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Research Article

THE COMET ASSAY A METHOD TO MEASURE DNA DAMAGE IN ORAL SUBMUCOUS FIBROSIS AND ORAL LEUKOPLAKIA PATIENTS: A CASE CONTROL STUDY

M.Shanmuga sundaram[1]*, K.Saraswathi Gopal[2], B.G.Harsha Vardhan[3]

[1]Department of Oral Medicine and Radiology Meenakshi Ammal Dental College, Chennai, Tamilnadu, India.
[2]Professor and Head of the Department, Department of Oral Medicine and Radiology Meenakshi Ammal Dental College, Chennai, Tamilnadu, India.
[3]Professor, Department of Oral Medicine and Radiology Meenakshi Ammal Dental College, Chennai, Tamilnadu, India.

ABSTRACT

The aim of the study is to evaluate the DNA damage in oral submucous fibrosis and leukoplakia patients and to correlate with the normal population. The present study, "The comet assay a method to measure DNA damage in Oral submucous fibrosis and Oral leukoplakia patients; A case control study" was conducted in the Department of Oral Medicine and Radiology and the Central Research Laboratory, Meenakshi Ammal Dental College and Hospital, MAHER University, Chennai. The clearance for conducting the study was obtained from Ethical Committee, MAHER University following the regular protocols. The patients were divided into 3 groups. Group I consists of 20 Oral submucous fibrosis patients, group II consists of 20 Leukoplakia patients and group 3 consists of 20 control patients. Buccal exfoliate cells have been collected from all the patients in the group and the DNA damage is evaluated by the method of Single cell gel electrophoresis technique/ comet assay technique. The mean tail length of the DNA obtained from the buccal epithelial cells of the Oral submucous fibrosis subjects(group I) was 9.14(±2.08), leukoplakia subjects(group II) was 11.83(±1.21) and control subjects(group III) was 3.81(±0.49) respectively. The mean tail length of DNA obtained from the buccal epithelial cells of leukoplakia which was 11.83(±1.21) which was significantly higher when compared to the epithelial samples obtained from the oral submucous fibrosis group and control group with a P value <0.001. Also the tail length of the DNA obtained was compared with the duration of the habit and types of habits which has a positive co relation between the tail length damage to type of habit and duration of the habit. We conclude that analysing DNA damage using comet assay for potentially malignant disorder patients is a useful tool in dentistry for early identification of the malignant transformation of the affected patients. Hence, this non-invasive technique is required for screening the patients with potentially malignant disorders.

Abbreviations: OSMF- oral submucous fibrosis, PBS – phosphate buffer saline, DNA - Deoxyribonucleic acid, PMD – Potentially malignant disorders, WHO – world health organization.

Key words:- Oral submucous fibrosis, Leukoplakia, Buccal exfoliated cells, Comet assay, Tail length, DNA damage.

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Corresponding Author

M.Shanmuga sundaram
Email: - shanmugadr@gmail.com

INTRODUCTION

The potentially malignant disorders such as leukoplakia, characterized by a white patch, mainly associated with tobacco smokers, and oral submucous fibrosis, characterized by deposition of collagen and marked blanching of oral cavity, leading to inability to open mouth, mainly associated with smokeless tobacco and betel quid chewers, have a greater prevalence in our country. Both of these conditions have high cancer turnover potentiality and if detected early can be prevented and treated successfully. With present advances in genotoxicological studies, extent of DNA damage provides a platform to determine the cancer progression. One of the important hallmarks for cancer progression is DNA damage, resulting either from various carcinogens accumulating from etiologic influences or due to genetic errors. If detectable and quantifiable the damage of DNA, these may contribute toward an early detection and prediction system for oral cancer development.

MATERIALS AND METHOD

The present study "The comet assay a method to measure DNA damage in Oral submucous fibrosis and Oral leukoplakia patients; A case control study" was conducted in the Department of Oral Medicine and Radiology and the Central Research Laboratory, Meenakshi Ammal Dental College and Hospital, MAHER University, Chennai. The clearance for conducting the study was obtained from Ethical Committee, MAHER University following the regular protocols. The patients were divided into 3 groups. Group I consists of 20 oral submucous fibrosis patients, group II consists of 20 Leukoplakia patients and group 3 consists of 20 control patients. After explaining about the study and about DNA protocols (i.e. about discarding the DNA sample after the results) consent form was obtained from each patient.

