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Original Article

DNA damage in buccal cells in oral PMDs and malignant disorders by comet assay: a comparison with blood leukocytes

Garima Rawat[1], Aadithya B Urs[2], Anita Chakravarti[3], Priya Kumar[2,*]

[1] Dharamshila Narayana Hospital, New Delhi

[2] Department of Oral & Maxillofacial Pathology, Maulana Azad Institute of Dental Sciences, New Delhi, India

[3] Department of Microbiology, Maulana Azad Medical College, New Delhi, India

Corresponding author:
Dr. Garima Rawat, M.D.S. Oral & Maxillofacial Pathology, Dharamshila Narayana Hospital, New Delhi
Email id- garima3103@gmail.com
Phone number-+91-9873776634

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Aim: DNA damage associated with Oral Squamous Cell Carcinoma (OSCC) and potentially malignant disorders (PMDs) is produced due to carcinogenic agents or increased oxidative stress. Comet assay can assist in early detection and evaluation of the amount of DNA damage; lymphocytesare the most commonly used cells for performing comet assay. Utilisation of buccal epithelial cells in comet assay can be a minimally invasive and rapid method. The present study compared the efficacy of comet assay in assessing DNA damage in buccal cells over peripheral blood leucocytes (PBLs) in oral potentially malignant and malignant disorders. **Methods:** The study included fifty five patients each of Leukoplakia, Oral Submucous Fibrosis (OSMF) and OSCC along with fifty five healthy individuals as control. Buccal epithelial cells were collected from all the selected subjects. DNA damage was evaluated bymeasuring the mean tail length (μm). **Results:** A significantly increased mean tail length (μm) and higher DNA damage were found in OSCC (26.1096 + 1.84355) and there was a progressive stepwise increase in mean tail length from control (8.4982 + 0.93307) to PMD [leukoplakia (14.6105 + 0.71857); OSMF (12.5009 + 1.12694)] to OSCC.The mean tail length in different habit groups was greater than controls, though no significant difference was noted between habit groups. The mean tail length of buccal cells was significantly greater than the mean tail length of PBLs in all study groups and controls. **Conclusion:** Hence, use of comet assay on buccal epithelial cells can prove to be beneficiary for evaluation of DNA damage.

Keywords: Comet assay. DNA damage. Epithelial cells. Leukocytes. Mouth neoplasms.

Introduction

The most common potentially malignant disorders (PMDs) affecting the oral cavity include leukoplakia, erythroplakia and oral submucous fibrosis (OSMF)[1,2]. 30-80% of the oral malignancies arise from PMDs like leukoplakia and OSMF. Malignant transformation rates for leukoplakia and OSMF range from 0.13-17.5% and 2.3-7.6% respectively[3]. Oral squamous cell carcinoma (OSCC) is the most common malignant disorder of the oral cavity, accounting for over 90% of all malignant neoplasms[4-6].

Etiopathogenesis of OSCC is multifactorial including tobacco either smokeless or smoked with or without alcohol which has synergistic role, some viruses, genetic and epigenetic factors and also, gene-environmentinteractions[7]. The oral deleterious habits stated earlier lead to oxidative stress or reactive oxygen species (ROS) generation which in turn produces damage of DNA at cellular level. These factors cause base alteration promoting break in the DNA helix, these double-strand breaks (DSBs) are lethal to cells and lead to loss of genetic information. DNA damage incurred may promote genomic instability and aid the development of disease, including cancer[8-10].

