival sulcus depth≤ 3 mm) and who have never smoked are considered for this study as a control group. (Group A) Patients with chronic generalised periodontitis (more than 30% sites involved) with clinical evidence of bleeding on probing and periodontal pockets measuring ≥5 mm (AAP classification 1999.) Were included in the periodontitis group. (Group B) Current smokers according to CDC Classification were included in the smoking group. (Group C).

Sample collection: Sample collection was made at the standardized time according to the diurnal cycle between 8 am to 10 am. Care was taken not to expose the patient to direct light before sample collection. Subjects were instructed not to eat or rinse within 60 minutes prior to sample collection. Around 2 ml of whole unstimulated saliva was collected simply by drooling into a sterilized vial with the forward tilted head or by allowing the saliva to accumulate in the mouth and then expectorate into a vial. The resulting saliva was centrifuged immediately at 3000 rpm for 10 min. The clear supernatant was collected and stored in aliquots at -20°C until the determinations were performed.[6]

Determination of melatonin :

Enzyme linked immunosorbant assay (ELISA) was used for determination of Melatonin in the unstimulated whole saliva. A commercially available kit (IBL, Germany) was used for this purpose. The assay procedure follows the basic principle of competitive ELISA whereby there is competition between a biotinylated and a non-biotinylated antigen for a fixed number of antibody binding sites. The amount of biotinylated antigen bound to the antibody is inversely proportional to the analyte concentration of the sample. When the system is in equilibrium, the free biotinylated antigen is removed by a washing step and the antibody bound biotinylated antigen is determined by use of streptavidin-peroxidase as marker and TMB as substrate. Quantification of unknowns is achieved by comparing the enzymatic activity of unknowns with a response curve prepared by using known standards.

Statistical analysis

One way ANOVA test was done to compare the difference of age, probing depth and Melatonin levels. Posthoc

tukey test was used for multiple comparisons. P<0.001 was considered as statistically significant.

RESULTS:

All samples in each group showed the presence of melatonin. Mean value for salivary melatonin levels (SML) are depicted in Table 1.

Not unexpectedly, patients with periodontitis exhibited statistically significant higher values of probing depths (5.45±0.605) than healthy controls. (2.45±0.605mm). Also, the probing depths of smokers with periodontitis (7.05±1.276) were significantly higher than the other two groups. (Table 2) This suggests that smoking may increase periodontal breakdown.

Patients with ages ranging from 30-50 yrs were chosen for this study and were randomly allocated to a particular group according to clinical findings. There was no statistically significant difference seen in the mean ages between the subjects of the three groups. (Table 2) mean ages being (35.2±4.336) for group A, (39.85±6.209) for group B and (37.25±4.983) for group C.

As for the salivary melatonin levels, the highest levels of salivary melatonin were seen in healthy subjects (4.097422±0.226737). The levels reduced in patients with chronic periodontitis (2.450833±0.38212) and further reduced in patients with chronic periodontitis who smoked. (1.237223±0.186318)

DISCUSSION:

Saliva is a complex secretion whose components exert a well documented role in health and disease and its diagnostic use is spreading (Bald and Glowacki, 2005). Moreover, saliva contains various antioxidants, Including melatonin.[2]

Melatonin, chemically N- acetyl- methoxy-tryptamine has recieved considerable attention recently because of its effect in the oral cavity as an anti-oxidant and free radical scavenger, an immune modulater and as a promoter of bone formation.

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. [4]

There is an abudance of literature (Hajati et al,2011, Antonio et al,2006, Mhaske et al,2006 etc) [6,7,8] proving that melatonin levels decrease when the periodontal condition worsens. Also, There are studies suggesting that smoking causes oxidative stress and decreases blood melatonin levels. (Ozguner F et al, Ursing et al) [9,10] A study in 2006 (N. Garg et al) showed that smoking increases the levels of free radicals in tissues, which may be responsible for the periodontal destruction [11]. Ko et al. Showed that reactive oxygen species (ROS) do not exist in either unburned tobacco leaves or in cigarette ash. ROS in CS are created through combustion and exist in the gas

phase. Saliva is the first biological fluid that encounters the inhaled CS. [12]

This study aimed to co-relate the salivary melatonin levels in smokers as compared to non smokers suffering from periodontal disease. Guentsch et al. Reported that patients with periodontitis demonstrate more lipid peroxidation (i.e. More oxidative stress) than healthy subjects, and this effect is enhanced by smoking [13] Similar to this, we also measured lower Melatonin levels in periodontitis patients (2.450833±0.38212) compared to healthy controls (4.097422±0.226737), and Melatonin concentrations in smokers with periodontitis were significantly lower (1.237223±0.186318) than those in non-smoking controls. This suggests that melatonin possess the ability to fight against infection and inflammation and neutralising ROS. This may be explained by the fact that Melatonin possesses an electron-rich aromatic indole ring and functions as an electron donor, thereby reducing and repairing electrophilic radicals (Martinez *et al., 2005).* It is irreversibly oxidized hence it is considered as a suicidal or terminal antioxidant. [14]

Thus, smoking may enhance the effect of ROS in periodontitis, thereby increasing the tissue destruction resulting from oxidative stress ultimately leading to a decrease in the total Melatonin levels in saliva.

