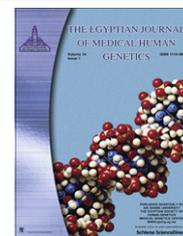




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ORIGINAL ARTICLE

Evaluation of micronucleus frequency by acridine orange fluorescent staining in buccal epithelial cells of oral submucosus fibrosis (OSMF) patients

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Abstract Oral submucosus fibrosis (OSMF) is a collagen-related disorder seen in habitual betel quids and smokers. This is a high risk precancerous condition in which the connective tissue fibers of the lamina propria and deeper parts of the mucosa becomes stiff with restricted mouth opening. Patients with severe cases have symptoms like difficulties in chewing, swallowing and speaking. In the present study 25 individuals were gutkha chewers and 25 were OSMF patients (chewing gutkha along with smoking) and 25 individuals were taken as controls. A significant increase in the frequency of micronuclei was observed in OSMF patients (34.4 ± 1.79) as compared to gutkha chewers (14.4 ± 0.73) and controls (4.36 ± 0.27). The number of micronucleated cells in OSMF, gutkha chewers and control groups were 19.84 ± 0.69 , 12.6 ± 0.51 and 4.20 ± 0.27 , respectively and are significantly different at $p < 0.05$. Acridine orange is used due its fluorescence nature and easier visibility of the micronucleus present in the buccal epithelial cells. It is concluded that chewing gutkha along with smoking is more dangerous for human health as it hastens the incidence of OSMF.

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1. Introduction

The increased popularity of commercially available arecanut types i.e. pan masala and gutkha among young people of India has increased the incidence of oral submucosus fibrosis (OSMF) [1,2]. OSMF was known in Indian medical literature since the time of Sushruta – a great Indian physician who lived in the era 2500–3000 B.C. It was first described in the modern literature by Schwartz and Joshi [3,4]. This disease occurs mostly in South East Asia, but some cases were also reported

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from countries like Kenya, China, UK, Saudi Arabia and a few parts of the world [5]. In India about 5 million people are suffering from OSMF [6]. These chewables (pan masala, gutkha, etc.) contain various substances that irritates the delicate skin of the mouth (mucosa). As a result of long time use of these chewables, the skin loses its elasticity. The disease is known as oral (i.e. related with mouth) submucous (meaning skin below the delicate skin of the mouth) fibrosis (i.e. hardening and scarring) [7]. OSMF starts with a burning sensation in mouth and ultimately leads to the total closure of the mouth [8]. It is observed that the onset of OSMF takes about 2–20 years to present with its signs and symptoms [9,10]. In regular gutkha users, OSMF was seen at earlier ages as compared to traditional betel quid users. A gutkha sachet weighs ~3.5 g and contains 7% moisture, whereas the net weight of a betel quid is nearly 4 g (with ~1.14 g of tobacco) and contains 70% moisture. Due to the consumption of more dry weight of tobacco, areca nut and slaked lime gutkha users are exposed to OSMF at earlier ages compared with the other types of betel quid users [11]. Various studies approved that the regular intake of areca nut was the vast etiological factor in the formation of OSMF [12,13]. The major alkaloids present in arecanut i.e. arecoline on hydrolysis, produces arecaidine, which affects fibroblasts. When slaked lime i.e. $\text{Ca}(\text{OH})_2$ is added to areca nut in a pan, then the hydrolysis of arecoline to arecaidine facilitates fibroblastic proliferation and increases the formation of collagen [14]. But the exact role of areca nut in the development of OSMF is not defined. There may be a HLA-linked genetic susceptibility for areca nut alkaloids and tannins in patients with OSMF condition [15]. Many studies are going on in searching for the cause of fibrosis due to arecanut. Recent findings showed that upregulation of the copper-dependent extracellular enzyme lysyl oxidase by fibroblasts in OSMF is important, resulting in the excessive crosslinking and accumulation of collagen [16]. The frequency of micronucleus (MN) in OSMF patients was significantly higher as compared to the healthy individuals [17]. The micronucleus assay is a mutagenic test system for the detection of chemicals that induces the formation of small membrane bound DNA fragments i.e. micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes which are not able to migrate with the rest of the chromosomes during the anaphase of the cell cycle. The purpose of the micronucleus assay is to detect those agents which modify the chromosome structure and segregation in such a way so as to lead to the induction of micronuclei in the interphase cells. Micronucleus assay is helpful in the detection of the extent of DNA damage in a cell and gives us information about cancer formation in an individual. The aim of the present work is to make individuals aware about the mutagenic and carcinogenic effects of smoking and gutka chewing and their progression toward oral submucous fibrosis (OSMF). Acridine orange is a nucleic acid selective fluorescent dye that emits green radiation at 525 nm, making the scoring of MN in stained cells easy and with less error.