For evaluation of DNA damage various methods known as genotoxicity assays are known such as single cell gel electrophoresis (SCGE) or comet assay and micronucleus assay (MN assay). Comet assay (SCGE) is most widely used as an *in vitro* and *in vivo* genotoxicity test[9,11]. Comet Assay was first developed in 1984 by Ostling and Johansson and later in 1988, it was modified by Singh et al. It is commonly utilised for quantification and analysis of DNA damage in individual cells and depends on partial unwinding of the supercoiled DNA inagarose-coatedslides. This allows the DNA to be attracted towards the anode underelectrophoresis, forming 'comet-like' images as seen underfluorescence microscopy. The relative amount of DNA inthe comet tail indicates DNA break frequency[12-15]. A cell with DNA damage appears as a 'comet' and undamaged cell appears as a 'halo'. The head of the comet is composed of intact DNA while the tail is composed of damaged DNA. The length of the comet tail formed is directly proportional to the amount of DNA damage present in that cell[16]. Any eukaryotic cell type that can be obtained as a single cell or nuclear suspension assay can be used to perform this assay[13]. Amongst eukaryotic cells, lymphocytes are the most commonly used cells. Using buccal cells in comet assay to assess DNA damage can be a minimally invasive and rapid method[11]. The aim of the present study was to evaluate the efficacy of comet assay in assessing DNA damage in buccal epithelial cells in oral potentially malignant disorders and OSCC and comparing the findings with those of peripheral blood leucocytes (PBLs).

MATERIALS AND METHODS

Ethics

This study was conducted in the Department of Oral Pathology and Microbiology, Maulana Azad Institute of Dental Sciences, New Delhi, India. The study was approved by the Institutional Ethical Committee Board.

Study design

This prospective study was designed to evaluate the efficacy of comet assay in buccal mucosal cells over PBLs in assessing DNA damage in oral potentially malignant and malignant disorders.

Part I of this project evaluated the efficacy of comet assay in assessing DNA Damage in Peripheral Blood Leukocytes (PBLs) in Oral Potentially Malignant and Malignant Disorders.

Subjects of study

Clinically and histopathologically confirmed patients with leukoplakia, OSMF and OSCC in the age range from 18 years to 80 years were included in the study. The study sample comprised of 220 patients including leukoplakia(Group B; n=55), Oral Submucous Fibrosis (Group C; n=55), Oral Squamous Cell Carcinoma (Group D; n=55) along with healthy age and sex matched individuals without any habit history (control, Group A; n=55) were included in the study.

The patients with habits were categorised into three habit groups:

- Smokeless group (usage of Tobacco, Gutkha, Pan or Supari)
- Smoked group (usage Cigarette or Bidi)
- Mixed group(usage of both smoked and smokeless forms)

Patients not willing to participate, suffering from any infectious or contagious disease, with any other white patch such as candidiasis, oral lichen planus and lichenoid reaction and previous history of surgery, radiotherapy or chemotherapy, or any vitamin, or dietary supplement use were not included in the study.

Sample collection

Buccal mucosal cells- Exfoliated buccal cells were collected using cytobrush from patients (site of lesion) and controls (buccal mucosa). Buccal cells were stored in RPMI-1640 medium at -80º C untilprocessed.

Preparation of buffers

Lysis Buffer: Ingredients per 1000 mL were 2.5 M NaCl - 146.1 gm, 100 mM EDTA - 37.2 gm, 10 mM Trizma base -1.2 gm, 1% Triton X- 100 - 10 mL, 10% DMSO - 100 mL. Ingredients were added to 700 mL dH2O, and the mixture was stirred to dissolve the constituents. The pH was adjusted to 10.0 using concentrated (1N) HCl or NaOH. TheTriton X-100 and DMSO were added and the final volume was made to 1 litre with dH_2O. The buffer was stored at room temperature.

Alkaline Buffer: pH > 13: Ingredients per 1000 mL were 300 mM NaOH - 12 gm and 01 mM EDTA – 0.3 gm

Neutralising Buffer: pH=7.5: Ingredients per 1000 mL were 0.4 M Tris - 48.45 gm. This was added to ~800 mL dH2O, pH was adjusted to 7.5 with concentrated

(1N) HCl and the final volume was made to 1 litre. The buffer was stored at room temperature.

Trypsin solution: Ingredients per 1000 mL were 0.25% trypsin and 1 mM EDTA in PBS - 0.03 gm.

Staining solution: Ethidium Bromide dye (Sigma Aldrich): 2 mg is dissolved in 100 mL of distilled water and used for staining.

