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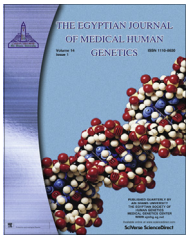

ORIGINAL ARTICLE

Assessment of DNA damage by panmasala, gutkha chewing and smoking in buccal epithelial cells using alkaline single cell gel electrophoresis (SCGE)

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KEYWORDS

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Buccal epithelial cells

Abstract In the present study the comet assay was performed in buccal epithelial cells to evaluate DNA damage among pan masala or gutkha chewers and smokers. The assay is a rapid, suitable and sensitive method for detecting various forms of DNA damage at individual cell level. The study comprises 300 individuals of which 50 individuals were gutkha chewers along with smoking, 50 individuals were pan masala chewers along with smoking, 50 individuals were gutkha chewers, 50 individuals were pan masala chewers, 50 individuals were smokers and 50 individuals were non-users (control) or not having any addiction. Comet tail length was observed to measure the extent of DNA damage. In all groups a significant increase in the tail length was observed as compared to the non-users (control). The highest tail length was observed among gutkha chewers along with smoking (36.9 ± 3.60). The results of the present study suggest that the panmasala and gutkha are genotoxic agents and induce DNA damage.

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1. Introduction

Gutkha and pan masala are popular among all age groups in India. Various studies have shown that the chewing of tobacco with lime or betel quid with tobacco and areca nut causes cancer in humans [1]. These dry products generate reactive oxygen species (ROS) in the buccal cavity of chewers [2]. These smokeless products are related to the genotoxicity affecting the DNA repair pathways [3]. DNA repair mechanism plays an important role in the maintenance of DNA integrity and prevention of cancer. If DNA remains unrepaired, it will lead to mutagenesis, genetic instability and ultimately cell death [4]. Single-cell gel electrophoresis (SCGE) or comet assay is used to monitor genotoxicity in the exposed population [5]. It detects different kinds of DNA alterations, single strand breaks,

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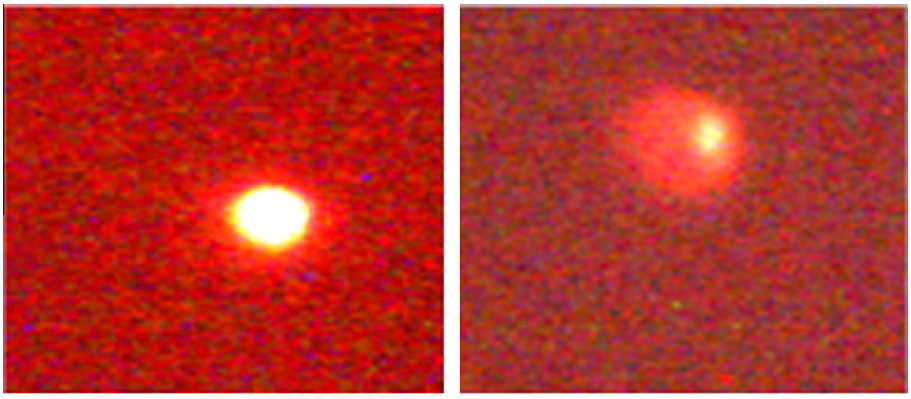

Figure 1 (A) Normal undamaged DNA of buccal epithelial cells in healthy individuals and (B) damaged DNA of the buccal epithelial cell of Gutkha chewers along with smoking.

Table 1 Showing comet tail length in different groups.

Groups	Number of individuals	Age range	Age (mean ± SE)	Comet tail length (mean ± SE)
Control	50	22–69	29.7 ± 1.41	3.41 ± 0.41
Smokers	50	23–65	40.1 ± 1.71	14.9 ± 0.79[a]
Pan masala chewers	50	26–58	30.0 ± 1.42	29.3 ± 3.41[a,b]
Gutkha chewers	50	21–62	32.5 ± 1.63	31.6 ± 3.52[a,b]
Pan masala + smoking	50	23–56	32.2 ± 1.18	33.6 ± 3.59[a,b]
Gutkha + smoking	50	20–67	37.7 ± 1.50	36.9 ± 3.60[a,b]

[a] Significant as compared to control ($p < 0.05$).
[b] Significant as compared to smokers ($p < 0.05$).

double strand breaks, alkali-labile sites, cross-links and incomplete repair sites [6,7]. This assay can be applied to proliferating cells, like buccal cells and nasal cells that are susceptible to carcinogenic and mutagenic agents [8]. In the comet assay for buccal cells, the cells are embedded in agar gel and cell membranes are removed by using a lysing solution, and the DNA is allowed to unwind by performing electrophoresis at alkaline pH. DNA loops around strand breaks are in a relaxed state, and are pulled towards the anode, giving a comet tail like appearance. Undamaged DNA remains in the nucleoid or comet head [9,10]. The aim of the present study is to make individuals aware about the consequences of pan masala or gutkha chewing as well as smoking that can cause DNA damage leading to oral submucosis fibrosis (OSMF).

