Original Article

The comet assay a method to measure DNA damage in oral submucous fibrosis patients: A case-control study

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ABSTRACT

Context: Oral submucous fibrosis (OSF) is a chronic, progressive condition and rise in incidence of this disease has been attributed to an increased uptake of betel nut and its products, especially among young people. Due to its potential for malignant transformation, easy and reliable techniques for its early detection are needed. Comet assay is a simple technique to detect and quantify single strand breaks, double strand breaks and alkali labile damages. **Aims:** The present pilot study was undertaken to assess the deoxyribonucleic acid (DNA) damage in OSF and healthy groups using comet assay. **Settings and Design:** The hospital-based study was conducted by evaluating buccal mucosal cells of 50 individuals in the age range of 16-47 years and comet assay was carried out for all the two groups under alkali conditions. **Materials and Methods:** Oral epithelial cells were obtained from buccal mucosa of each 25 cases of healthy and OSF groups were subjected to comet assay. DNA damage was evaluated by measuring the tail length. **Statistical Analysis Used:** Student's *t*-test and Turkeys-multiple *post-hoc* procedures were used to statistically analyze the obtained data. **Results:** Increase in the tail length in buccal epithelial cells of OSF group when compared with the healthy group was noted. Furthermore, there was a significant increase in the DNA damage with duration of habits. **Conclusions:** We conclude that increase in the tail length formation in OSF groups when compared to healthy groups; this indicates DNA damage in the oral epithelial cells. The comet assay is a relatively simple, but sensitive and well-validated tool for measuring strand breaks in DNA in single cells.

Key words: Betel nut, comet assay, deoxyribonucleic acid, nitrosamines, oral submucous fibrosis

INTRODUCTION

Oral submucous fibrosis (OSF) is a potentially malignant disorder (PMD) and crippling condition of the oral mucosa. Pathogenesis of OSF is believed to be multifactorial and the factors that trigger the disease include consumption of chewing areca nut, chilies, nutritional deficiencies and immunologic processes.[1] World-wide 2.5 million people are suffering from OSF. This number has risen to 5 million in the Indian subcontinent itself. The malignant transformation rate of OSF has been found to be 4-13% world-wide, whereas it is 7.6% in Indian population.[2]

Environmental factors, radiation, tobacco products are known induce genotoxicity and alters the structure of deoxyribonucleic acid (DNA). DNA damage can be estimated by using methods such as micronucleus assay, comet assay, and challenge assay, by using oral exfoliated epithelial cells and indirectly by using surrogate cells like leukocytes and lymphocytes.[3] Among the assays; "comet assay," also called "single cell gel electrophoresis," is a sensitive and rapid technique for quantifying and analyzing DNA damage in individual cells, originally developed by Ostling and Johansson in 1984. Comet assay has advantages of being sensitive, time saving and does not require proliferating cells to study DNA damage at the single cell level.[3] The cell with DNA damage appears in the form of "comet" while undamaged cell appears as a halo. The head is composed of intact DNA, while the tail consists of damaged or broken pieces of DNA.[4]

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The evaluation of DNA damage in PMDs is important, to identify the individuals who are at risk for malignant transformation. Hence, we hypothesize tobacco chewing either in the form of betel quid and gutkha induces the DNA damage and also high-risk individuals suffering from OSF patients can be identified.

MATERIALS AND METHODS

The present pilot study was conducted in the Department of Oral Pathology and Microbiology, SDM College of Dental Sciences and Hospital, Dharwad, in association with Department of Biochemistry, SDM College of Medical Science and Hospital, Dharwad.

The sample size of 25 OSF groups and 25 healthy groups were selected with 5% α error and 95% power of the test. Buccal exfoliated cells were obtained from these 50 individuals in the age range of 16-47 years visited to our college. The detail methodology explained to individuals and written informed consent was obtained.

