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The Dental Caries Experience in Relation to Salivary Flow Rate, salivary melatonin and Mutans Streptococci Bacteria in Smoker and Non-Smoker Patients.

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ABSTRACT

Dental caries is a localized, progressive, destructive, irreversible microbial based disease of multifactorial nature; these factors include (host, microbes and food) they influence differently on the initiation and progression of dental caries. The aims of the study: was to evaluate the effect of smoking on salivary flow rate, salivary melatonin level and viable count of mutans streptococci (M.S) bacteria in oral cavity and there relation to dental caries experience. The samples were collected from 80 male students ranging in ages from 18-22 years old .Where they divided in to two groups, 40 non-smokers (control group) and 40 smokers (study group). Unstimulated salivary samples were collected. Salivary flow rate were estimated and viable count (CFU/ml) of mutans streptococci was determined. The diagnosis and recording of dental caries was done according to WHO, 1987 criteria and the level of salivary melatonin was determined by ELISA. the result revealed that the salivary flow rate and salivary melatonin level were lower in smoker group than non-smoker , while the means value of dental caries experience Decay ,Missing and Filling tooth (DMFT) and (CFU/ml)of M.S were higher in oral cavity of smoker group than non-smoker group. the smoking has negative effect on salivary flow rate, salivary melatonin and increase the viable bacterial count of M.S and dental caries in smoker patients.

Keywords: dental caries, salivary flow rate, mutans streptococci, melatonin

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INTRODUCTION

Smoking cigarette is a wide spread health problem and a major cause of mortality; people continue to consume cigarettes daily, smoking combine with several biochemical and microbial alterations of saliva that could effect on dental caries severity among smoking individuals.

Dental caries is an irreversible microbial disease of the calcified tissues of the teeth, characterized by demineralization of the inorganic portion and destruction of the organic substance of the tooth, which often leads to cavitation(1) .

Mutans streptococci belong to the family describe as lactic acid bacteria, lactic acid bacteria characteristically ferment sugar through the glycolytic pathway to form pyruvate, which is convert to lactate(lactic acid) ,the amount of lactic acid produce by the individual bacteria depend upon the environmental condition e.g. PH ,oxygen, amount of sugar present and competition with other microorganism, they also depend upon complement of enzymes present within the bacteria that produce alternative fermentation end products , like acetate (acetic acid) ,butyrate and ethanol (2,3).

The pineal gland has been called a neuroendocrine transducer because of its important role in photoperiodism. The major hormone of the pineal gland is N-acetyl-5-methoxy-tryptamine or melatonin which is synthesized from the amino acid tryptophane. Melatonin has the highest levels in plasma during nighttime, regulation of the melatonin secretion is under neural control, sympathetic innervation seems to play a major role via its release of noradrenaline ,a ltered patterns and/or levels of melatonin secretion have been reported to coincide with sleep disorders, “jet lag“, depression, stress, schizophrenia, hypothalamic amenorrhea, pregnancy, anorexia nervosa, some forms of cancer, immunological disorders as well as control of sexual maturation during puberty, most of the circulating melatonin is metabolized in the liver to 6-hydroxymelatonin and subsequently to 6-sulfatoxymelatonin which is excreted into the urine. (4,5).

MATERIAL AND METHODS

Samples distribution

Eighty dental students (male only) were divided in to two groups, 40 smoker and 40 non -smoker, their age ranges between 18-22 years old. The participants were healthy with no sign and symptoms of any systemic disease.

Samples collection and bacteriological work

Saliva Sample collection was made in early morning at time between 8A.M to 9A.M ,subject was instructed not to eat or drink in same day prior to sample collection. Around 1-3 ml of whole un-stimulated saliva was collected simply by drooling into an autoclaved sterilized glass graduated tubes, with the forward tilted head or by allowing the saliva to accumulate in the mouth and then expectorate into a tube, while the determination of salivary flow rate done by using the graduated glass tube, the rule used was (volume of sample collected divided on time needed for collection ml /min.). Then serial dilution was prepared by using sterile saline (10^3,10^5) .The 0.1 ml of dilution was spread on mitis salivaris bacitracin selective media (HiMedia, India), and the inoculated plates was incubated anaerobically by using the gas bag for 48 hours in $37C^0$, the identification of mutans streptococci done by detecting colony morphology using dissecting microscope, gram stain (6) and biochemical tests (6), the viable bacterial count expressed as colony forming unit per ml of saliva.

