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ORIGINAL ARTICLE

ESTIMATION OF PLASMA LYCOPENE IN ORAL LEUKOPLAKIA

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ABSTRACT:

Aim - To estimate and compare plasma lycopene levels in patients with clinically/ histopathologically proven leukoplakia with that of control group (smokers/non-smokers) **Materials and Methods-** Total of 90 individuals were included in the study with two main groups namely cases and controls: clinically/ histopathologically proven cases of oral leukoplakia (30) and age and sex matched control group divided into two categories non-smokers (30) and smokers without leukoplakia (30). Diagnosis of oral leukoplakia was first made on the basis of history and clinical features. Determination of lycopene in blood plasma by high performance liquid chromatography. **Results-** The age of the patients varied between 32-65 years. In the study group out of 30 patients of leukoplakia 16 patients were with clinically homogenous variety of leukoplakia and 14 with the speckled variety. Histopathologically out of 30 patients 13 had mild dysplasia and 17 with moderate dysplasia. The mean plasma lycopene level values of non smokers, smokers without leukoplakia and smokers with leukoplakia were 137.4±62.745, 40.07±23.956 and 29.46±17.497 respectively. Plasma lycopene level was significantly higher in non-smokers than smokers without leukoplakia and smokers with leukoplakia at 99% confidence level i.e. p-value less than 0.001 but there is non-significant difference in smokers without leukoplakia and smokers with leukoplakia (p-value=0.156). **Conclusions-** From the analysis of the result, and within limitations of the present study, the following conclusions can be drawn leukoplakia by itself do not cause reduced lycopene levels however, the lycopene levels are further reduced in oral leukoplakia when compared to non smoker controls its levels are inversely proportional to severity of leukoplakia, frequency and duration of habit.

Keywords- Leukoplakia, Plasma Lycopene

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INTRODUCTION:-

Major oral health problems till date are related to teeth and gums but other mucosal diseases are prevailing, which also include potentially malignant disorders. According to study conducted in south India the prevalence of leukoplakia, OSF and oral lichen planus was 0.59%, 0.55%, and 0.15% respectively. The prevalence of smoking, drinking alcoholic beverages and chewing tobacco was 15.02%, 8.78% and 6.99% respectively. Smoking and chewing were significant predictors of leukoplakia in their population.[1] Oral leukoplakia frequently precedes oral cancer and has similar etiologic factors. According to WHO 1997 Leukoplakia is defined as " a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion".[2] It is now categorized under potentially malignant disorders.[3]

Studies have shown that process of carcinogenesis occurs by generation of reactive oxygen species (ROS) , which act by initiating lipid peroxidation (LPO).[4] Antioxidants especially lycopene exhibits highest physical quenching rate constant with singlet oxygen. Lycopene has been found to be least 3-fold more effective than ß- carotene in preventing cell death by quenching of NOO radicals. It also protects DNA damage induced by 1-methyl 3-nitro-1-nitrosoguanidine and H_2O_2.[5]

Lycopene is a red colored fat soluble carotenoid, discovered by Ernest et al in 1959, which gives tomatoes and several other fruits their deep red color.[6] In general, tomato based food products provide 85% of dietary lycopene and remaining 15% is obtained from watermelon,

pink grapefruit, guava, papaya and other sources.[6] The mean plasma level of lycopene ranges from 0.22 to 1.06 nmol/ml and it contribute to about 21% to 43% of the total carotenoids.[7]

Humans are not able to synthesis lycopene *de novo*, we can only obtain it from the diet. The level of lycopene in human body is affected by several biological and lifestyle factors.[8] It is absorbed better from heat-processed food sources and lipid-rich diets than from raw food[9,10] Lycopene is most predominant carotenoid in human plasma.[8] Once ingested it appears in plasma, initially in the very-low-density lipoprotein (VLDL) and chylomicron fractions and later in low-density lipoprotein (LDL) and high- density lipoprotein (HDL) fractions. Owing to its lipophilic property, lycopene is found to concentrate in LDL and VLDL.[11] Other than human serum, lycopene can also distribute in various human tissues, particularly in the adrenal gland, liver, prostate gland, and testes, where it is the most predominant carotenoid.[8,12]

Lycopene is not a necessary nutrient. However, it may be very important for optimal health. The use of antioxidants for treatment of oral leukoplakia is not new, however a specific correlation between a particular antioxidant level in the plasma and susceptibility to develop leukoplakia/severity of disease has not been clearly elucidated. Further, lycopene dosages used in different studies for the treatment of various oral diseases (including oral submucous fibrosis, oral lichen planus, leukoplakia etc) have been empirical and not clearly established. Human oral leukoplakias have been treated with a range of retinoids and carotenoids. Although β-carotene has been shown to be protective and known to produce sustained remissions in patients with oral leukoplakia[13], the importance of other carotenoids, whose levels in blood are particularly correlated with those of β-carotene, has not been adequately explored in precancerous states.[14] In view of the forgoing a need to carry out a study to determine the level of plasma lycopene in oral leukoplakia and ascertain its correlation with disease severity, if any, is warranted.