Comet assay was carried out for all the two groups under alkali conditions according to the procedure explained by Singh e*t al*.[4] Cells from the oral cavity collected by using sterile wooden spatula and were transferred to sterile plastic (eppendorf[c]) tubes containing phosphate buffer saline medium and centrifuged for 15 min to get cell suspension. After draining supernatant, 70 μl of low melting agarose was added and homogenized in a mechanical vibrator. 5 μl of the prepared homogenate was sandwiched between a layer of 110 μl regular agarose and a top layer of 70 μl of low melting agarose on fully frosted slides. The slides were kept on ice during the polymerization of each layer. After solidification, the slides were immersed in lysing solution (146.4 g of NaCl, 37.2 g of ethylenediaminetetraacetic acid [EDTA], 1.2 g of Tris, 1% Triton X-100,4 ml of dimethyl sulpha oxide). The slides were then washed in Tris buffer and placed in the electrophoresis buffer (200 g of NaOH, 7.4 g of EDTA, pH >13) for 20 min. for DNA to unwind. The submarine electrophoresis was performed at 400 mA and 20 volts in a horizontal electrophoresis platform for 15 min. The slides were neutralized with Tris buffer (48.5 g of Tris, pH 7.5) [laboratory technician] and stained with ethidium bromide for 10 min. Each slide was analyzed using a fluorescent microscope. DNA damage was evaluated by measuring the tail length. Using image analyzer the total length and diameter was measured and the tail length was calculated by subtracting the diameter from the total length [Figure 1]. A total of 25 randomly selected cells per slide were analyzed, mean was calculated and obtained results were statistically analyzed by using SPSS 16 (SPSS Inc., Chicago, Illinois, USA) Student's *t*-test and Turkeys-multiple *post-hoc* procedures were used.

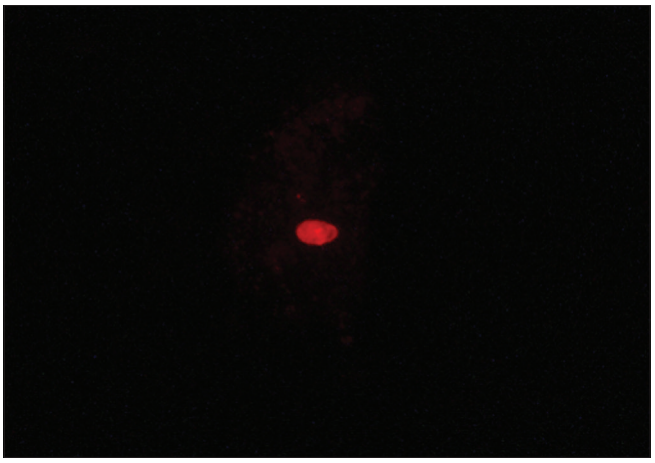


Figure 1: Tail length in buccal epithelial cell of oral submucous fibrosis patient stained with ethidium bromide stain (×40)

RESULTS

Mean age (mean ± standard deviation) of OSF and healthy groups were 27.24 ± 8.19 and 23.24 ± 2.31 years [Table 1]. 19 patients belonged Grade II, six patients were at Grade III and no cases were belonging to Grades I and IV [Table 2]. Clinical grading was done as per the grading system proposed by Ranganathan *et al*. (2001). Distribution of the habit among the patients was seven patients reported with betel quid chewing, whereas 10 and 8 patients had gutkha habit and more than one habit respectively [Table 3].

The mean tail length (in μm) of OSF group was 12.92 ± 0.90 and the mean tail length of healthy group was 8.34 ± 0.36. Statistically significant difference ($P < 0.05$) was evident in the mean tail length of OSF and healthy group [Table 4]. DNA damage was significantly higher in OSF group than the healthy group.

Table 2 shows the comparison of clinical grading with respect to tail length by *t*-test. The mean tail length in OSF clinical Grade II was 12.93 ± 0.99 and in Grade III was 12.92 ± 0.61. Statistical difference was not observed between OSF clinical Grades II and III with tail length ($P > 0.05$). This is probably because of uneven distribution of sample in the study. The mean tail length in betel quid, gutkha and mixed habits were 12.8814 ± 0.5371, 13.2020 ± 0.8871, 12.6150 ± 1.1475 respectively. There was no statistical significance was observed between the different types of habits and tail length [Table 3]. Tail length formation was seen irrespective of type habits used. However, significant and positive correlation was noted between the mean tail length and duration of habits at 10% level of significance ($P < 0.10$). The duration of habit and tail length is dependent on each other [Graph 1]. Regression graph showed a gradual increase in the tail length with duration of the habits and is irrespective of types of habit.