Group I	Group II	Group III
20 OSMF subjects	20 Leukoplakia subjects	20 control subjects

Software used

"Casplab software" which is used to analysed the DNA tail length in Windows OS.

Study group.

Inclusion Criteria:

Persons who are willing for the study.
Persons in the age group above 20 years.
Subjects with past or present history of tobacco or areca nut chewing and/alcohol consumption.
Exclusion Criteria:
1. Subjects without past or present history of tobacco or areca nut chewing and/alcohol consumption.
2. Subjects with systemic diseases and disorders, as they may show some extent of DNA damage. (Diabetes mellitus, hypertension, Rheumatoid Arthritis etc.)

Inclusion Criteria:

Persons who are willing for the study
Healthy individuals in age group above 20 years.
Persons who have never used cigarettes or other forms of tobacco during their life time.

Exclusion Criteria:

1. Subjects with past or present history of tobacco or areca nut chewing and/alcohol consumption.
2. Subjects with systemic diseases and disorders, as they may show some extent of DNA damage.(Diabetes mellitus, hypertension, Rheumatoid Arthritis etc.)

Collecting the exfoliate cells.
The patient was advised not to take any food for atleast half an hour before the procedure.
The patient is seated comfortably in a calm, unstrained position.
A sterile swab was dipped into the Phosphate buffer saline.
The exfoliate cells was collected by scraping the swab against the buccal mucosa were the lesion was evident.
After collecting the cells the swab was replaced in the container.

Evaluation of DNA damage

The DNA damage is evaluated by the method of Single cell gel electrophoresis technique/ comet assay technique. A commercially available kit (TREVIGEN) was used for evaluating the DNA damage.
Cut the swab and place it in a 2ml of centrifuge tube containing 1ml of 1 X sterile phosphate buffer saline (PBS).
Gently tap and vortex sample.
Centrifuge for 12000 rpm for 2 min
Discard supernatant.
To the pellet add 10 µl of 1 X sterile PBS
Use cell in ration 10 : 1.
Combine cells at 1 x 105/ml with molten Low Melting Agarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipette 50 µl onto Comet Slide. If necessary, use side of pipette tip to spread agarose/cells over sample area to ensure complete coverage of the sample area. If sample is not spreading evenly warm the slide at 37°C before application.
Place slides flat at 4°C (e.g. place in refrigerator) for 10 minutes. Increasing gelling time to 30 minutes improves adherence of samples in high humidity environments. Immerse slides in 4°C lysis Solution for 30-60 minutes. Drain excess buffer from slides and immerse in freshly prepared Alkaline Unwinding Solution, pH>13.

Immerse Comet Slide in Alkaline Unwinding Solution for 20 minutes at room temperature or 1 hour at 4°C

Add 850 ml 4°C Alkaline Electrophoresis Solution, place slides in electrophoresis slide tray and cover with Slide Tray Overlay. Set power supply to 50 volts for 30 minutes. Gently drain excess electrophoresis solution, gently immerse twice in distilled water for 5 minutes each, then in 70% ethanol for 5 minutes.

Dry samples at 37°C for 10-15 minutes. Drying brings all the cells in a single plane to facilitate observation. Samples may be stored at room temperature. After drying, the slides are stained with a fluorescent dye (ethidium bromide), and DNA "comet" was easily visualized using a fluorescent microscope.

The broken DNA fragments or damaged DNA undergoing electrophoresis migrate away from the nucleus. In this process, the smallest fragments travel the farthest. The extent of DNA liberated from the head of the comet is directly proportional to the amount of DNA damage. Using image analysis software, comet tail length was calculated by subtracting the diameter from the total length.