Clinical part -Dental caries index (DMFT)

DMF: Decay ,missing and filling is universally adopted index was used according to WHO[7].To detect the distribution of dental cries for each subject, DT (decay and left untreated) MT (missing extract due to caries) FT (filled tooth) , the total number of effected tooth by dental caries is a summation of DT+MT+FT which is known as DMFT value, the dental examination was done by using diagnostic dental instrument (probe and dental mirror) on dental chair.

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Determination of salivary melatonin by ELISA

Test principle

The assay procedure follows the basic principle of competitive ELISA whereby there was 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 was inversely proportional to the analyte concentration of the sample. When the system was in equilibrium, the free biotinylated antigen was removed by a washing step and the antibody bound biotinylated antigen was 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.

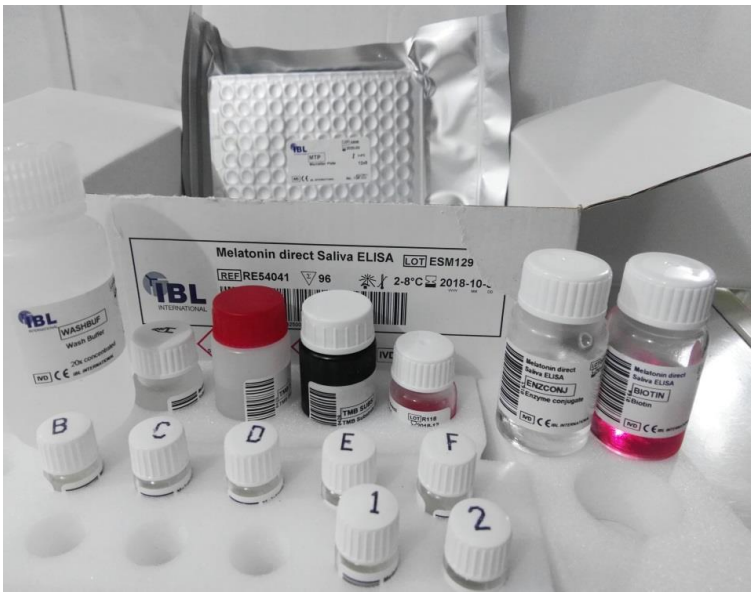

Figure 1: Salivary melatonin ELISA kit

Statistical analysis

Mean value of each variable, standard error (SE), standard deviation (SD), correlation coefficient and independent t-test were done between variables in two groups , All analyses were performed by using SPSS version 24.

RESULTS

The mean value of salivary flow rate in non-smoker group (0.1925) ml/min was higher than the mean value of smoker group (0.1550) ml/min., with significant difference ($p < 0.05$) between both groups (table 1).

The mean value of viable count (CFU/ml) $\times 10^3$ of mutans streptococci in non-smoker group (164.975) CFU/ml was less than that of smoker group (171.325) CFU/ml. and the statistical analysis revealed non-significant difference between two groups ($p > 0.05$).

According to dental caries index tooth (DMFT), the mean value in non- smoker group (8.600) was less than smoker group (10.150). The statistical analysis showed non- significant difference among the two groups ($p > 0.05$).

The Mean value of salivary melatonin (pg/mL) (2.082) was lower in smoker group than non-smoker group mean value (2.645) ,with significant difference ($p < 0.05$) among two groups

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Table (1) the statistical analysis of salivary flow rate, viable count of mutans streptococci, Dental caries index DMFS and salivary melatonin among non-smoker and smoker groups.

Variables	Non-smoker 40			Smoker 40			Statistical analysis		
	mean	SE	SD	mean	SE	SD	t-test	pvalue	Sig.
salivary flow rate	0.192	0.135	0.859	0.155	0.112	0.714	-2.199	.034	S
CFU of M.S	164.975	25.456	160.998	171.325	27.6621	174.950	.176	.861	NS
DMFT	8.600	.850	5.377	10.150	.940	5.950	1.235	.224	NS
Salivary melatonin	2.645	0.187	1.188	2.082	0.202	1.293	2.235	.031	S

DMFT= decay, missing, filling tooth, CFU=colony forming unit, SE =standard error , SD= standard deviation , P ≤0.05 Significant , P>0.05 Non significant

Identification of Mutans Streptococci

Identification of Mutans Streptococci was carried out by three stages which are

a. Colony Morphology:

The colonies of Mutans Streptococci are cultured on selective media (mitis salivaris bacitracin agar plate), incubated at 37 C^0 for 48 hr. and appear, either light blue in color about 1-2 mm in diameter (Smooth type) or appeared as irregular colonies with rough or frosted glass surface (Rough type) as shown in fig.(2-A). Most of mutans streptococci colonies had a depression at the middle with a drop of polysaccharide in it, or sometimes the whole colony submerged in a pool of polysaccharide fig.(2-B).