Procedure for Comet assay in buccal cells

Buccal epithelial cells were collected from patients using a cytobrush gently from the buccal mucosa or the lesion (in study groups). The brush was then swirled into an eppendorf tube containing RPMI-1640 medium and centrifuged at 2000 rpm for 10 min. The supernatant was removed and 300µl of trypsin solution was added to the buccal cells and incubated for 30 min at 37°C. Then the cells werecentrifuged and the supernatant was discarded. The cells werethen washed thrice by centrifugation at 2000 rpm for 10 min in cold PBS. About 40µl of cell suspension and 60µl of 0.5% LMPA were mixed and placed on frosted slides previously coated with 1% NMPA. To the solidified agarose, a third layer of 1% LMPA was applied and the slides were dipped in cold lysis buffer and refrigerated for 24 hours. Following lysis, the slides were placed in the cold alkaline buffer (pH > 13) for 20 min to unwind DNA strands and expose the alkali labile sites ALS (alkali unwinding). Then the slides were subjected to electrophoresis under alkaline conditions (pH >13) at 300mA and 15V for 25 min. After this, the same procedureas used for PBLs was followed.

Statistical analysis

The mean values, standard deviation and ranges (maximum and minimum) were calculated for each variable. The resulting data was analyzed using SPSS software, version 20 (Armonk, NY: IBM Corp). Data was expressed as mean ± standard deviation. Differences between different variables were analyzed using parametric Student t-test and Analysis of variance (ANOVA). Correlation was calculated using the Pearson's correlation.

A P value ≤ 0.05 was considered to be statistically significant.

RESULTS

The results for mean tail length of PBLs in different study groups and habit groups were obtained from the previous part of this study and then these were compared with the results of buccal epithelial cells.

Distribution of patients and controls according to age, gender and habit was studied. [Table 1]

Comet assay was performed on peripheral blood leucocytes of all the subjects. The DNA damage (mean tail length) in the buccal cells in leukoplakia, OSMF, OSCC and control groups were assessed and results of each are illustrated in figures 1, 2, 3 and 4 respectively. The mean tail length (µm) buccal cells in leukoplakia, OSMF and OSCC were compared with controls. [Table 2]

Table 1. Table showing demographic data of patients included in the study

Group	No of patients	Age	Gender (M:F)	Sample collected	Habit
Group A (Control)	55	15-76	41:14	Both buccal cells and PBLs	-
Group B (Leukoplakia)	55	20-70	50:5	Both buccal cells and PBLs	Mixed - 14
					Smoked - 14
					Smokeless - 27
Group C(OSMF)	55	18-73	39:16	Both buccal cells and PBLs	Mixed - 10
					Smokeless - 45
Group D(OSCC)	55	28-80	44:11	Both buccal cells and PBLs	Mixed - 16
					Smoked - 5
					Smokeless - 34