Statistical analysis

The collected data was analysed with SPSS 16.0 version. To describe about the data descriptive statistics frequency analysis, percentage analysis was used for categorical variables and for continuous variables the mean and S.D were used. To find the significance in categorical data Chi-square test was used. In the above statistical tool the probability value 0.5 was considered as significant level.

In this study, we have evaluated the DNA damage in patients with OSMF, leukoplakia and compared with the controls. We have also compared the DNA damage with the type of habits and duration of the habits.

The collected data has been tabulated for statistical analysis.

- Total samples (n=60),
- study group I (n=20),
- study group II (n=20),
- Healthy control group III (n=20)

Statistical analysis used was

- Sample t test
- Chi square test
- Pearson Correlation test

GRAPH – 1

Table - 4 & graph - 1 inference: Among 20 cases of OSMF, 20 cases of Leukoplakia and 20 cases of controls, difference noted between them with gender was found not to have any statistical significant ($p<1.5$)

The number of subjects included in this study is 60, of which group I clinically diagnosed and histopathologically proven Oral submucous fibrosis subjects consist of sample size 20, group II clinically diagnosed leukoplakia consist of sample size 20 and group II control subjects consist of 20 samples. Group 1 consist of 19 males and 1 female : group II consist of 20 males: and group III consist of 19 males and 1 female respectively. (Table 4 and graph 1)

Table -5 & graph - 2 inference: Among 20 cases of OSMF, 20 cases of Leukoplakia and 10 cases of controls, the age difference noted between them was found to be significant. ($p<0.001$)

The mean age of the Oral Submucous Fibrosis (group 1) was 36.00 (± 7.739) years, Leukoplakia (group 2) was 44.15(±12.67) years and control was 40.65(±1.823) years. Among 20 cases of OSMF, 20 cases of leukoplakia and 20 cases of controls, the age difference noted between them was found to be significant (Table 5 & graph - 2).

Table-6 & graph - 3 inference: Among 20 cases of OSMF, 20 cases of Leukoplakia and 20 cases of controls, the difference noted between them with tail length obtained from the DNA extracted from the buccal epithelial cells was found to be highly significance ($p<0.001$)

The mean tail length of the DNA obtained from the buccal epithelial cells of the Oral submucous fibrosis subjects (group 1) was 9.14(±2.08), leukoplakia subjects (group 2) was 11.83(±1.21) and control subjects (group 3) was 3.81(±0.49). The mean tail length of DNA obtained from the buccal epithelial cells of leukoplakia which was 11.83(±1.21) was significantly higher when compared to the epithelial samples obtained from the oral submucous fibrosis group and control group. P value is <0.001. (Table 6 & graph -3)

Table- 7 & graph - 4 inference: Among the duration of the habit, the difference noted between the tail length damage with duration of the habits was found to be highly significance ($p<0.001$)

The duration of the habits were recorded in years for smoking, chewing and alcohol consuming were calculated for each group and has been compared. The number of years for duration of the habits has a direct co-relation with increased tail length damage hence the difference noted between the groups with duration of the habits were highly significant. (Table -7 & graph -4)

The Post Hoc test with multiple comparison statistics for the study groups and healthy control group (n=60) was analysed. The mean difference between the groups OSMF, Leukoplakia and controls were highly significant with p value (<0.001) which is 99.9% significant. (Table – 8)

Table-9 & graph - 6 inference: This shows that the maximum number of subjects with leukoplakia are smoker and the maximum number of subjects with OSMF are chewers and alcoholic.

The subjects were analysed with their types of habits such as smoking: chewing: smoking and alcohol: smoking and chewing: chewing, alcohol and smoking,

chewing and alcohol. The data's were analysed which revealed maximum tail length damage was observed in smoking and chewing subjects, with tail length damage of, 12.24 which was significantly higher.(Table -9 & graph- 6).

Table-10 & graph- 7 inference: Analysing the subjects on educational status revealed that maximum number of subject were educated only till higher secondary.

Table-11 & graph - 8 inference: analysing the subject with their profession reveals that most of the OSMF patient and Leukoplakia patients are drivers and most of the control patients are employed in an corporate based jobs.