2. Subjects and methods

The study comprised 25 cases of oral submucous fibrosis (OSMF) patients and 25 cases of gutkha chewers along with 25 healthy individuals as the control group. The patients were

selected from among those visiting the Outpatient Department of the Ziauddin Ahmed Dental College and Hospital, Aligarh, India.

2.1. Chemicals

Trizma hydrochloride ethylene diamine tetra acetic acid (EDTA) from SRL, India. Acridine Orange stain, sodium chloride, methanol, glycerol and sodium hydroxide pellets from Merck (India). To make the buffer solution 0.1 M EDTA, 0.001 M Tris HCl and 0.02 M NaCl were dissolved in sterile 1 l distilled water. The pH of the buffer was adjusted to 7.0 with NaOH.

2.2. Oral mucosa cell collection and processing

Oral mucosa cells were collected from each subject using a soft toothbrush gently from the oral mucosa of the cheeks [18]. The brush was then swirled into a centrifuge tube containing a buffer solution of pH 7. Oral mucosa cells were washed thrice by centrifugation at 1500 rpm for 10 min in the buffer solution [18]. A volume of 25 ml of buffer in a 50 ml conical tube was used in every washing step. Washing with the buffer leads to the inactivation of endogenous DNAases present in the oral cavity, removes bacteria and cell debris that would complicate the scoring [19]. Gentle pipetting of the cells into a buffer solution reduces the clumping and lyses of the cells. The cell density was then checked with a phase contrast microscope. The cell solution was either concentrated by centrifugation or diluted in the buffer as required. Once the cell density ($1.5\text{--}2 \times 10^6/\text{ml}$) was reached, 50–100 μl of the cell suspension was laid and spread well on a clean, pre-heated (37 °C) glass slide and allowed to air dry for 5–10 min. The slides were then fixed in methanol, stained with acridine orange [20] and observed under a microscope. About 2000 oral mucosal cells were scored per individual.

3. Results and discussion

Fig. 1, shows the micronucleus in OSMF patients. A significant increase in the frequency of micronucleus was observed in the OSMF patients (34.4 ± 1.79) as compared to gutkha chewers (14.4 ± 0.73) and the control group (4.36 ± 0.27) (Table 1). The number of micronucleated cells in OSMF, gutkha chewers and control group were 19.84 ± 0.69 , 12.6 ± 0.51 and 4.20 ± 0.27 , respectively (Table 1). The micronuclei frequencies in single cells are 3 and 4 i.e. higher in OSMF patients as compared other groups. About 19 patients of OSMF were having 4 micronuclei per cell. Among gutkha chewers 12 patients showed 2 micronuclei per cell (Table 2). The age distribution of OSMF patients, gutkha users and the control group is presented in Table 3. It shows that the higher use of these products was found among the age group of 25–40 years. The people of this age group have more addiction of these harmful products. The frequency of micronuclei was found to be more in the age group of 25–40 years (Table 3). The mean values of total micronucleus are shown in the form of a box plot (Fig. 2).