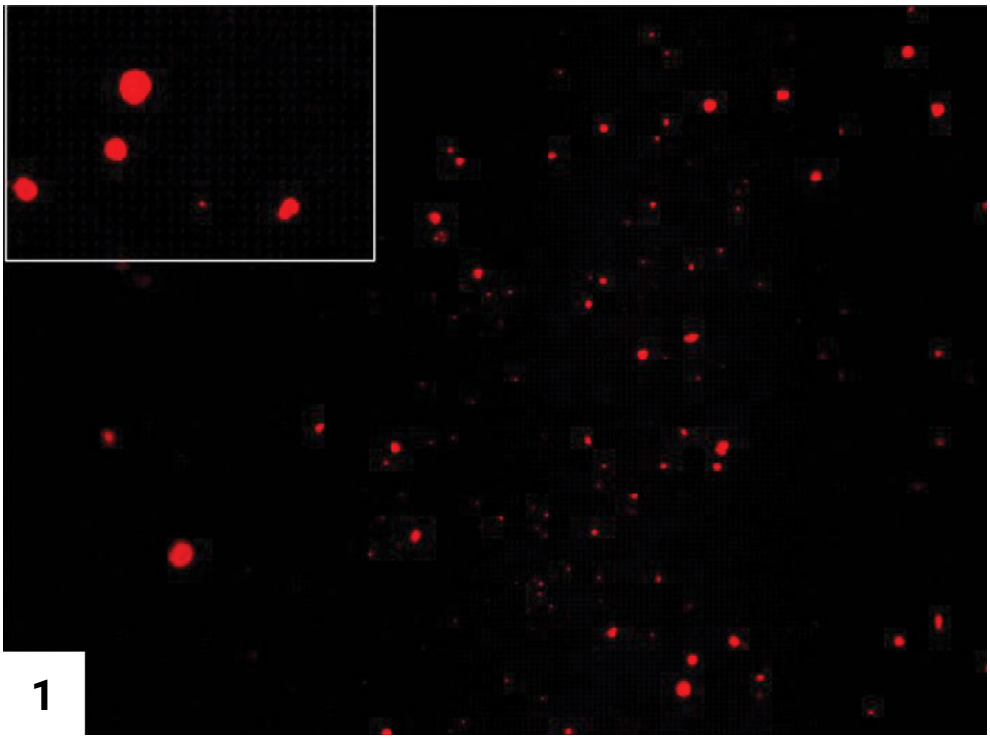

Figure 1. Non-fragmented and undamaged DNA in buccal cell samples of control. (Inset- Magnified view of single cell without DNA damage)

Comparison of mean tail length of PBLs and Buccal Cells in Different Study Groups was done using ANOVA. [Table 3] The mean tail length (μm) of buccal cells was significantly more than that of PBLs in all study groups compared to controls.

Comparison of mean tail length of buccal cells between different habit groups and control was done using Student t- test. [Table 4] Though the mean tail length was

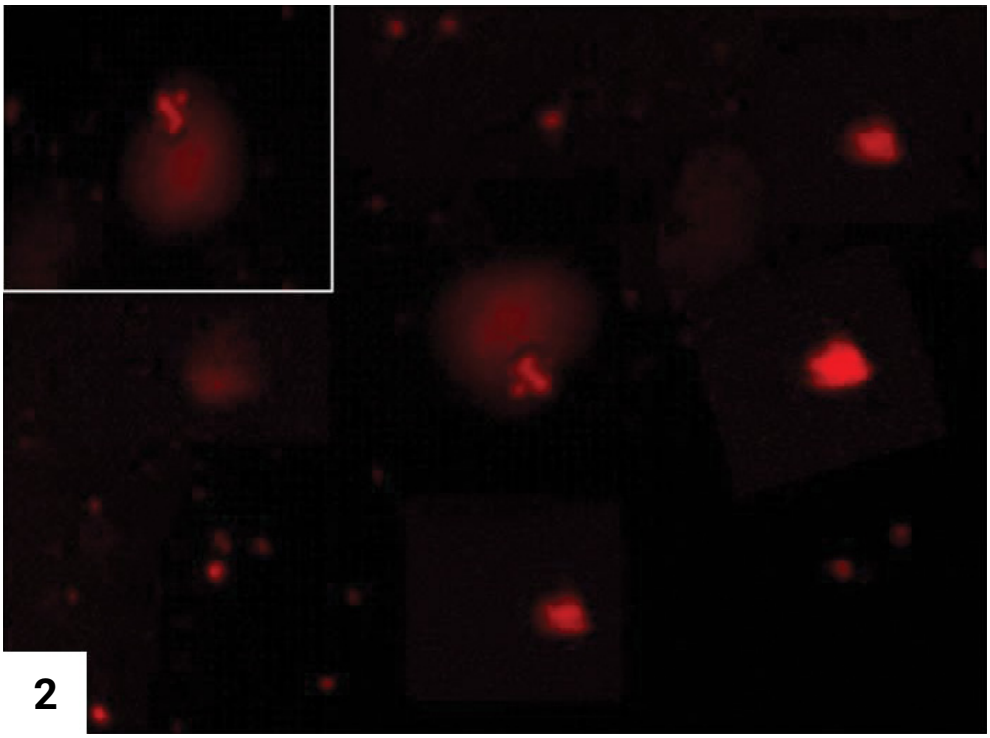

Figure 2. DNA damage in the form of comet in buccal cell samples of leukoplakia. (Inset- Magnified view of single comet)