2. Subjects and methods

2.1. Study design and sample

The study comprises of 300 individuals (50 gutkha chewers, 50 gutkha chewers along with smoking, 50 pan masala chewers, 50 pan masala chewers along with smoking, and 50 smokers and 50 controls). A written consent was taken from each individual, and the samples were taken from the Department of the Ziauddin Ahmad Dental College and Hospital, A.M.U., Aligarh, UP. The period of the study was almost 8 months.

2.2. Single cell gel electrophoresis (SCGE)

Buccal epithelial cells were collected from subjects by using a soft bristle tooth brush gently from the oral mucosa of the cheeks. The brush was then swirled into a tube containing cold phosphate buffered saline (PBS) and centrifuged at 2000 rpm for 10 min. The supernatant was removed and 300 µl of trypsin solution (0.25% trypsin, 1 mM EDTA in PBS) was added to the buccal cells and incubated for 30 min at 37 °C. The cells were centrifuged and the supernatant was discarded. The cells were then washed thrice by centrifugation at 2000 rpm for 10 min in cold PBS. About 40 µl of cell suspension and 60 µl of 0.5% low melting agarose (LMA) were mixed and placed on frosted slides previously coated with 1% normal melting agarose. To the solidified agarose, a third layer of 1% low melting agarose was applied and the slides were dipped in freshly prepared cold lysing solution (2.5 M NaCl; 100 mM EDTA; 10 mM Trizma base; 1% Triton X; 10% DMSO) for 24 h. [7] Then the slides were subjected to electrophoresis (300 mM NaOH/1 mM EDTA) (pH > 13), followed by neutralization (0.4 M Tris–HCl) and stained with ethidium bromide (20 µg/ml) [11]. Three slides were prepared per individual and a total of 50 randomly captured comets per slide, under a fluorescence microscope were analysed for scoring comet tail length by using comet score 1.5 software (TriTek corporation).

2.3. Statistical analysis

Statistical analysis was carried out by the Student's '*t*' test and regression using commercial software Statistica Soft Inc.

Table 2 Frequency of cigarette, gutkha and pan masala intake by various groups.

Groups	Duration of addiction (years) (mean ± SE)	Cigarettes/day (mean ± SE)	Gutkha/day (mean ± SE)	Pan masala/day (mean ± SE)
Control	–	–	–	–
Smokers	9.0 ± 0.60	7.06 ± 0.14	–	–
Pan masala chewers	9.04 ± 0.46	–	–	8.52 ± 0.08
Gutkha chewers	9.80 ± 0.62	–	8.76 ± 0.09	–
Pan masala + smoking	9.58 ± 0.63	5.92 ± 0.28	–	8.32 ± 0.06
Gutkha + smoking	9.62 ± 0.58	5.86 ± 0.27	9.16 ± 0.09	–

3. Results and discussion

The effect of gutkha and pan masala chewing along with and without smoking was studied in buccal epithelial cells using single cell gel electrophoresis (SCGE) (Fig. 1). A total of 300 subjects corresponding to 50 for each group were recruited for this study. Table 1 represents the mean of the age and tail length. The mean values for the duration of addiction of chewing/smoking, as well as pan masala, gutkha and cigarettes taken per day are presented in Table 2. The mean tail length in smokers (14.9 ± 0.97), pan masala chewers (29.3 ± 3.41), pan masala + smoking (33.6 ± 3.59), gutkha chewers (31.6 ± 3.52), and gutkha chewers + smoking (36.9 ± 3.60) was significantly higher ($p < 0.05$) as compared to non-users (control) (3.41 ± 0.41) (Table 1, Fig. 2). The mean duration of addiction is almost the same in all the studied groups (Table 2) and the highest tail length was observed in the gutkha + smoking group (Fig. 2). We correlated the mean age and tail length and found that the tail length is not related with the age ($r = 0.11790$; $p < 0.8162$). Hence the addiction is directly related to the tail length. The highest DNA migration was found among gutkha chewers with smoking habit. The increase in the mean comet tail length was observed as: Non-users < smokers < pan masala chewers < gutkha chewers < pan masala + smoking < gutkha + smoking (Fig. 2). The regression analysis shows the value of R^2 (0.138) (Fig. 3). SCGE or Comet assay in buccal epithelial cells is easier and a safe method to detect DNA damage among humans [12]. When the amount of ROS generated in cells increases from the capacity of the normal detoxification system then oxidative stress leads to cellular damage, along with the DNA damage [13]. DNA damage can occur as single-stranded (ss) breaks or doublestranded (ds) breaks [14]. The main objective of the study was to evaluate the extent of the DNA damage due to various addictions. In the present study the higher values in comet tail length were observed among gutkha chewers along with smoking. The tobacco present in cigarette/beedi induces DNA adducts and oxidative DNA damage in human tissues. The formation of carcinogens may lead to DNA mutation and by disturbing the protein function may lead to cancer [15,16]. The tobacco-specific nitrosamines can induce miscoding in the DNA that could result in the tumourigenic process in the oral cavity [17]. Pan masala or smokeless tobacco causes genotoxicity that affects DNA repair pathways [3]. In smokers, comet tail length was found to be more as compared to the non-users (control) group which may be due to oxidative stress in smokers. This causes an imbalance between the formation of reactive oxygen species (ROS) and the ability to neutralize ROS [18]. The formation of the DNA adduct is the initiating step in the process of carcinogenesis. Pan masala and gutkha also contain various irritating substances that make the skin lose its elasticity [19]. The main carcinogens in gutkha are derived from their ingredients (arecanut, catechu, and tobacco). A high level of nitrite and nitrate reductase activity has been reported in the saliva of gutkha chewers [20,21]. There are reports for the generation of ROS by the aqueous extract of arecanut and catechu leading to the genotoxic damage in buccal epithelial cells [22]. The occurrence of oral cancer has been well documented independently in association with oral habits such as smoking, betel quid chewing and tobacco chewing [23,24]. These oral habits have also been associated with DNA damage. Comet assay is used for the