The socioeconomic status of the subjects was also included in this study by knowing the education, occupation and income of the subjects. Kuppuswamy socioeconomic scale was employed for the same, it revealed maximum number of the subject were lower middle class.(Table 10,11 & graph 7,8).

RESULTS

Table 1. Master Chart – Study Group-I (Oral Submucous Fibrosis)

S.NO	Age/Sex	Grade OSMF	Tail length (µm)
1	50/F	IV	5.4
2	35/M	IV	10.9
3	36/M	IV	12.9
4	40/M	IV	6.3
5	42/M	IV	11
6	24/M	IV	7.8
7	42/M	IV	8.1
8	52/M	IV	9.1
9	28/M	IV	10.6
10	34/M	IV	9.4
11	43/M	IV	8.2
12	26/M	IV	6.9
13	26/M	IV	7.4
14	31/M	IV	8.3
15	42/M	IV	7.8
16	37/M	IV	12.2
17	30/M	IV	9.1
18	30/M	IV	9.5
19	37/M	IV	9.1
20	35/M	IV	12.9

Table 2. Master chart – study group-II (Leukoplakia)

S.No	Age/Sex	Diagnosis	Tail length (µm)
1	43/M	Leukoplakia In Right Buccal Mucosa And Right Lateral Border Of The Tongue	11.2
2	21/M	Leukoplakia In Left Buccal Mucosa	8.5
3	47/M	Leukoplakia In Left Buccal Mucosa	12.4
4	60/M	Leukoplakia In Right Buccal Mucosa	13.1
5	48/M	Leukoplakia In Left Buccal Mucosa	11
6	46/M	Leukoplakia In Left Buccal Mucosa	11.5
7	35/M	Leukoplakia In Right And Left Buccal Mucosa	12.6
8	30/M	Leukoplakia In Left Buccal Mucosa	11.8
9	50/M	Right And Left Commissure Of The Lip	11
10	54/M	Left Commissure Of The Lip	13.1
11	36/M	Right Commissure Of The Lip	13.1
12	36/M	Leukoplakia In Left Buccal Mucosa	11.7
13	18/M	Leukoplakia In Left Buccal Mucosa	13.9
14	65/M	Leukoplakia In Left Buccal Mucosa	11.3
15	67/M	Right Commissure Of The Lip	11.6
16	43/M	Leukoplakia In Right And Left Buccal Mucosa	10.3

17	45/M	Leukoplakia In Right And Left Buccal Mucosa	10.9
18	43/M	Leukoplakia In Right And Left Buccal Mucosa	12.6
19	45/M	Leukoplakia In Right And Left Buccal Mucosa	12.7
20	51/M	Right And Left Commissure Of The Lip	12.3

Table 3. Master Chart Group-III Healthy Control

S.NO	AGE/SEX	Tail length(µm)
1	45/M	3.8
2	48/M	4.1
3	37/M	3.1
4	43/M	3.2
5	38/M	3
6	41/M	3.5
7	39/M	4.1
8	46/M	4.2
9	45/M	4.3
10	41/M	3.6
11	44/M	4.1
12	46/M	3.9
13	37/M	4.1
14	26/F	3.7
15	41/M	3.6
16	38/M	4.1
17	43/M	3.8
18	35/M	4.3
19	39/M	4.1
20	41/M	3.7

Table 4. Gender:

		Gender		Exact Sig (2-sided)	Total
		MALE	FEMALE		
Groups	OSMF	19	1	0.418	20
	Leukoplakia	20	0		20
	Control	19	1		20

Table 5. Age

Group	Mean Age	SD
OSMF	36.00	±7.739
Leukoplakia	44.15	±12.67
Control	40.65	±1.823

Table 6. Tail Length

Group	Mean Tail Length (µm)	SD
OSMF	9.14	±2.08
Leukoplakia	11.83	±1.217
Control	3.81	±0.49

Table 7. Duration of the Habits and Tail Length

Duration of the Habit (Years)	Tail Length in OSMF (µm)	Tail Length in Leukoplakia (µm)
0-10	6.5	10.3
11-20	8.5	11.4
21-30	11.6	12.1
31-40	12.1	12.9