Aim: To estimate and compare plasma lycopene levels in patients with clinically/ histopathologically proven leukoplakia with that of control group.

Objectives: -To determine the correlation; if any between plasma lycopene levels and oral leukoplakia with reference to disease severity

-To compare and correlate plasma lycopene levels in smokers with oral leukoplakia and without oral leukoplakia and in non-smokers

MATERIALS & METHODS:

The present study was conducted in the Department of Oral medicine and Radiology of Sri GuruGovind Tricentenary Dental College in collaboration with Jagsonpal Pharmaceuticals Ltd Faridabad. The study was approved by ethical committee of Sri GuruGovind Tricentenary Dental college prior to its commencement.

Study Subjects

Study group was screened from the patients attending the out patient department of Oral Medicine and Radiology, SGT Dental College Budhera, Gurgaon. 30 such patients, who were clinically diagnosed with oral leukoplakia and fulfilling the inclusion criteria were included for the study. And the age and sex matched healthy individuals comprised the control group.

Selection criteria:-

Inclusion criteria: 30 clinically/histopathologically proven cases of oral leukoplakia, 30 age and sex matched healthy individuals without any oral deleterious habits and with no known systemic diseases or precancerous lesions/conditions (controls) and 30 age and sex matched healthy individuals with habit of smoking without any known systemic diseases or precancerous lesions/conditions (controls)

Exclusion criteria: subjects on oral antioxidants, patients with other mucosal disease (eg. Oral cancer, oral lichen planus, oral submucous fibrosis) and patients known to have other systemic disease (screened on the basis of their medical history/questionnaire).

Method of collection of data:

The study includes two main groups namely cases and controls.

1. Clinically/ histopathologically proven cases of oral leukoplakia (30)
2. Age and sex matched control group divided into two categories: Non-smokers (30) and Smokers without leukoplakia (30)

Diagnosis of oral leukoplakia was first made on the basis of history and clinical features described as follows:

A. i. Homogenous

ii.Non Homogenous which includes: Speckled, Nodular, Verrucous or Proliferative verrucous leukoplakia.[3]

B. Histopathological grading according to severity of dysplasia as follows: Mild/Moderate/Severe epithelial dysplasia or Carcinoma in situ.[15]

After explaining about the study to the subjects, an informed consent was obtained from the patients. For all the subjects in both the groups detailed case history was taken along with thorough oral examination. The patients clinically diagnosed with leukoplakia underwent incisional/ excisional biopsy and the estimation of plasma lycopene levels were done for both the groups.

Method of collection of blood sample

After obtaining detailed case history patients were asked to report next day morning with fasting for 8 hour before arrival**.** This was to avoid any dietary influence on plasma lycopene levels. Then 4ml of venous blood was withdrawn and put into sodium citrate vial (3.2%) of AkuSet™ and was assigned a number with the help of marker. Blood samples were stored on ice until centrifuged for 10 min at

4000 rpm, plasma was separated[16] and collected in a new vial assigned a same number. Vials were then wrapped with the tin foil and blood samples chilled in ice[17,18] packs were transported on the same day after collection for analysis and all analysis were done under dim light.

Determination of lycopene in blood plasma by High performance liquid chromatography (HPLC)

a. Preparation of standard solution:

Accurate amount of lycopene was weighed (so that the final dilution should be 1 ppm) in 50ml volumetric flask, dissolve in chloroform and make the volume 50 ml with chloroform. 1 ml of it was taken in 25 ml volumetric flask and the volume was completed with mobile phase and 20 micro liter of standard solution was injected.

b. Preparation of test solution:

1ml of plasma is taken into tube and 1ml of alcohol was added and mixed in vortex mixer for 1 minute, 2 ml of petroleum ether was added and again mixed in vortex mixer for 1 minute. The petroleum ether layer was separated and evaporated under nitrogen, and 0.5 ml of absolute alcohol was added and mixed properly 20 microliter of sample was injected.