b. Gram Stain Characteristics

Mutans Streptococci bacteria when subjected to Gram's stain, the result were be gram positive, appear in small ovoid or spherical shape in short or long chains as in fig.(3) .In addition mutans Streptococci was recognize as non-motile when examined under microscope by direct smear.

c. Biochemical Tests

All colonies of mutans Streptococci were catalase negative, tested for catalase production by addition of 2-3 drops of 3% H_2O_2 to a few isolated colonies on clean slide. and had a positive reaction in fermentation of mannitol , which is indicated by the changing the indicator color from red to yellow through the formation of acid after incubation as shown in fig. (4).

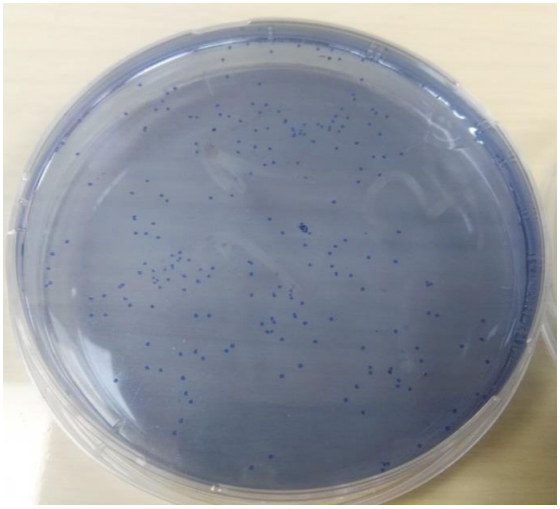

Figure 2-A: Colonies of Mutans Streptococci on MSB agar

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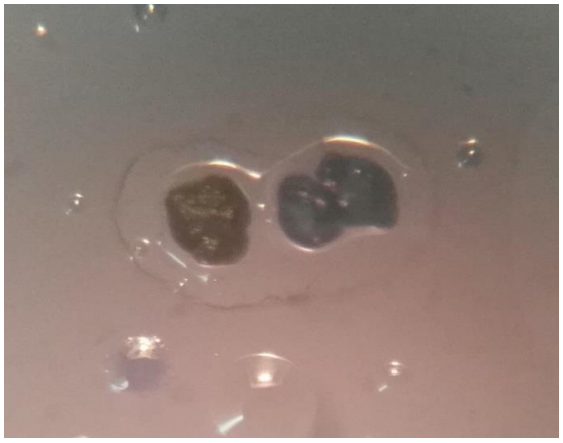

Figure 2-B: Mutans Streptococcus bacteria on mitis salivaris bacitracin agar (15× magnification)

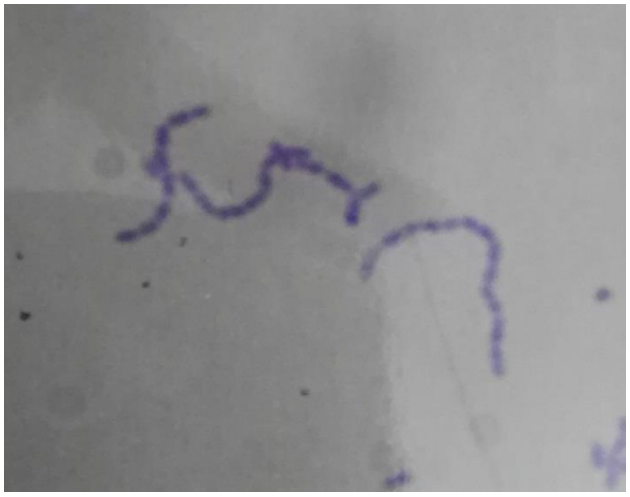

Figure 3: Gram's stain of Mutans Streptococci cell from pure culture (1000× magnification)

Figure 4: Mannitol fermentation test of Mutans Streptococci

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A : Positive control tube(agar and bacteria without mannitol).
B : Study tube (agar and mannitol inoculated with MS).
C : Negative control tube (agar and mannitol).

The correlation coefficient between the variables in both groups

1- The correlation coefficients between the salivary flow rate and viable count of mutans streptococci.

The result in table (2) showed the correlation between salivary flow rate (ml/min) and CFU /ml $\times 10^3$ of M.S in non-smoker group which was negative, non-significant ($p > 0.05$). While the result showed negative high significant correlation was seen in smoker group ($p<0.001$).