Table 8. Comparision Between Groups

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Diagnosis	(J) Diagnosis	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
age	Leucoplakia	OSMF	8.15000*	2.99526	.024	.9011	15.3989
		Control	17.85000*	3.66843	.000	8.9719	26.7281
	OSMF	Leucoplakia	-8.15000*	2.99526	.024	-15.3989	-.9011
		Control	9.70000*	3.66843	.029	.8219	18.5781
	Control	Leucoplakia	-17.85000*	3.66843	.000	-26.7281	-8.9719
		OSMF	-9.70000*	3.66843	.029	-18.5781	-.8219
years	Leucoplakia	OSMF	6.95000*	2.45658	.018	1.0048	12.8952
		Control	20.85000*	3.00868	.000	13.5686	28.1314
	OSMF	Leucoplakia	-6.95000*	2.45658	.018	-12.8952	-1.0048
		Control	13.90000*	3.00868	.000	6.6186	21.1814
	Control	Leucoplakia	-20.85000*	3.00868	.000	-28.1314	-13.5686
		OSMF	-13.90000*	3.00868	.000	-21.1814	-6.6186
TailLength	Leucoplakia	OSMF	2.68500*	.49088	.000	1.4970	3.8730
		Control	8.10000*	.60120	.000	6.6450	9.5550
	OSMF	Leucoplakia	-2.68500*	.49088	.000	-3.8730	-1.4970
		Control	5.41500*	.60120	.000	3.9600	6.8700
	Control	Leucoplakia	-8.10000*	.60120	.000	-9.5550	-6.6450
		OSMF	-5.41500*	.60120	.000	-6.8700	-3.9600

Table 9. Types of Habits and Tail Length

Habits	Tail Length in OSMF (µm)	Tail Length in Leukoplakia (µm)
Smoking		11.25
Chewing	8.14	
Smoking and alcohol		12.02
Smoking and chewing	8.85	12.24
Chewing and alcohol	10.46	

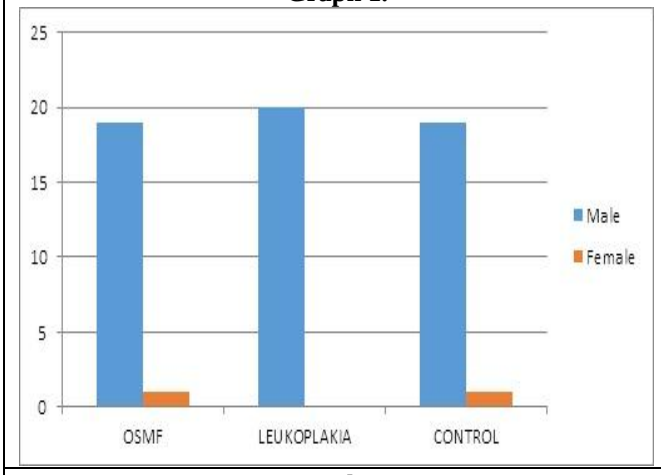
Table 10. Socioeconomical Status

Crosstab							
			Education				Total
			Uneducated	School	1st Degree	2nd Degree	
Diagnosis	Leucoplakia	Count	5	15	0	0	20
		% within Diagnosis	25.0%	75.0%	.0%	.0%	100.0%
		% within education	71.4%	50.0%	.0%	.0%	40.0%
	OSMF	Count	2	12	6	0	20
		% within Diagnosis	10.0%	60.0%	30.0%	.0%	100.0%
		% within education	28.6%	40.0%	54.5%	.0%	40.0%
	Control	Count	0	13	5	2	20
		% within Diagnosis	.0%	30.0%	50.0%	20.0%	100.0%
		% within education	.0%	10.0%	45.5%	100.0%	20.0%
Total		Count	7	40	11	2	60
		% within Diagnosis	14.0%	60.0%	22.0%	4.0%	100.0%
		% within education	100.0%	100.0%	100.0%	100.0%	100.0%