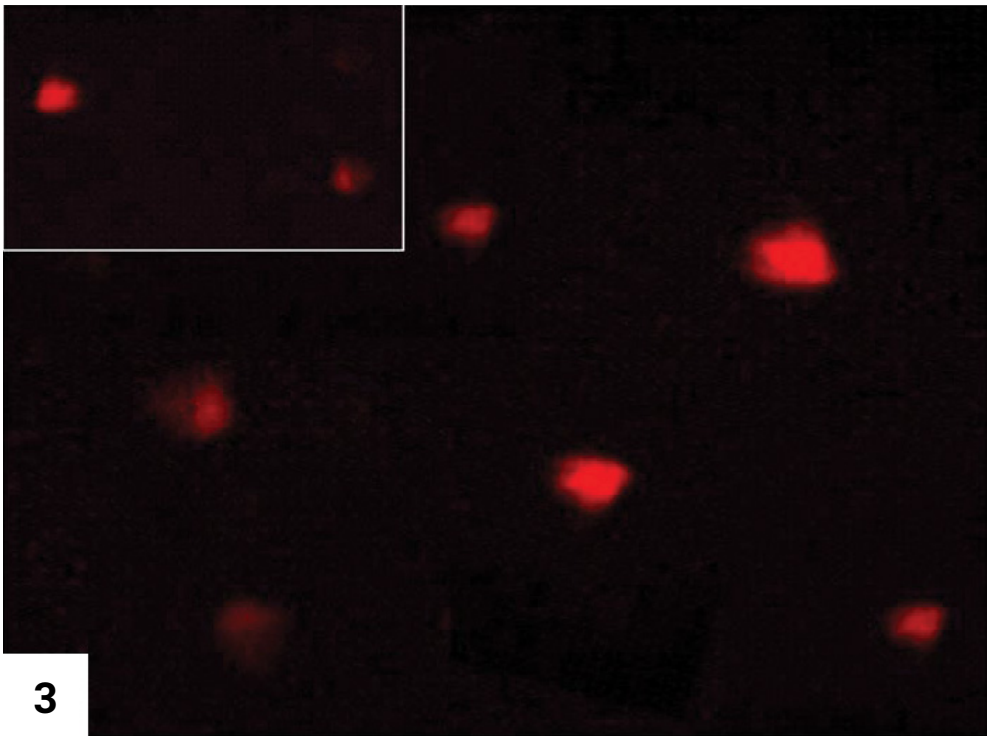

Figure 3. DNA damage in buccal cell samples of OSMF. (Inset- Magnified view of single cell with DNA damage)