Chromatographic conditions: Mobile phase: 47: 47: 06 (Acetonitrile: Methanol: Chloroform),Wave lengh: 472 nm, Column: Novapek $C_{16,}$ Flow Rate: 1.5 ml/liter, Plasma lycopene level values were given in ng/ml , 1 µmol/l × 536 = 1ng/ml, 536 is the molecular weight of lycopene

RESULTS:

The study was designed to assess the Plasma lycopene levels in patients with oral leukoplakia and to compare the results with age/sex matched controls. The control group was further divided into smokers and non smokers. Total of 90 individuals were included in the study. Out of which 30 patients were of clinically/histopathologically proven oral leukoplakia with the positive history of smoking and all the patients were males. The age of the patients varied between 32-65 years. In the study group out of 30 patients of leukoplakia 16 patients were with clinically homogenous variety of leukoplakia and 14 with the speckled variety. Histopathologically out of 30 patients 13 had mild dysplasia and 17 with moderate dysplasia. The age and gender matched control group of 60 males without oral premalignant lesions/ conditions were divided into two groups. One with the habit of smoking i.e. 30 and another of non smokers also comprised of 30 individuals.

In table 1.1 the mean age of non smoker was 47.97 ± 9.144 and for smokers without leukoplakia was 48.17 ± 9.029 and smokers with leukoplakia was 47.37 ± 10.132. In table 1.2 the mean plasma lycopene level values of non smokers, smokers without leukoplakia and smokers with leukoplakia were 137.4±62.745, 40.07±23.956 and 29.46±17.497 respectively. In table 2 when annova test was applied for comparing the mean values between three groups and within three groups for age than p value is not significant. But for plasma lycopene levels it is highly significant. To see the multiple comparisons between two groups, post-hoc test is followed. In table 3 when Post-hoc was applied then, plasma lycopene level is significantly higher in non-smokers than smokers without leukoplakia and smokers with leukoplakia at 99% confidence level i.e. p-value less than 0.001 but there is non-significant difference in smokers without leukoplakia and smokers with leukoplakia (p-value=0.156).

Table 4,5,6 and 7 compares the mean values between the two groups of smokers without and with leukoplakia, unilateral and bilateral distribution of leukoplakia, homogenous and speckled leukoplakia, mild and moderate dysplasia. On comparison of mean frequency of bidi smoking, duration of habit, plasma lycopene levels and age in all the group none of p-value is significant. The statistical analysis was done using SPSS (statistical package for social sciences) Version 16.0 statistical analysis software.

Table 1.1: Descriptive Statistics of Age

		N	Mean	Std. Deviation
Age	Non-Smokers	30	47.97	9.144
	Smokers Without Leukoplakia	30	48.17	9.029
	Smokers With Leukoplakia	30	47.37	10.132
	Total	90	47.83	9.348

Table 1.2: Descriptive Statistics of Plasma Lycopene Levels

		N	Mean	Std. Deviation
Plasma lycopene Levels	Non-Smokers	30	137.4	62.745
	Smokers Without Leukoplakia	30	40.07	23.956
	Smokers With Leukoplakia	30	29.46	17.497
	Total	90	70.74	64.232

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Table 2: ANOVA to Compare Mean values between three groups

ANOVA						
		Sum of Squares	df	Mean Square	F	p-value
Age	Between Groups	10.4	2	5.2	0.058	0.943
	Within Groups	7766.1	87	89.266		
	Total	7776.5	89			
Plasma lycopene Levels	Between Groups	212357.268	2	106178.634	66.127	<0.001**
	Within Groups	139694.51	87	1605.684		
	Total	352051.778	89			

** The p-value is significant at 5% level

Table 3: Post-hoc Dunnett t3 for Plasma Lycopene Levels

(I) Group	**(J) Group**	**p-value**
Non-Smokers	Smokers Without Leukoplakia	<0.001
	Smokers With Leukoplakia	<0.001
Smokers Without Leukoplakia	Smokers With Leukoplakia	0.156

The p-value is significant at 5% level

Table 4: Mean Comparison between Smokers without Leukoplakia and Smokers with Leukoplakia.