Table (2) the correlation coefficient between the salivary flow rates (ml/min.) and (CFU /ml) of mutans streptococci and lactobacilli bacteria

Variable	Salivary flow rate(ml/min)					
	Non-smoker			Smoker		
	r value	P value	Sig.	r value	P value	Sig.
MS count (CFU/ml)	-0.238	0.138	NS	-0.538	.000	HS

P ≤0.05 Significant , P>0.05 Non significant

2- The correlation coefficient between salivary flow rate and DMFT

In table (3) the correlation coefficient between the salivary flow rate and (DMFT) was negative with highly significant correlation in both groups as ($p<0.001$).

Table (3) the correlation coefficients between the salivary flow rate (ml/min) and dental caries index in smoker and non-smoker groups

Variable	Salivary flow rate (ml/min)					
	Non-smoker group			Smoker group		
	r value	P value	significance	r value	P value	significance
DMFT	-0.556	.000	HS	-0.629	.000	HS

P ≤0.05 Significant , P>0.05 Non significant

3-The correlation coefficient between the salivary flow rate and salivary melatonin

The result in table (4) revealed that relation between the salivary flow rate and salivary melatonin was positive significant correlation in non-smoker group (P ≤0.05) and highly significant in smoker group ($p<0.001$).

Table (4) the correlation coefficient between salivary flow (ml/min) rate and salivary melatonin (pg/mL)

Variable	Salivary flow rate (ml/min)					
	Non-smoker			Smoker		
Ssalivary melatonin	r value	P value	Sig.	r value	P value	Sig.
	.518	.001	S	.777	.000	HS

P ≤0.05 Significant , P>0.05 Non significant

4-The correlation coefficients between the CFU/ml of mutans streptococci and DMFT among the non-smoker and smoker group

Table (5) revealed a positive significant correlation between viable count of mutans streptococci and DMFT, ($p<0.001$) in both groups.

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Table (5) correlation coefficient between the CFU/ml of mutans streptococci and DMFT among the non-smoker and smoker group

Variable	Mutans streptococci (CFU/ml)					
	Non-smoker			Smoker		
	r value	p value	Significance	r value	p value	significance
DMFT	0.596	.000	HS	0.694	.000	HS

P ≤0.05 Significant , P>0.05 Non significant

5- The correlation coefficient between the salivary melatonin and Colony Forming Units (CFU/ml) of salivary Mutans Streptococci

Table (6) showed positive high significant relation between salivary melatonin and M.S viable count in both group (, (p<0.001).

Table (6) the correlation coefficient between the salivary melatonin (pg/mL) and Colony Forming Units (CFU/ml) of salivary Mutans Streptococci

Variable	Mutans streptococci (CFU/ml)					
	Non-smoker group			Smoker group		
	r value	P value	significance	r value	P value	Significance
Salivary melatonin	-0.651	.000	HS	-0.704	.000	HS

P ≤0.05 Significant , P>0.05 Non significant

6- **Correlation coefficient between the Salivary melatonin and DMFT among non-smoker and smoker groups**

The result in table (7) illustrates a negative highly significant correlation between salivary melatonin and DMFT in both groups, the (p<0.001).

Table (7) correlation Coefficient between the salivary melatonin (pg/ml) and DMFT among the non-smoker and smoker group

Variable	Salivary melatonin (pg/ml)					
	Non-smoker			Smoker		
	r value	p value	Significance	r value	p value	significance
DMFT	-.676	.000	HS	-.760	.000	HS

P ≤0.05 Significant , P>0.05 Non significant

DISCUSSION

1-The salivary flow rate in relation to dental caries experience

In the present study the mean value of salivary flow rate in smoker group was lower than non-smoker, the difference between two groups was statistically significant, and this result means that smoking has negative effect on salivary flow rate. The explanation of this reduction may be due to the effect of smoking on taste receptors which is consider as a primary receptor site in the oral cavity that exposed constantly to the tobacco particles, generally the use of tobacco decreases the sensitivity of taste receptors with subsequent depression in salivary reflex, presumably, this might lead to change the taste receptors response and hence alter in salivary flow rate, the importance of saliva is due to its role in maintaining a healthy oral environment , clearing of cariogenic foods from oral cavity, buffer capacity (8) .The mean value of DMFT was higher in smoker group as compare with the non-smoker group, the result of current study agree with other previous studies (9,10). The statistical analysis revealed no significant differences between two groups. The increased of dental caries index in smoker group may be attributed to the deficient in the salivary flow rate which is lead to deficiency in clearing capacity of the cariogenic food from the mouth and deficiency

in neutralizing effect and buffer capacity of acids produced by cariogenic bacteria (11) , or due to the shifting of the bacterial population towards *lactobacillu*s and the cariogenic streptococci in smokers all might argue for increased dental caries(12) .The essential role of salivary flow rate against cariogenic bacteria and caries process confirm by the result of current study that detect the correlation between the salivary flow rate and DMFT, in both smoker and non-smoker group which was negative relation, this result agree with AL-Saadi, 2009(13), which said that salivary flow rate was negatively associated with dental caries.

