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Research Article

Evaluation of oxidative DNA damage in gingival epithelial cells of patients with oral sub mucous fibrosis

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ABSTRACT

Objective: The study was undertaken to determine oxidative DNA damage and free radical levels in patients with oral submucous fibrosis.
Methods: The DNA damage was evaluated using comet assay and free radicals including nitric oxide and malondialdehyde levels were estimated using biochemical methods. Statistical analysis was performed using student t-test.
Results: Comet assay revealed an increased frequency of comets in patients when compared to controls. The mean levels of nitric oxide and lipid peroxidation were significantly increased in patients when compared to controls.
Conclusion: The increased free radical generation and increased frequency of comets in patients indicating increased oxidative DNA damage. This might be due to the presence of alkaloids and other carcinogenic constituents present in betel quid and tobacco. The cumulative effects of betel quid and tobacco components may play a synergistic role in causing disease in South Indian population.

Key words: Oral submucous fibrosis; DNA damage; comet assay; gingival epithelial cells, nitric oxide, malondialdehyde.

1. INTRODUCTION

Oral submucous fibrosis (OSF) an insidious, precancerous and chronic disease that may affect the entire oral cavity and has been reported to be associated with environmental and genetic factors. The most common oral pre malignancies appear to be related to the habit of chewing betal quid, tobacco smoking and heavy alcohol consumption.[1,2,3] In addition, the inherited genetic susceptibility may also play an important role in causing oral tumorigenesis. OSF is found in 4/1000 adults in rural India and above 5 million young Indians are suffering from this precancerous condition. This is a result of increased popularity of the habit of chewing pan masala, a mixture of spices including betal nuts, catechu, menthol, cardamom, lime etc. It has a mild stimulating effect and is often eaten at the end of the meal to help digest food and as a breath mint. A variety of etiological factors affecting OSF includes capsaicin, betal nut alkaloids, hypersensitivity, auto immunity and genetic pre disposition [4]

Recent epidemiological studies have provided evidence that areca nut is the main etiological factor for causing oral submucous fibrosis. A clear dose-dependent relationship was observed for both frequency and duration of chewing areca nut in the development of the disease[5]. Commercially available freezed products like pan masala, guthka and mava (areca and lime) are having higher concentrations of areca nut and are appear to cause OSF more rapidly than by self-prepared conventional betal quid that contain smaller amounts of areca nut. Among the chemical constituents of betal quid, alkaloids are the main components play a synergistic role. Betal quid products have been strongly implicated and associated with the incidence of oral submucous fibrosis, which is extremely debilitating and has no known cure and has a high rate of malignant transformation. The use of tobacco with lime, betel quid with tobacco, betel quid without tobacco and areca nut have been classified as carcinogenic to humans.[6]

According to WHO the generalized pathological state of the oral mucosa associated with a significantly increased risk of cancer. Development of the pathological process is closely related to cell injury. One of the important mechanisms of membrane damage by free radicals.[7] Accumulation of excess amount of these free radicals causes destructive effects on lipid biomembrane, sulfhydril bonds of proteins and nucleotides of DNA. Further, it has been hypothesized that excess free radical generation leads to cancer.[8]

During the last few years there has been increasing interest in buccal epithelial cells for cytogenetic evaluation of different materials. In the present study the gingival epithelial cells were used for cytogenetic evaluation with comet assay (Single cell gel electrophoresis or SCGE). This technique detects DNA strand breaks in the individual cells in alkaline conditions.

2. MATERIALS AND METHODS

2.1. Study subjects

A total of hundred OSF patients with the age group of 15-55 years and hundred controls with same age, socio-economic status and who are not having any history of OSF were selected for the study. OSF patients with no systematic disease were included. Patients who enrolled into the clinical trial and with acute illnesses or infections or under retinoid or carotenoid therapy for 6 months prior to study were excluded. Patients with prior history of oral cancer were also excluded from the study. For the study informed consent was taken from each participant and approval for conducting research on OSF cases was obtained from the Institute's ethical committee.

2.2. Data and sample collection

The study group was clinically examined by the dentists of Osmania Govt. Dental College and Hospital, Hyderabad and information on their age, nature of job, personal habits like smoking (which included number of cigarettes per day, duration of smoking), pan chewing (quantity, frequency, duration and intensity) consumption of alcohol (quantity, frequency and duration) health status, medical history and reproductive history was collected using a standard questionnaire.

Gingivial epithelial cells were collected in phosphate buffered saline (PBS) from both the groups using brush biopsy and also a total of 5 ml of blood was drawn and transferred to heparinized vaccutainers to prevent coagulation. The samples were kept in ice flask to maintain temperature and transported to Institute of Genetics and Hospital for Genetic Diseases, Osmania University for further analysis. The blood samples were centrifuged at 2000 rpm for 10 minutes and plasma was separated. Erythrocyte suspension was washed twice with normal saline and kept in -20^0 C for further estimation of antioxidants while plasma was used for the estimation of nitric oxide and lipid peroxidation.