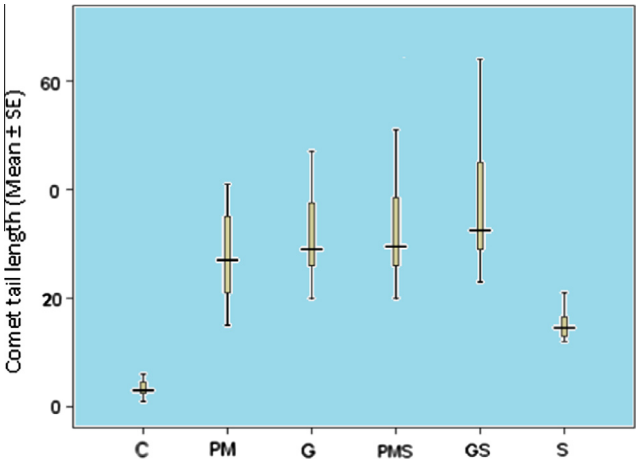

Figure 2 Comet tail length among various groups. C, control; PM, pan masala; G, gutkha; PMS, pan masala with smoking; GS, gutkha with smoking; S, smoking.

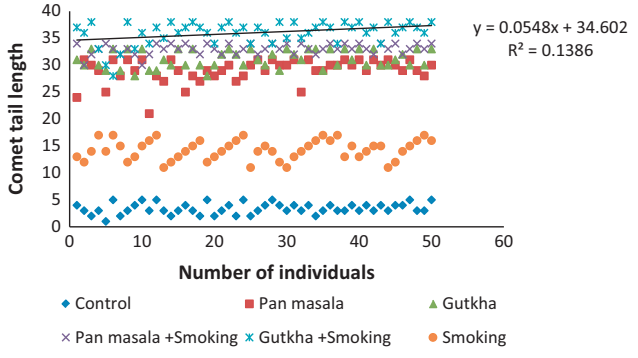

Figure 3 Comet tail length among individuals of different groups and regression analysis.

biomonitoring study and the tail length has been the most commonly used parameter for DNA damage measurement [25]. In our present study the highest DNA damage was observed among the gutkha + smoking group. Gutkha is a mixture of arecanut, catechu, lime, cardamom, unspecified flavouring agents, and tobacco. Arecanut is the main component of gutkha responsible for the oral submucosis fibrosis (OSMF) [26]. In our earlier studies the high frequency of micronucleus was found among gutkha users [27]. The high frequency of micronucleus was also found among OSMF patients (gutkha chewers) [28]. However, earlier studies have shown that the ROS produced by arecanut is responsible for the initiation of OSMF [29]. The aqueous extract of *N*-nitroso compounds related to arecanut, that is, 3-(methylnitrosamino) proprionitrile is highly cytotoxic and genotoxic in cultured human buccal epithelial cells, responsible for the induction of tumours among betel quid chewers[30].

4. Conclusion

Single cell gel electrophoresis (SCGE) is of potential value for human biomonitoring against the harmful agents such as pan masala or gutkha chewing and smoking. This study shows the genotoxic effect of panmasala or gutkha chewing and smoking. Gutkha chewing along with smoking are most dangerous for health, so it is important to increase the awareness programmes to make the people aware of the consequences and possible risks associated with these addictions.

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