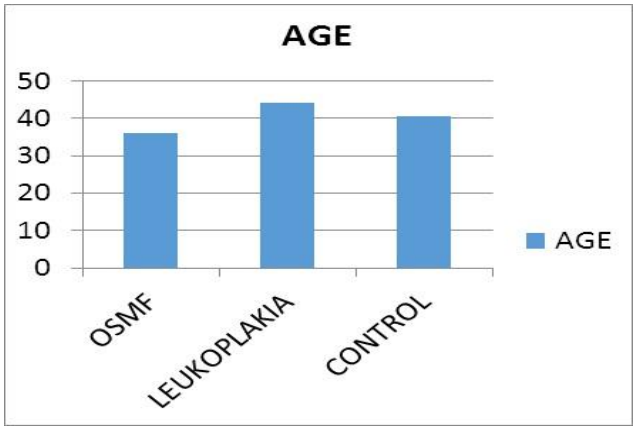
Table 11. Socioeconomical Status

Crosstab						
			Job			Total
			Driver	Labour	Others	
Diagnosis	Leukoplakia	Count	9	4	7	20
		% within Diagnosis	45.0%	20.0%	35.0%	100.0%
		% within job	40.9%	36.4%	41.2%	40.0%
	OSMF	Count	11	5	4	20
		% within Diagnosis	55.0%	25.0%	20.0%	100.0%
		% within job	50.0%	45.5%	23.5%	40.0%
	Control	Count	2	12	6	20
		% within Diagnosis	20.0%	20.0%	60.0%	100.0%
		% within job	9.1%	18.2%	35.3%	20.0%
Total		Count	22	21	17	60
		% within Diagnosis	44.0%	22.0%	34.0%	100.0%
		% within job	100.0%	100.0%	100.0%	100.0%

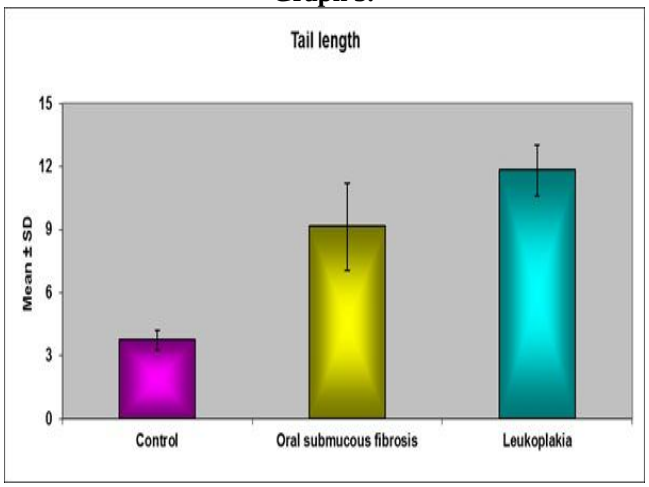
Graph 1.



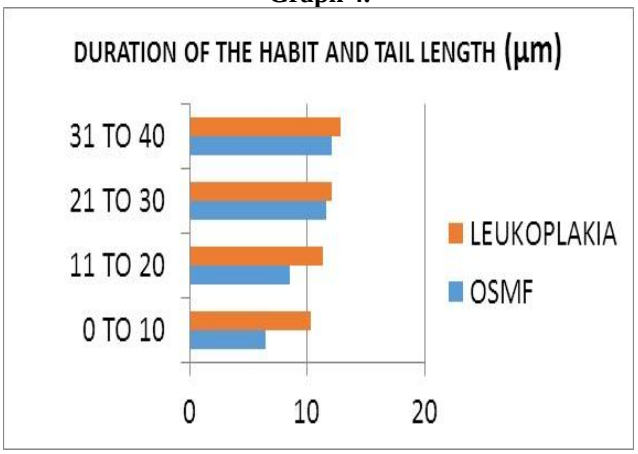
GRAPH 2.