2.3. Estimation of nitric oxide in plasma

The nitrite concentration in the plasma was determined by using Griess reaction[9] in which NO_2 reacts with 3% sulfanilamide in 0.3% naphthalene ethylene diamine dihidrochloride, forming chromophore. 0.5 ml of plasma was precipitated with 50 µl of 70% sulphosalicylic acid (SSA) (Merk) and mixed well for 5 minutes and vortexed and then centrifuged at 3000 rpm for 20 minutes. 200 µl of supernatant was taken and to which 10% NaOH, tris buffer and Griess reagent were added and incubated for 10 min in dark. The absorbance was read at 540 nm. The concentration of nitrite in plasma was determined using the standard curve. A standard curve was prepared by using known amounts of sodium nitrite ($NaNO_2$).

2.4. Estimation of lipid per oxidation

Lipid peroxidation products were quantified by thiobarbutyric acid (TBA) method[10]. Malondialdehyde, the end product of lipid peroxidation present in the biological samples, which reacts with thiobarbituric acid (TBA) under acidic conditions at 95^0 C to form a pink colored product. 0.5 ml of plasma was made upto 1ml with normal saline and an equal volume of trichloroacetic acid (TCA) was added, and incubated at 37^0 C for 20 min and centrifuged at 3000 rpm for 10 minutes. To 1 ml of TCA extract, 0.25 ml TBA was added and heated in a water bath at 95^0 C for 1 hr until a faint pink colour appeared. After cooling the colour was extracted in 1ml of butanol and the intensity was read at 532 nm. 1,1,3,3 tetra ethoxy propane (1-100 nmol/ml) was used as the standard.

2.5. Evaluation of DNA damage using comet assay

Gingival epithelial cells were centrifuged for 10 minutes at 1000 rpm. The supernatant was discarded and leukocytes were used for the comet assay. Comet assay was carried out according to the method described by Singh et al[11] with slight modifications. Frosted microscopic slides were washed and cleaned with ethanol (90%) and air dried in dust free environment. Slides were prepared in duplicate for each sample and the complete experiment was carried out in 4-80C temperature. Pre-coating of microscopic slides was carried out by dipping into 1% molten agarose prepared in phosphate buffer saline (PBS pH-7.4). The slides were immediately wiped off on backside, and covered with a 24 x 60 mm cover slips and were allowed to form the gel at 4°C for 10 min. Ten micro liters of cell suspension was mixed with 110µl (0.5%) of low melting agarose prepared in PBS. The mixture was immediately coated onto microscopic slide, covered with cover slip and placed on ice for 5 min for allowing agarose to form gel. After carefully removing the cover slips a third layer of 110µl of low melting agarose was coated onto the slides and allowed to gel at 4°C for 10 min. After removing cover slips, slides were immersed and incubated in freshly prepared ice cold lysing solution(2.5M NaCl, 100mM EDTA, 10mM Tris-HCl, and 1% Triton X-100) for overnight and washed with distilled water. The lysed cells were incubated for 30 minutes to unwind the DNA with denaturation solution (0.3M NaOH, 10mM EDTA pH 13.1). Electrophoresis was carried in horizontal submarine gel electrophoresis unit at 25V (1.5V/cm) and 300mA for 15 min using alkaline electrophoresis solution (0.3M NaOH, 10mM EDTA pH 13.1). Slides were then drained, washed twice in distilled water for 5 min and incubated in neutralizing solution (0.4M Tris - HCl, pH 7.5) and washed twice with distilled water. Slides were dehydrated in absolute methanol for 10 min and were allowed to dry at room temperature. The whole procedure was carried out in incandescent light to minimize artifactual DNA damage. The slides were silver stained[12] as it has sensitivity for visualization of DNA damage on light microscope.

2. 6. Scoring of Comets

A total of 50 individual cells per subject were screened and the ratio of comets indicated the measure of DNA damage. The DNA damage was measured using an ocular micrometer fitted in the eyepiece of the transition microscope. Undamaged cells showed an intact nucleus and damaged cells have the appearance of a comet with prominent head and tail. The mean DNA damage (Mean ± SD) was calculated along with the controls.

2. 7. Statistical Analysis

Statistical analyses were performed using Student *t*-test and $p<0.05$ was considered statistically significant. Values are given as Mean ± Standard Deviation.

3. RESULTS AND DISCUSSION

In the present investigation biochemical and cytogenetic studies were carried out to estimate free radicals like nitric oxide and lipid peroxides and comet assay in 100 patients and 100 controls. The results on nitric oxide, lipid peroxidation and comet assay were presented in a Table-1. The results showed an increased mean nitrite (2.89), MDA levels (4.46) and increased mean frequency of comets (4.97) in patients when compared to (1.22), (2.24) and (1.49) in controls respectively. The mean nitrite and MDA levels and also the mean frequency of comets were statistically significant (P<0.05) in patients with oral submucous fibrosis when compared with the values observed in the control group (Table-1).