Table 1: Mean and SD of OSF group and healthy group

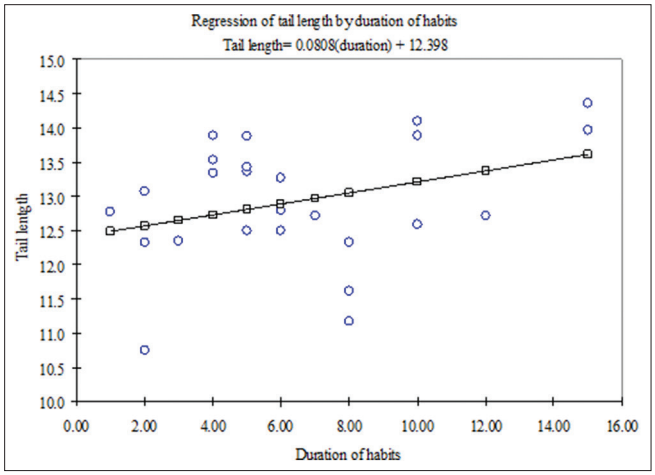
Group	Mean age	SD age
OSF group	27.24	8.19
Healthy group	23.24	2.31
Total	25.24	6.29

SD: Standard deviation, OSF: Oral submucous fibrosis

Table 2: Comparison of clinical grading with respect to tail length by *t* test

Clinical grading	Number of OSF cases	Mean of tail length (μm)	SD of tail length	*t* value	*P* value
II	19	12.93	0.99	0.0185	0.9854
III	6	12.92	0.61		

SD: Standard deviation, OSF: Oral submucous fibrosis



Graph 1: Correlation between duration of habits with tail length

DISCUSSION

We assessed the DNA damage of buccal exfoliated epithelial in OSF and healthy groups by using alkaline comet assay. All OSF patients were male with the mean age group of 27.24 years and youngest patient recorded was 16 years and oldest was 47 years [Table 1]. The reasons for the rapid increase of the disease in younger individuals is reported to be due to an increase in the popularity of commercially prepared areca nut preparations like gutkha, and an increased uptake of this habit by young people.[5] Sinor *et al*.[6] in their study have reported 79% of the OSF cases occurred in less than 35 years and Shah and Sharma[7] reported that the majority of cases were seen in between 21 and 40 years of age.

The duration and frequency of habit and the development of OSF is well documented.[8,9] The average frequency of 8 times daily consumption and habitual use of betel quid for at least 5 years predisposes the oral mucosa to oral premalignant disorders including OSF.[8] In the present study, the patients started the habit in early age that is an average of 6 years before initial manifestation of disease.

Table 3: Pair wise comparison of habits with respect to tail length by Turkeys-multiple *post-hoc* procedures

Type of habit	Number of cases	Mean of tail length	SD of tail length
Betel quid	7	12.8814	0.5371
Gutkha	10	13.2020	0.8871
Mixed	8	12.6150	1.1475

SD: Standard deviation

Table 4: Tail length in OSF group and healthy group by *t* test

Group	Number of cases	Mean tail length (μm)	SD	*t* value	*P* value
OSF	25	12.92	0.90	23.5970	0.0000
Healthy	25	8.34	0.36		

P<0.05. SD: Standard deviation, OSF: Oral submucous fibrosis

In the present study, 19 cases were Grade II and six cases were Grade III OSF group. Since patients rarely report at such an early stage (Grade I) and OSF with malignant change (Grade IV) were not reported during the study period. The possible reason for most of the patient reporting in Grades II and III is due to a burning sensation and restricted mouth opening [Table 2]. Clinical grading is more important for the assessment of progression of disease and treatment plan as repeated biopsy would lead to further fibrosis. Hence in the present study, we correlated the clinical grading, different types of habits and DNA damage.

A significant proportion of cases in our study group were habitual chewers of betel quid, gutkha and combination of more than one habit [Table 3]. There is sufficient evidence to show that habitual gutkha and betel quid are associated with the occurrence of several oral mucosal disorders, including OSF and oral cancer.[6,8,10,11]

Several studies have shown the evaluation of buccal epithelial cells is a good biomarker to know early damage in target tissues.[4,12,13] Buccal epithelial cells have revealed significantly higher tail length formation compared with the leucocytes.[3] As the deleterious effect of habit is in direct contact with buccal epithelial cells than surrogate cells to study DNA damage in PMDs and oral squamous cell carcinoma.

We used alkaline comet assay in exfoliated buccal cells from healthy individuals and OSF patients with an assumption that buccal epithelial cells could show the DNA damage in OSF patients.