	Group	**N**	**Mean**	**Std. Deviation**	**p-value**
Frequency	Smokers Without Leukoplakia	30	11.2	4.421	0.588
	Smokers With Leukoplakia	30	11.87	5.05	
Duration	Smokers Without Leukoplakia	30	19.13	5.519	0.528
	Smokers With Leukoplakia	30	20.17	7.003	
Plasma lycopene Levels	Smokers Without Leukoplakia	30	40.07	23.956	0.055
	Smokers With Leukoplakia	30	29.46	17.497	
Age	Smokers Without Leukoplakia	30	48.17	9.029	0.748
	Smokers With Leukoplakia	30	47.37	10.132	

Table 5: Mean Comparison between Unilateral and Bilateral

	Distribution	**N**	**Mean**	**Std. Deviation**	**p-value**
Frequency	Unilateral	16	10.62	5.62	0.153
	Bilateral	14	13.29	4.046	
Duration	Unilateral	16	18.06	5.579	0.078
	Bilateral	14	22.57	7.861	
Plasma lycopene Levels	Unilateral	16	31.59	18.712	0.486
	Bilateral	14	27.03	16.339	
Age	Unilateral	16	46.06	10.03	0.461
	Bilateral	14	48.86	10.413	

Table 6: Mean Comparison between Homogeneous and Speckled.

	Clinical Type	**N**	**Mean**	**Std. Deviation**	**p-value**
Frequency	Homogeneous	16	9.88	4.951	0.018
	Speckled	14	14.14	4.258	
Duration	Homogeneous	16	17.38	7.154	0.017
	Speckled	14	23.36	5.458	
Plasma lycopene Levels	Homogeneous	16	35.95	18.813	0.027
	Speckled	14	22.05	12.791	
Age	Homogeneous	16	45.5	10.218	0.289
	Speckled	14	49.5	9.967	

The p-value is significant at 5% level

Table 7: Mean Comparison with respect to Histopathological Grading

	Histopathological Grading	N	Mean	Std. Deviation	p-value
Frequency	Mild	13	9.62	4.805	0.03
	Moderate	17	13.59	4.651	
Duration	Mild	13	17.54	7.902	0.071
	Moderate	17	22.18	5.67	
Plasma lycopene Levels	Mild	13	40.49	17.987	0.001
	Moderate	17	21.04	11.75	
Age	Mild	13	45.31	10.483	0.339
	Moderate	17	48.94	9.877	

The p-value is significant at 5% level

DISCUSSION:-

The association between oral leukoplakia and tobacco habits is well established in numerous epidemiologic studies. The association has generally been found to be strong and therefore the habits of tobacco smoking and chewing are accepted as the principal aetiologic factors for oral leukoplakia (Gupta PC, 1980)[19] Reports indicate that 15.8-48.0% of Oral squamous cell carcinoma patients were associated with Oral leukoplakia when diagnosed.[20]

Various treatment modalities have been suggested in the literature for management of leukoplakia by various authors. These include both pharmacological and non pharmacological approaches for the same. As the association of leukoplakia and tobacco has been strongly suggested so the free radical scavengers should be the necessary part of the treatment regimen in tobacco chewers or smokers to prevent the formation and to induce remission or inhibition of progression of precancerous lesions into malignany.[5]

It is well known fact that tobacco is addictive, and its use is harmful to health in many ways. Both smoked and smokeless tobacco contain the alkaloid ie nicotine, which is the main addictive agent. Smoked as well as unburnt tobacco contain thousands of chemical compounds. Many of these compounds are not only irritants and toxins but they are also carcinogens. The most potent carcinogens in tobacco are the tobacco-specific nitrosamines, polyacrylic aromatic hydrocarbons, and many others.[21] Lycopene, the carotenoid that gives the ripe tomato its bright red color, is a very effective natural antioxidant and quencher of free radicals.[5]

Carotenoids are naturally occurring plant pigments. Humans ingest many different carotenoids in the diet, with the primary dietary sources being fruits and vegetables. Plasma concentrations of carotenoids can be readily measured. Such analysis have indicated that plasma carotenoids are correlated with consumption of fruits and vegetables, with plasma carotenoid concentrations considered one of the best biomarkers of fruit and vegetable intake currently available.[22,23] Carotenoids and α-tocopherol are among the most widely studied compounds in various populations, for both serum and plasma concentrations and dietary intake. Examination of specific micronutrients levels in serum and plasma therefore will advance our knowledge on putative anti-carcinogenic agents.

The present study is a biochemical study and an attempt was to analyse plasma lycopene levels in patients with oral leukoplakia and compare with normal healthy individuals thereby analyzing the oxidative stress. An attempt was also to compare the same between different clinical types of leukoplakia and also according to histopathological grading of dysplasia. In the present study the plasma lycopene levels of clinically and histopathologically proven cases of leukoplakia were determined and compared to those with controls by High performance liquid chromatography undertaken at Jagsonpal Pharmaceuticals.