Table-1: Results on Free radicals and Oxidative DNA Damage in patients with oral submucous fibrosis

Group	Mean Nitrite Levels	Mean MDA Levels	Mean Frequency of Comets
OSF Patients	2.19*±0.94	4.46*±1.09	4.97*±1.26
Controls	1.22±0.42	2.24±0.56	1.49±0.02

*P<0.05

Oral sub mucous fibrosis (OSF) is a chronic disease of the oral cavity, characterized by inflammation and progressive mucosal fibrosis.[13] This may be a result of either direct stimulation from exogenous source of environmental toxicants like areca alkaloids or by changes in tissue antigenicity that may lead to an auto immune response.[14] Oral submucous fibrosis is caused by the use of areca nut in various forms. All areca nut products were associated with OSF, more with pan masala. Earlier study from Chennai confirmed that strong association between areca nut use and OSF and the increasing use of pan masala. [15]

Betal quid chewing is a popular habit and more common in Hyderabad, which is a major metropolitan city and capital of Andhra Pradesh, India and is located at Deccan Plateau. Hyderabad is currently the second largest metropolis in India, covering an area of 621.48 sq. km.[16-18] It is the sixth most populous city and the sixth-most populous urban agglomeration in India.[19] In this city most of the people fall into tension relieving habits like alcoholism, smoking, pan, and tobacco or betel quid chewing. These habits not only relive tension to some extent but also produce bad effects on human body apart from being addictive. Oral sub mucous fibrosis is one of the conditions resulting from these tension relieving habits. It is well known that tobacco and betal quid components are as important risk factors for causing oral disorders or oral cancer.

The toxic components of betal quid (BQ) and tobacco can interact with cellular constituents like proteins, lipids and DNA. Different types of primary lesions induced by chemical mutagens in cellular DNA and chromosomes. To understand the role of betel quid in the pathogenesis of oral sub mucous fibrosis (OSF), we used comet assay, cytotoxicity test, and oxidative stress markers, to see the free radical generation and their consequent effects on DNA damage and lipid per oxidation. Malondialdehyde (MDA) has been demonstrated to play a vital role in the pathogenesis of several diseases and inflammatory processes. Results showed an increased frequency of comets in OSF patients indicating DNA damage. The increased oxidative stress is indicated by the elevated levels of nitric oxide and lipid peroxidation, which can be used as reliable markers for assessing oxidative damage. Similarly an earlier study by Renuka et al [8] demonstrated high levels of MDA and low levels of beta carotene in OSF patients when compared to controls. In general, it is accepted that chromosomal aberrations and DNA damage are casual events in causing and developing cancers. DNA damage plays a vital role in the activation of proto-oncogenes, and their interactions leading to cause malignancy. In the present study increased DNA damage was observed in target tissue (i.e., gingival epithelial cells), which may be an indication of an enhanced cancer risk. Earlier reports showed an increased frequency of DNA damage in circulating lymphocytes and stated that the carcinogenic agent can produce damage not only in the target tissue but also in other host cells such as circulating lymphocytes.[20] In other study the micronuclei and other nuclear anomalies reflected genetic damage and cytotoxicity, indicated association with tobacco and areca nut consumption.[21] Increased cytogenetic damage has been reported by Fareed et al[22] in peripheral blood lymphocytes and exfoliated buccal mucosal cells of pan masala chewers.

Moreover, BQ extracts also inhibit DNA repair activity of O^6-methylguanine-DNA methyltransferase in buccal mucosal tissue and cell cultures *in vitro* .[23] Generation of reactive oxygen species (ROS) from betal quid and smoke components can oxidize DNA bases, e.g. deoxyguanosine to yield 8-oxo-dG (inducing G→T transversions), there by promoting the tumourigenic process in the oral cavity. Singh and Rao[24] reported reduced glutathione content and enhanced CYP450 activity, in the liver of mice treated with areca nut. Thus could cause increased oxidative metabolism of carcinogens and reduced detoxification. Glutathione depletion leads to increased oxidative stress that can cause DNA damage and can trigger the carcinogenic process. Glutathione *S*-transferases M1 and T1 are enzymes known to detoxify ROS, lipid peroxidation products and tobacco-derived carcinogens that have been found in the saliva of BQ/tobacco chewers. Nair *et al*[25] reported Null genotypes for *GSTM1* and *GSTT1* and their association with the increased risk of developing leukoplakia in chewers. So far, no similar studies on gene–environment interactions in *pan masala, gutkha* or areca nut chewers have been reported.

The results suggested that betal quid and tobacco are important etiological factors for causing and developing the disease. Further studies, are needed to clarify the role of betel quid without tobacco as a carcinogen alone and in combination with tobacco, as all the subjects were chewers.

Competing interests

The authors have declared that no competing interests exist.

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