The associated risk factor for OSF is chewing betel quid and gutkha containing areca nut. The amount of areca nut in betel quid, the frequency and duration of chewing are clearly related to the development of OSF.[14] In addition, habitual gutkha users have shown to present with OSF at earlier ages compared with traditional betel quid users. A gutkha

sachet weighs 3.5 g and contains 7% of moisture, whereas net weight of betel quid is nearly 4 g (with 1.14 g of tobacco) and contains 70% moisture. Because gutkha users tend to consume more dry weight of tobacco, areca nut and slaked lime, they might develop OSF at earlier ages compared to other types of betel quid users.[11]

Betel nut has a high potential for inducing genotoxicity and cytotoxicity.The carcinogenicity of areca nut without tobacco has been identified.[15] The recent IARC monograph on betel quid identifies areca nut as a "group one carcinogen."[14] The mutagenic potential of arecoline is enhanced significantly by other constituents of betel nut and the presence of other factors like the extent of alkalinity/acidity or the presence of alcohol. Alkaline pH is known to enhance the potential damaging actions of alkaloids.[14]

The incidence of OSF is a continuous upsurge condition and worldwide estimates in 1996 indicate that the disease affected 2.5 million people. In the Indian constituent alone, the statistics for OSF is about 5 million people (0.5%) of the population of India. The potential malignant rate transformation reported is 7.6% over a 17 years period in India.[9] Consequently the OSF is considered as a public health issue in the world-wide. The malignant transformation rate of OSF has been found to be 4-13% world-wide.[2]

OSF being a potential for malignant transformation, early detection at the cellular level helps to identify the individuals who are at high-risk. To study the cytogenetic changes of deleterious effects of habit either oral mucosal cells, urine sample or blood cells like lymphocytes and leukocytes can be used as a sample.[16] 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 cell to study as these cells are directly and chronically exposed to the habits like betel quid and gutkha.[4]

The pathogenesis of malignant transformation is not precisely known and is thought to be multifactorial. Dense fibrosis and less vascularity of the corium, in the presence of altered cytokine activity create a unique environment for carcinogens from both tobacco and areca nut to act on the epithelium.[17] These carcinogens accumulate in the connective tissue for longer duration. Decreased vascularity denies the quick absorption of carcinogens into the systemic circulation and results in longer duration of exposure to carcinogens.[5]

The presence of areca nut specific nitrosamines like N-nitrosoguvacoline 3-proprionaldehyde and 3-proprionitrite and increased level of reactive oxygen species in areca nut, is further enhanced by the presence of slake lime in quid has been implicated in oxidative DNA damage.[14] Additional factors such as nutritional deficiency, constant immunologic abnormalities, altered cytokine level, mechanical trauma and genetic vulnerability are been observed in OSF, further these increases the risk of malignant transformation.[18]

Exfoliated cells can be used for biomonitoring the effects of genotoxic endpoints. Exfoliative cytology is a simple and non-invasive technique used to biomonitoring and effect of carcinogenic, mutagenic compounds example by demonstrating micronuclei in these cells. The increased frequency of cells containing micronuclei in pre-cancer and cancer patients are been observed, which indicating DNA damage.[3]

Other method to study the genotoxic effect is sister chromatid exchange (SCE). The lymphocyte cells showed SCE in betel nut chewers, OSF and oral cancer patients in comparison to non-chewers. The frequency of SCE was increased in the OSF patients with habit of chewing pan parag. There was also a positive coefficient of SCE cells with frequency, duration, intensity and period of exposure of pan parag to the oral mucosa in OSF patients indicating the genotoxic effect of the betel nut content in the pan parag.[19]

Comet assay has the advantage over the other methods as it can be used at any phase of differentiation of cell, also the speed, simplicity, low cost, the small number of cells required (<10,000 cells), its sensitivity and widespread applicability with eukaryotic cells. Other advantage is genotoxic and genoprotective effects can be studied in the tissue or cell type specific.[20] Therefore, comet assay is used to investigating DNA damage and repair characteristics in a wide range of tumor cells in response to a variety of DNA damaging agents. These studies include both investigations on human tumor cell lines and on tumor cells extracted from cancer patients.[21] Single strand DNA breaks result from a variety of factors including ultraviolet light, X-rays, ionizing radiation, toxins, chemical or by reactive oxygen species.[22]

In the present study, mean tail length in OSF group was found to be 12.92 μm, in comparison to mean tail length of healthy group 8.34 μm. The mean tail length between both groups was found to be statistically significant ($P < 0.05$) [Table 4]. But we did not get the correlation between the clinical grades in OSF group with tail length [Table 2]. This is probably because of uneven distribution of sample in the study and once the disease is developed there will be the formation of tail length irrespective of clinical grading.