Raw and processed tomatoes are the main sources of lycopene in the human diet. However, the lycopene content of tomato products is highly variable, being affected by factors such as variety, ripeness, climate and geographical site of production and processing. The amount of lycopene present in the diet of an individual is therefore difficult to assess accurately and for this reason consumption of raw and processed form of tomatotes have been only taken into account in the present study, the individuals are from rural population and were generally vegetarian and the source of dietary lycopene is from the cooked vegetables. Raw tomatoes were rarely consumed and fruits were not consumed regulary. All the patients were having similar kind of dietary pattern and presumed to have uniform dietary source of lycopene.

In the present study mean plasma lycopene levels for the leukoplakia patients (smokers) was 29.46±17.497 ng/ml, and for controls who were smokers it was 40.07±23.956 ng/ml, however the mean levels for non-smokers was 137.4±62.745 ng/ml which was significantly more. This was supported by many studies that smokers have lower plasma concentrations of most carotenoids than non-smoker.[24] The possible explanation is that smoking is well known for introducing a source of free radicals, which can increase lipid peroxidation and DNA damaging. Among the cellular molecules, lipids that contain unsaturated fatty acids with more than one double bond are particularly susceptible to action of free radicals, The resulting reaction known as lipid peroxidation, disrupts biological

membranes and is thereby highly deleterious to their structure and function.[25]Lycopene may be used to neutralize the free radicals generated from smoking.

In the study conducted by Nagao T (2000)[14] the mean serum lycopene and β-carotene levels among male cases were significantly lower than those detected for controls. The results of the population-based study suggested that high serum levels of β-carotene and lycopene reduce the risk of leukoplakia in Japanese males. In their study this was confirmed in the bivariate analysis but the significance of lycopene was not apparent in the logistic regression analysis.

The present study reveal no significant difference in plasma lycopene levels of cases of leukoplakia when compared with age, gender, frequency and duration of associated habit matched controls. Whereas a definite significant difference in plasma lycopene levels is observed between smokers and non smokers, which shows inverse relation of smoking and plasma lycopene levels. Thus the role of lycopene in managing the oral leukoplakia is attributed to its role as antioxidant in decreasing free radical damage and its abilty to quench singlet oxygen, which is a reactive unstable molecule. Role of lycopene in leukoplakia is imperical but it should be supplemented in leukoplakia as leukoplakia is potentially malignant lesion and the role of lycopene in inhibition and progression of cancer has been proven.

SUMMARY AND CONCLUSION:

The present study was conducted to estimate plasma lycopene levels in oral leukoplakia. Estimation of plasma lycopene levels in 30 clinically and histopathologically proven cases of leukoplakia associated with smoking was done and compared with age and sex matched controls. The control group was further divided into two groups of smoker and non-smoker.

This study reaffirms the fact that smoking is inversely related to plasma lycopene levels. Statistical analysis of the data showed that plasma lycopene levels are significantly lower among leukoplakia patients when compared with non-smoker controls. To exclude disparity among cases and controls, an attempt was also made to compare cases with age, sex, frequency and duration of habit matched controls which revealed non significant difference between the two. In this study no correlation was found between the groups when frequency and duration of habit was also taken into consideration between cases and controls.

In the study an attempt was also made to compare lycopene levels among the cases with homogenous and speckled variety with mild and moderate dysplasia respectively. Which revealed significant difference in plasma levels in both. Speckled variety with moderate dysplasia showed decreased levels as compared to Homogenous with mild dysplasia. But when the frequency and duration of habit was taken into account for both. The frequency and duration of smoking was found to be more in speckled variety with moderate dysplasia which possibly could explain the variation of lycopene levels among the cases.

From the analysis of the result, and within limitations of the present study, the following conclusions were drawn

i. It could be concluded that smoking causes reduced lycopene levels.
ii. Leukoplakia by itself do not cause reduced lycopene levels however, the lycopene levels are further reduced in oral leukoplakia when compared to non smoker controls
iii. Lycopene levels are inversely proportional to severity of leukoplakia, frequency and duration of habit.

Lycopene being important carotenoid, further studies with large sample size are necessary to investigate lycopene levels and its association with leukoplakia associated with various habits, Also according to clinical and histopathological types and impact of dietary sources is required as not much of the work has been done on plasma lycopene levels and its relation with oral leukoplakia.

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