CONCLUSION:

Saliva can be used as a diagnostic tool in periodontal diagnosis. Although melatonin is present in a very minimal concentration compared to other biomarkers of periodontal disease, taking its anti-inflammatory, bone forming and antioxidant properties into account, it may still be considered a marker of periodontal disease. Inflammatory diseases like periodontitis reduces the antioxidant levels of melatonin and environmental conditions like smoking reduce it even furthur. Hence, cessation of smoking may be helpful in restoring to a certain limit, the balance between antioxidant and pro-oxidants in saliva.

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TABLES:

Table 1

ONE WAY ANOVA TEST TO COMPARE THE DIFFERENCE OF AGE , PD AND MELATONIN LEVELS

	GROUPS	N	Mean	Std. Deviation	Statistics/ mean squares	Df2(welch) / F(Anova)	P VALUE
Age	HEALTHY: NON SMOKERS	20	35.2	4.336	3.776	37.271	0.024
	PERIODONTITIS: NON SMOKERS	20	39.85	6.209			
	PERIODONTITIS: SMOKERS	20	37.25	4.983			
	Total	60	37.43	5.491			
PD	HEALTHY: NON SMOKERS	20	2.45	0.605	172.845	35.824	<0.001
	PERIODONTITIS: NON SMOKERS	20	5.45	0.605			
	PERIODONTITIS: SMOKERS	20	7.05	1.276			
	Total	60	4.98	2.111			
Melatonin level	HEALTHY: NON SMOKERS	20	4.097422	0.226737	932.752	35.85	<0.001
	PERIODONTITIS: NON SMOKERS	20	2.450833	0.38212			
	PERIODONTITIS: SMOKERS	20	1.237223	0.186318			
	Total	60	2.595159	1.213225			

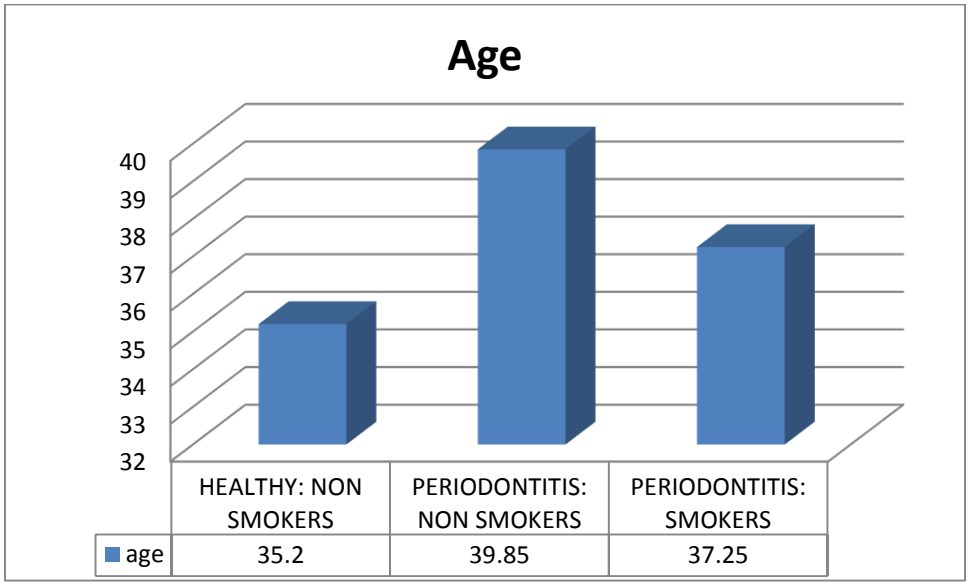
Table 2

POSTHOC TUKEY TEST

Multiple comparisons					
Tukey HSD					
Dependent variable	(i) group	(j) group	Mean difference (i-j)	Std. Error	Sig.
Age	HEALTHY: NON SMOKERS	PERIODONTITIS: NON SMOKERS	-4.650	1.655	**.018**
		PERIODONTITIS: SMOKERS	-2.050	1.655	.436
	PERIODONTITIS: NON SMOKERS	PERIODONTITIS: SMOKERS	2.600	1.655	.266
Pd	Healthy: non smokers	Periodontitis: non smokers	-3.000	.281	**<0.001**
		PERIODONTITIS: SMOKERS	-4.600	.281	**<0.001**
	PERIODONTITIS: NON SMOKERS	PERIODONTITIS: SMOKERS	-1.600	.281	**<0.001**
Melatonin level	Healthy: non smokers	Periodontitis: non smokers	1.64658900	.08796594	**<0.001**
		PERIODONTITIS: SMOKERS	2.86019890	.08796594	**<0.001**
	PERIODONTITIS: NON SMOKERS	PERIODONTITIS: SMOKERS	1.21360990	.08796594	**<0.001**