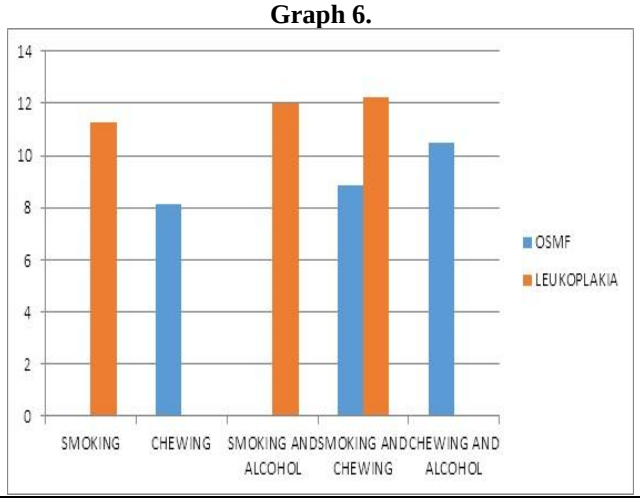
Graph 3.



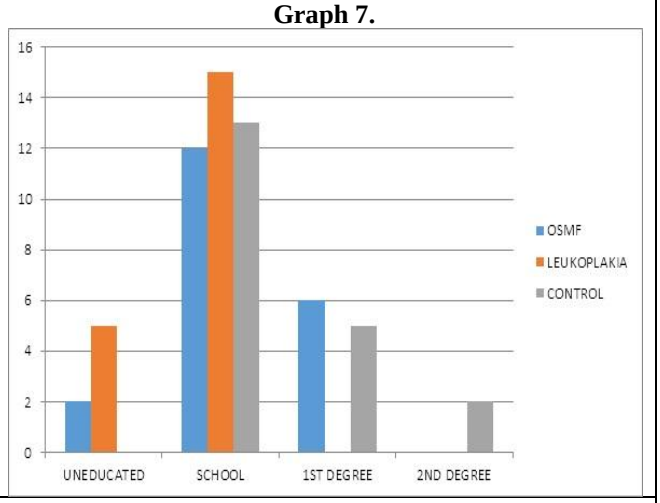
Graph 4.



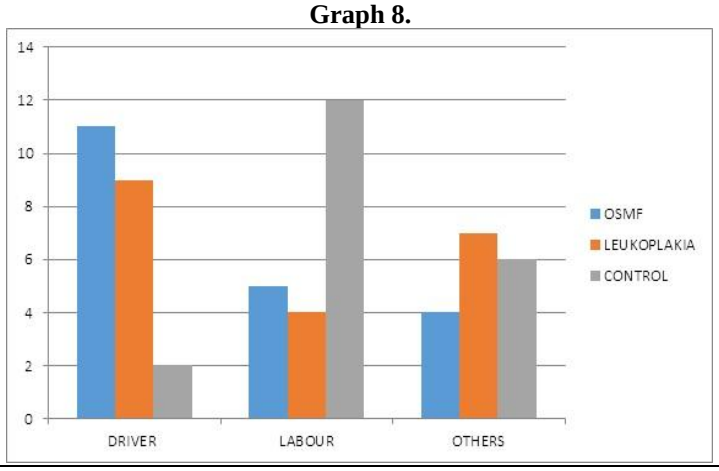
Graph 6.



Graph 7.



Graph 8.



DISCUSSION

The potentially malignant disorders such as leukoplakia, characterized by a white patch, mainly associated with tobacco smokers, and OSMF characterized by deposition of collagen and marked blanching of oral cavity leading to inability to open mouth, mainly associated with smokeless tobacco and betel quid chewers, have a greater prevalence in our country. Both of these conditions have high cancer turnover potentiality and if detected early can be prevented and treated successfully. With present advances in genotoxicologic studies, extent of DNA damage provides a platform to determine the cancer progression.[11] One of the important hallmarks for cancer progression is DNA damage, resulting either from various carcinogens accumulating from etiologic influences or due to genetic errors. If detectable and quantifiable, these may contribute toward an early detection and prediction system for oral cancer development[9].

Guptha et al. [1]. in 1992 proposed clinical staging for OSMF. Based on that in this study the OSMF group consists of all 20 patients with grade IV which reveals blanching with limited mouth opening and multiple vertical fibrotic bands.