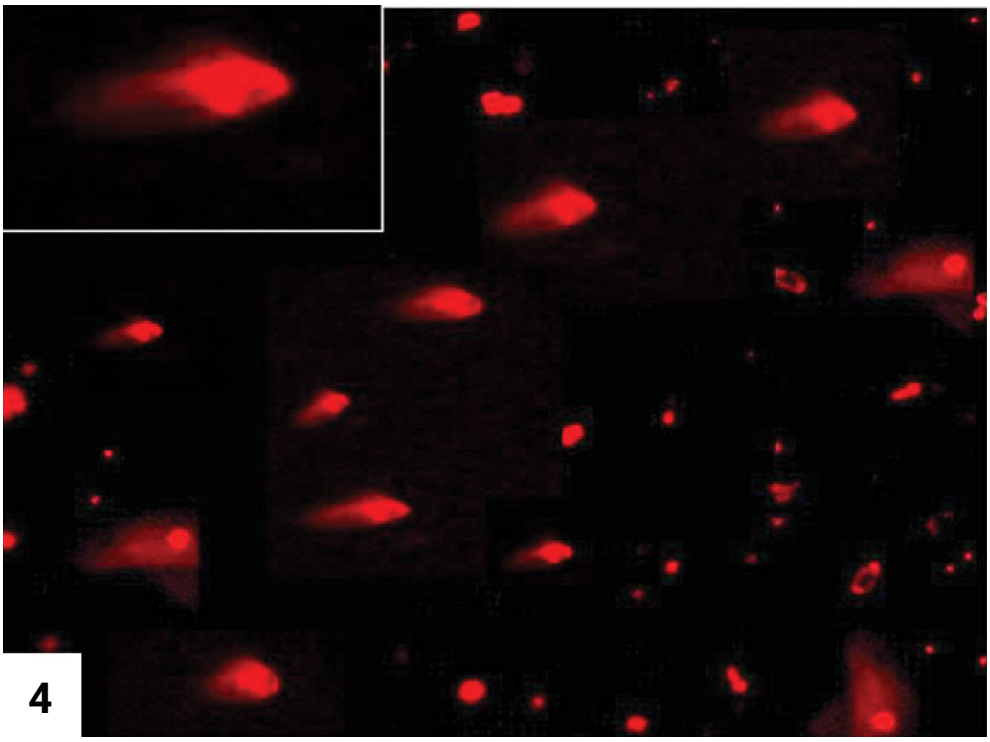

Figure 4. DNA damage in the form of comet in buccal cell samples of OSCC. (Inset- Magnified view of single comet with tail)

Table 2. Comparison of Mean Tail Length of Buccal Cells between Different Study Groups

CONTROL	STUDY GROUPS Mean tail length (µm) (mean + standard deviation)	p-Value	
GROUP A (8.4982 + 0.93307)	GROUP B (14.6105 + 0.71857)	0.000	p value < 0.05 is considered as statistically significant
	GROUP C (12.5009 + 1.12694)	0.000	
	GROUP D (26.1096 + 1.84355)	0.000	

On comparing the mean tail length of buccal cells by Student t-test between various study groups, it was found to be highest for OSCC followed by Leukoplakia and OSMF compared to the control. Highly significant difference was obtained between the study groups and control.

Table 3. Table showing p-values Obtained by Comparison of Mean Tail Length of PBLs and Buccal Cells in Different Study Groups

	STUDY GROUPS	Buccal cells	
		p-Value	
PBLs	GROUP A	0.000	p value < 0.05 is considered as statistically significant
	GROUP B	0.000	
	GROUP C	0.000	
	GROUP D	0.000	

On comparing the mean tail length of peripheral blood leucocytes and buccal cells of various study groups, the mean tail length of buccal cells was significantly more than that of PBLs in every study group.

Table 4. Comparison of Mean Tail Length of Buccal Cells between Different Habit Groups and Control

CONTROL	HABIT GROUPS Mean tail lengths (µm) (mean + standard deviation)	p-Value	
GROUP A (8.4982 + 0.93307)	SMOKELESS (17.3530 + 6.05758)	0.000	p value < 0.05 is considered as statistically significant
	SMOKED (17.6421 + 5.55230)	0.000	
	MIXED (18.8135 + 6.62379)	0.000	

On comparing the mean tail lengths of buccal cells by Student t-test between various habit groups, the tail lengths were significantly higher in the habit groups compared to control.

increased in the patients with habits, there was no significant difference between the various habit groups.

DISCUSSION

Prolonged exposure to carcinogenic agents induces oxidative stress or reactive oxygen species (ROS) generation that isgenotoxic and cytotoxic to human cells and causes damage leading to genetic alterations. Accumulation of these genetic alterations may initiate development of premalignant disorders and subsequently OSCC[8]. When the amount of ROS generated in the cells is significantlyhigh, it leads to cellular damage, as well as DNAdamage[8,9]. The ROS also affect the DNA repair mechanisms essential for maintenance of DNA integrity and prevention of cancer[8]. Thus, the progression of OSCC from potentially malignant disorders is a multistep process[3,5]. The common potentially malignant disorders like leukoplakia and Oral Submucous Fibrosis (OSMF) have malignant transformation rates in the range of 0.13-17.5% and 2.3-7.6% respectively[3].

The Comet assay or Single cell gel electrophoresis (SCGE) is a well known assay in assessing the DNA damage attributable to its rapidity, sensitivity, inexpensiveness and requirementof little biologicalmaterial[17-20]. The most commonly used cells in such assays are peripheral blood lymphocytes (PBLs) and there is a need to replace or complement lymphocytes with some other cell type.