2- Mutans streptococci in relation to salivary flow rate and dental caries experience

In present study the result showed that means value of CFU/ml of M.S bacteria was higher in smoker than non-smoker group, with no significant difference between two groups this was in agreement with other studies [(14)] .The explanation of this increased in count of cariogenic bacteria in oral cavity of smoker group may be due to either that tobacco smoking depress the immunoglobulin in oral cavity IgM , IgA, or could be due to reduced salivary flow rate and more carious teeth present in smokers than nonsmokers group which consider as retention site for bacteria [(9)] .The correlation coefficient in present study revealed positive highly significant relation between the (CFU/ml) of mutans streptococci and dental caries index(DMFT) in both groups, this finding agree with Al-mizraqchi,1998[(15)] .An explanation of this result due to that M.S are consider as highly cariogenic bacteria ,this is due to their several virulence factors mediated there carcinogenicity such as ,prevalent plaque adhesion-like cell surface proteins, acid production, tolerance, and production of glucosyl transferases, mutacin and intracellular polysaccharides (3).

The correlation coefficient between CFU/ml of M.S and salivary flow rate in smoker group was negative highly significant correlation and weak negative in non-smoker group, this lead to conclusion that high salivary flow rate represent as a protective factor against caries and cariogenic bacteria, since the role of saliva in maintaining a healthy oral environment which can be summarized by: diluting and eliminating sugars and other substances ,buffer capacity , balancing demineralization-reminerlization and the antimicrobial action of saliva in maintaining the equilibrium of the oral ecosystems, this is essential for dental caries control, the saliva is able to perform its function of maintaining the oral microbiota balance because it contains certain proteins, which are possess an antimicrobial effect because some of them are capable of modifying the bacteria's metabolism and inhibit their ability to adhere to the surface of the tooth, the most important proteins involved in oral ecosystem maintenance are proline -rich proteins, lysozyme, lactoferrin, peroxidases, agglutinins and histidine , as well as secretory immunoglobulin A and immunoglobulins G and M(3).

3-Salivary melatonin in relation to salivary flow rate and dental caries experience

The result of present study showed that salivary melatonin level was lower in smoker group than non-smoker group ,and the difference was statistically significant between two groups , this mean that the tobacco smoking is effect negatively on melatonin concentration in saliva, this is may be due to the role of melatonin in oral cavity which serves as an important antioxidant and free radical scavenger and act to get rid of negative effect of smoking particles and ROS (16)the result of current study agree with other previous study(17) .The correlation of MT with DMFS and DMFT were negative high significant relation, that's mean melatonin play an important role in reduction of dental caries this is may be due the role of melatonin against bacteria which consider one of the main etiological factors causing dental caries, MT may bind to free iron in the cytoplasm of the cell and restrict the growth of bacteria via this mechanism (18,19). While the correlation coefficient of salivary flow rate with melatonin were positive significant relation in non-smoker group and positive highly significant in smoker group, the explanation of these result may be when salivary flow rate decreased in smoker group , the clearance and washing action of saliva of all toxic products including ROS and bacterial biofilm will be reduced resulting in higher ROS level and bacterial count in oral cavity, so the available salivary antioxidants (MT) would be exhausted in reaction with the elevated free radicals and bacterial count in oral cavity .

4- Salivary melatonin in relation to mutans streptococci

In present study the coefficient correlation between the salivary melatonin and cariogenic bacteria (mutans streptococci) show negative, high significant relation in smoker and non-smoker group. Some study reported that melatonin have an antimicrobial activity against different microorganism (18, 19, 20) .In addition

to the in vitro studies virtually all studies performed in vivo with melatonin in infectious disease models documented it as a successful therapy(21,22). The explanation of the high inhibitory activity of melatonin against bacteria could be due to high metal binding capacity of MT including zing, copper, iron, melatonin can passes easily from all biological barriers including bacterial cell wall, it may bind with free iron in the cytoplasm and restrict bacterial growth via this mechanism (23).

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