Kuo MYP et al., (1995) Utsunomiya H et al.,(1998) [2,3]. have conclude the male to female ratio for OSMF was about 6:1 which has male predilection. In our study the male to female ratio was 19:1 which shows that male are even more commonly affected.

Axéll T et al. (1996) [4]. have concluded that smokers are more commonly affected with leukoplakia when compare to non-smokers. In accordance with the author in our study most of the patients diagnosed with leukoplakia was smokers.

Petti S et al.(2007) Napier SS et al.(2008) [5,6] had concluded that the onset of leukoplakia usually takes place after the age of 30 years; resulting in a peak incidence above the age of 50 years and that is strong male predilection. In accordance to the authours our study also has male predilection with the mean age of 44.15 years.

To study the cytogenetic changes of deleterious effects of habit either oral mucosal cells, urine samples or blood cells like lymphocytes and leukocytes can be used as a sample. Peripheral blood leucocytes in cancer is a choice to examine for increased levels of DNA damage and also serves as suitable surrogate cells where target tissue is not attainable. Buccal epithelial scraping cells can be the representative cells to study as these cells are

directly and chronically exposed to the habits like betel quid and gutkha.

Several studies have shown the evaluation of buccal epithelial cells is a good biomarker to know early damage in target tissues. Buccal epithelial cells have revealed significantly higher tail length formation compared with the leucocytes. In our study also we have taken buccal epithelial cells for the DNA analysis since it has highly significant tail length.

Saran *et al.*(2008) [7]. observed stepwise increased in micronucleus frequency from control to pre-cancer patients and from pre-cancer to cancer patients. In our study also we found step wise increase in the DNA tail length damage from control to OSMF and leukoplakia. The extent of DNA damage was more in leukoplaia when compare to control and OSMF.

Sanjit Mukherjee et al, (2011) [8]. conducted a study with Leukoplakia and oral squamous cell carcinoma in which the mean tail length of Leukoplakia was 12.96µm and 24.95 µm respectively. In our study the mean tail length of leukoplakia patients was 11.83µm.

Smita Jyoti et al. (2013) [9]. assessed the DNA damage in panmasala, gutkha chewing and smoking in buccal epithelial cells and found that more DNA damage in gutkha chewers with smoking habit. In our present study that patients with smoking habits has increased DNA damage followed by gutkha with alcohol consumers.

According to Vijayalakshmi madalli[10] (2014) the majority of the patients diagnosed with OSMF are between 20 -40 years of age. In our study the mean age of OSMF patients was 36 (±7.739) years.

In a study conducted by Ravichandra Udupa, (2014) [11] with 25 Oral submucous fibrosis patients the mean tail length was 12.92µm and there was no significant changes in the grading. In our study the mean tail length was 9.14µm for OSMF group which was significant when compare with the controls.

N. Madhulika et al(2015) [12]. recently conducted a study between lichen planus and lichenoid reaction to detect the extent of DNA damage in oral lichen planus and oral lichenoid reactions using comet assay and found the lichenoid reaction has more tail length when compare to lichen planus and also there is significant difference between the control and the study group. The study was not in accordance with our study since our study group was OSMF and leukoplakia but there was a positive co-relation that potentially malignant disorders have significantly increase tail length damage when compare with the healthy control groups.

CONCLUSION

Oral submucous fibrosis and leukoplakia are the most common potentially malignant disorders of the oral cavity which has higher malignant transformation. Early detection of the lesion and by knowing their ability to malignant transformation we can reduce the mortality and morbidity. Many methods are used in early detection of these lesions and there potential malignant transformation. We have done this study with comet assay which is more accurate and compared the DNA damage with the control patients to evaluate the amount of damaged DNA in patients with potentially malignant disorders. Also we compared the results with the type of habits and duration of the habits and found that there was a significant co relation between the DNA damage with type of habit and duration of the habit. We also conclude that DNA damage was significant method to evaluate the malignant transformation of the potentially malignant disorders such as OSMF and Leukoplakia. We suggest future studies to be carried along with Oral lichen planus to evaluate the damage of DNA towards malignant transformation. This can be incorporated into clinical practice to improve outcome and survival of persons at high-risk for oral cancer.

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