When tail length was compared with different types of habits, it was observed that the formation of tail length irrespective of different types of habits. The mean tail length in betel quid, gutkha and mixed habits were 12.8814

µm, 13.2020 µm, 12.6150 µm respectively [Table 3]. Studies showed that increase in tail length formation with betel quid, gutkha chewing and smoking habits.[13,22,23] We also observed that the gradual increase in the tail length with duration of the habits [Graph 1].

Lymphocytes showed increased DNA damage in individuals with chewing tobacco and smoking habit.[23] In oxidative stress induced DNA damage length was significantly increased in smokers group than in the non-smokers group in buccal exfoliated cells.[24] Furthermore, Mozaffarieh *et al*.[22] demonstrated double the amount of single strand DNA breaks in their circulating leucocytes than non-smokers. Moreover, smoking half pack a day could interfere with DNA integrity.

Polymorphism of DNA repair gene XRCC 1399 and HOGG 1326 were observed in smokers tobacco users. Also there was a significant difference in micronuclei, comet scores among the tobacco users and control subjects. These findings indicate, there is a polymorphism in DNA repair genes tends to makes the individual susceptible for the development of cancer in tobacco habitual.[25]

Saran *et al*.[3] observed stepwise increased in micronucleus frequency from control to pre-cancer patients and from pre-cancer to cancer patients. Similarly significant stepwise increase in DNA damage (basal/MNNG-treated/post-repair) was observed in buccal epithelial cells and peripheral blood leukocytes. Extent of DNA damage was demonstrated in leukoplakia, OSF, cancer patients in comparison to normal individuals. Carcinoma had greater tail length than pre-cancerous and controls, whereas the extent of DNA damage was greater in leukoplakia than OSF. DNA damage for people with any oral habit was significantly greater than those with no habits.[4]

Similar to buccal mucosa, the gingiva is also directly exposed to the chewing tobacco habits. All patients with OSF showed comet cells in a larger number than the controls.[26] Various studies have shown DNA damage which is significantly increased in patients suffering from different type of cancers including head and neck, breast, renal, colon and colorectal. Many of these studies have used extracted peripheral blood lymphocytes from cancer patients and compared to controls. Also epithelial cells or tumor cells were obtained by fine needle aspirate to study the DNA damage.[21,27]

Comet assay showed higher values of all irradiated blood samples when compared to control. For both tail length and tail movement, dose effect relationship was found to be linear in a dose range of 0.5 Gy and 10 Gy. It can be used predict the tumor response to chemotherapeutic drug the comet assay can be a clinical assay which helps in measuring the sensitivity of cells to chemical mutagens as well as their ability to repair the damage. The limitation of the assay is not all the drugs produce the sort of damage that can be measured by the comet assay, but it is a useful tool in assessing the sensitivity of cancer cells to particular compounds.[28]

Also the comet assay used as a tool for environmental bio monitoring, for example, pesticides, viscose factory workers occupationally exposed to carbon disulfide using buccal cell and lymphocyte respectively.[12,20] Another chemical isomalathion showed significant changes in the comet length of the lymphocytes throughout the range of concentrations tested over the malathion.[20] Comet assay can be used for screening of environmental genotoxic substances like coal and its derivatives, as it contains polycyclic aromatic hydrocarbons. Fossorial rodents reside closer to strip coal mine of Southern Brazil, lymphocytes showed significant DNA damage than non-coal mine areas. Thus, comet assay is an ideal approach for environmental evaluation using animals in their habitat and without sacrificing them.[29]

CONCLUSION

We have been able to demonstrate in this study that OSF patients had DNA damage which showed an increase in tail length and also that tail length is associated with deleterious oral habits. Measuring the extent of tail length in oral epithelium would provide direct evidence of association of DNA damage in oral epithelium with these deleterious oral habits. This can be incorporated into clinical practice to improve outcome and survival of persons at high-risk for oral cancer.

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