The age of patients in the study ranged from 18 years to 80 years with the mean age for controls, leukoplakia, OSMF and OSCC being 36.33 years, 43.54 years, 38.95 years and 49.05 years respectively. 79 % of the patients included in the study were males, these findings were comparable to other studies[3,7].

Amongst the patients, apredominance of smokeless tobacco usage (48.2%) was observed, while mixed tobacco (both smokeless and smoked) and smoked form accounting for 18.2% and 8.6% of the patients respectively.

In the *buccal cells*, the mean tail length (µm) was found to be significantly increased in OSCC (26.1096 + 1.84355µm), leukoplakia (14.6105+ 0.71857µm) and OSMF (12.5009 + 1.12694µm) compared to controls (8.4982 + 0.93307µm). The comet tail lengths were found to be greater in all study groups compared to controls, which symbolises the presence of increased DNA damage in the buccal cells of these

patients. The amount of DNA damage was most in OSCC patients as this group had the greatest mean tail length followed by leukoplakia and OSMF. In malignancies, there is loss of genomic stability or increased genomic instability, which can be either inherent or induced by external agents. The basal DNA damage that is observed in normal healthy individuals due to exposure to risk factors also plays an important role in carcinogenesis as well as in progression of the disease. The level of DNA instability of an individual at baseline level is critical in cancer predisposition and progression. Thus, an increase in mean tail length which is observed from healthy individuals to precancer to cancer may be due to progressive increase in genomic instability[21,22].

Udupa et al.[23] reported that the mean tail length of buccal cells in OSMF group (12.92 ± 0.90 μm) was significantly higher ($p< 0.05$) compared to the healthy group (8.34 ± 0.36 μm), indicating OSMF patients had increased DNA damage. In OSMF patients, the buccal epithelial cells are in direct contact with the deleterious effect of betel quid and gutkha containing areca nut in comparison to other surrogate cells. The generation of ROS by the aqueous extract of arecanut leads to the genotoxic damage in buccal epithelial cells, therefore, accounting for increased tail length and DNA damage[24].

Studies using alternative epithelial cells to assess DNA damage in other types of cancers of the body have been published in literature. Increased mean basal DNA damage was discerned by Udumudi et al.[25] in epithelial cells of patients with cervical cancer and cervical dysplasia compared to controls.

The authors of the present study in part 1 of this project had evaluated DNA damage in the *peripheral blood leucocytes* (PBLs) using comet assay in the same group of patients. These authors found that the mean tail length (μm) was significantly increased in OSCC (22.4335 + 1.52341), leukoplakia (13.0022 + 0.74316) and OSMF (10.6085 + 0.88140) compared to controls (6.8307 + 0.84261 μm). The increased comet tail lengths in all study groups compared to controls depicts presence of DNA damage in the PBLs of these patients[26,27]. The generation of ROS and exposure to genotoxins causes DNA breaks, reduced DNA repair capacity and oxidation of purines or pyrimidines. These genotoxins attack different sites on the DNA leading to the accumulation of DNA damage which increases the risk of cancer[18,28-29]. They also observed that the amount of DNA damage was greatest in OSCC patients as this group had the maximum mean tail length followed by leukoplakia and OSMF. There was significant stepwise increase in DNA damage in the PBLs from control to pre-cancer patients and from pre-cancer to oral cancer patients[26].

Cancer patients have maximum DNA damage as depicted by greatest mean tail length of comet in lymphocytes. This has been observed in cancers other than those of the oral cavity[25,29-32].

On comparing the results of the present study and the study done using PBLs by the current authors, it was found that the mean tail length of buccal cells was significantly more than PBLs in all study groups. The tail length of leukoplakia was closer to OSCC compared to OSMF suggesting that leukoplakia has severe DNA damage, which can be directly correlated with its high possibility to undergo malignant transformation[3].

Katarkar et al.[3] and Mukherjee et al.[7] in their respective studies also obtained significantly higher tail length in OSCC and leukoplakia compared to OSMF.