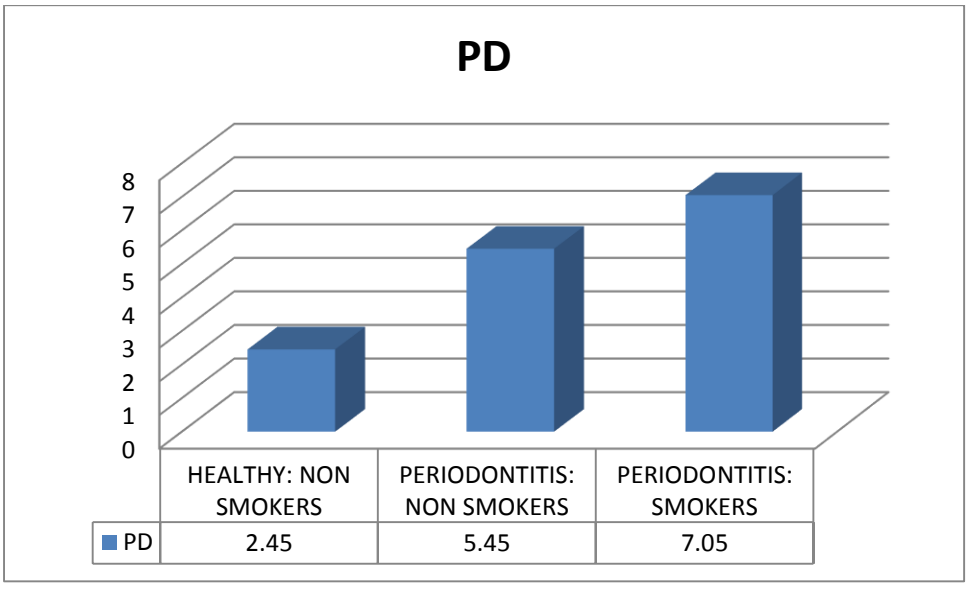
GRAPHS:

Graph 1



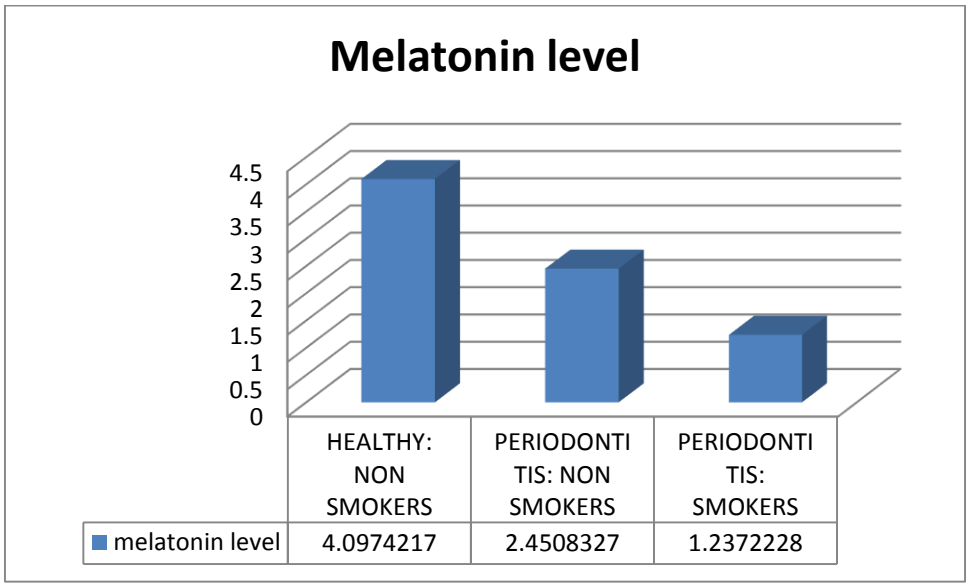
Comparision of age between group A, B and C

Graph 2



Comparision of probing depth between group A, B and C.

Graph 3



Comparision of Melatonin Levels between group A, B and C.

FIGURES :

Figure 1:Salivary melatonin ELISA kit used for estimation. (IBL, Germany)

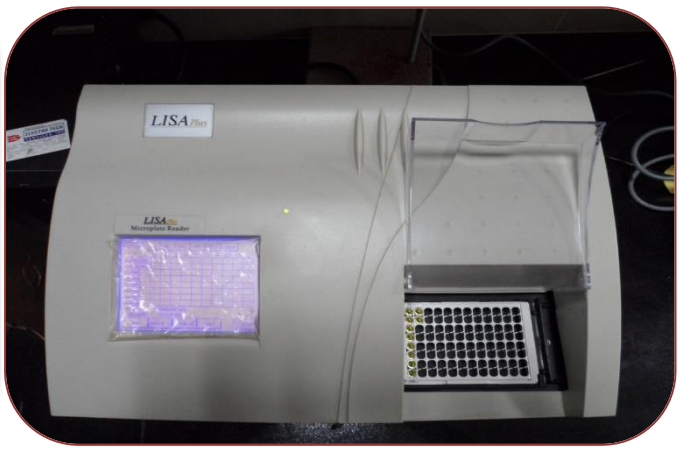


Figure 2:Samples in ELISA Reader