A thorough search of literature revealed that there is no study published till date comparing the mean tail length of PBLs and buccal cells using only comet assay in oral PMDs and OSCC. We hypothesize that the buccal epithelial cells come into direct contact with the mutagenic agents for prolonged duration as all the lesions of the study groups are habit associated. These cells are sensitive to DNA damage because of direct exposure to the carcinogen. Also, the buccal cells are short-lived cells (with renewal of 10–14 days) due to their continued renewal as compared to PBLs which are considered to be longer living cells. Hence, the presence of buccal cells with comet-like appearances is indicative of recent exposure to genotoxic agents. This may explain the increased mean tail length and higher levels of DNA damage that were observed in buccal cells compared to PBLs. Thus, comet assay of buccal cells can be a more sensitive biomarker to assess early damage in target tissue in comparison to PBLs.

Tobacco smoking and smokeless tobacco are important etiologic factors leading to oral cancer. These products are composed of carcinogens such as polyaromatic hydrocarbons nitrosamines and aromatic amines. These carcinogenic agents after deactivation in the liver are converted into electrophilic intermediates which in turn react with DNA to form covalently bound adducts. The formation of DNA adducts and the resulting mutations are responsible for oncogene activation and inactivation of tumor suppressor genes, leading to cancer. Few authors have reported presence of these DNA adducts in smokers[28,33-34].

In the current study, the mean tail length of buccal cells in different habit groups when compared with the control group of no habit showed highly significant results. The results of our study are comparable to those of Jyoti et al.[8] wherein gutkha chewers, gutkha chewers along with smoking, pan masala chewers, pan masala chewers along with smoking, and smokers had significantly increased ($p < 0.05$) tail length in buccal epithelial cells compared to the control group.

In the current study, no statistically significant difference was obtained in the tail length between different habit groups in both PBLs and buccal cells.

Udupa et al. also observed no statistically significant difference between the different types of habits and tail length of buccal cells[23], though the tail lengths were increased compared to no habit group as seen in our study. Thus, the increased tail length is associated with deleterious oral habits.

Jyoti et al.[8] observed that highest tail length of buccal cells was among gutkha chewers along with smoking (36.9 ± 3.60 µm) amongst all groups which was not observed in our study. These contrasting results may be due to non standardisation of duration and frequency of the habit in our study. Katarkar et al.[3] also showed that the patients with multiple habits had significantly increased ($p< 0.0001$) tail length compared to patients with single habit.

Similar results were obtained by Rawat et al.[26] on comparing the mean tail length of PBLs in different habit groups with the control group with no habit and obtained

highly significant results. Similar results have been stated by other authors also[28]. Many other investigators have investigated the effect of habit on mean tail length and have obtained comparable results[11,35]. However, Hoffmann et al.[36] showed no significant difference in DNA damage between smokers and non-smokers. These authors concluded that cigarette smoking had no effect on the amount of DNA damage in peripheral blood cells. No correlation between the length of thecomet and the number of cigarettes or the frequency of smoking was detected by Frenzilli et al.[37] and Mohankumar et al.[33]. These findings can be explained by assuming that the single DNA strand breaks can be induced in leucocytes also by free radicals generated due to the inflammation normally present in smokers. This reaction is independent of the amount of cigarette smoked and is related to individual susceptibility. The DNA single strand breaks (SSB) induced by agents like hydrogen peroxide are quickly repaired[16,34].

In the current study, we obtained a positive correlation between the mean tail length of PBLs and buccal cells in controls and the study groups as well as in the habit groups. Also, OSCC and other PMDs are epithelial in origin and hence, detection of DNA damage in this cell type can prove to be beneficial. This could be because the buccal epithelial cells are the first cells that come into direct contact with the carcinogenic agents and hence display maximum DNA damage. Thus, reflecting that buccal cells are more sensitive, non-invasive and effective indicators for evaluating DNA damage in potentially malignant and malignant disorders when compared to PBLs. Hence, with the present study we advocate the utilization of buccal cells for evaluation of genotoxicity during the earlier stages of carcinogenesis and should be considered as a replacement or alternative to lymphocytes.

Compliance with Ethical Standards:

No Funding

Conflict of Interest: No conflict exists

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

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