Chewables substances i.e. pan masala, gutkha, etc. contain various elements, which irritate the mouth (mucosa) skin. After a long time of its use the skin loses its elasticity [7]. A number of studies have shown that the formation of reactive

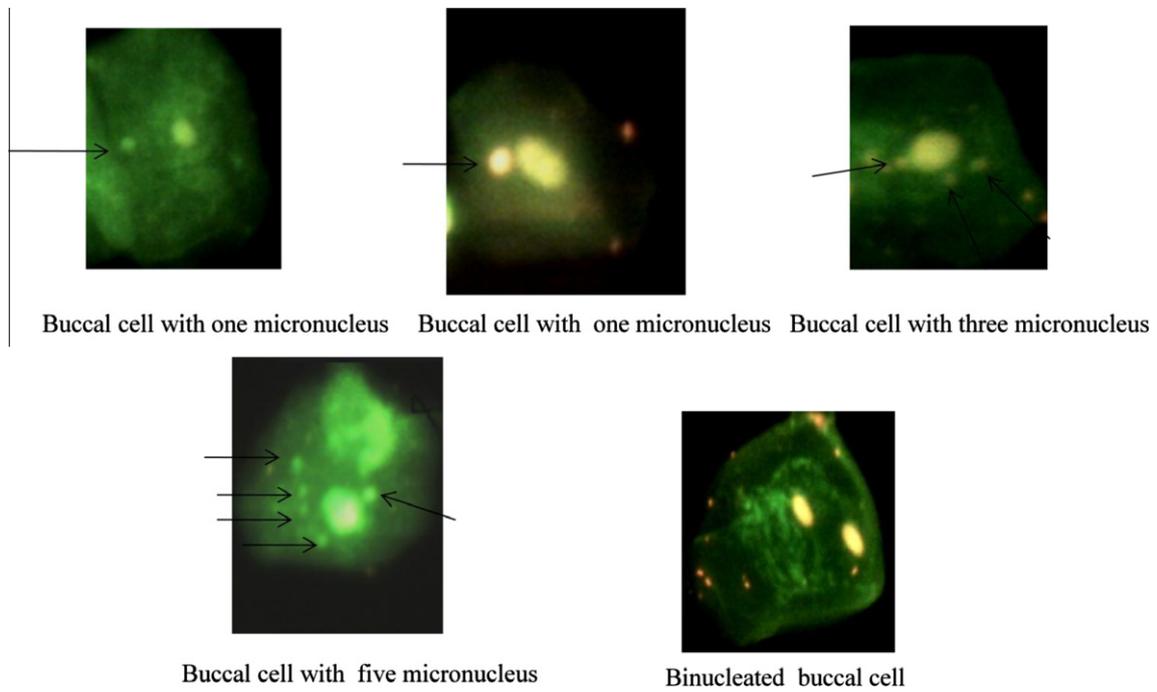


Figure 1 Buccal epithelial cells showing micronucleus stained with acridine orange.

Table 1 Total micronucleus frequency per 2000 cells per individual in buccal region of Control, Gutkha users and OSMF patients.

Groups	Number of Individuals	Age range	Age (Mean ± S.E)	MC (Mean ± S.E)	TM (Mean ± S.E)
Control (non-users)	25	15–60	34.0 ± 2.56	4.20 ± 0.27	4.36 ± 0.27
Gutkha chewers	25	14–65	31.5 ± 2.60	12.6 ± 0.51 ^a	14.4 ± 0.73 ^a
OSMF	25	17–53	30.5 ± 1.98	19.8 ± 0.69 ^a	34.4 ± 1.79 ^a

MC is the total number of micronucleated cells and TM is the total number of MN in group. OSMF: oral submucosus fibrosis.

^a Significant at $P < 0.05$ compared to control (non-users).

Table 2 Micronucleus distribution in various groups.

Number of micronucleus	Control group (non-users)	Gutkha chewers	OSMF patients
1	21	9	0
2	4	12	0
3	0	3	6
4	0	1	19
Total number of individuals	25	25	25

Table 3 Age distribution among various groups.

Age group	Control group (non-users)	Gutkha chewers	OSMF patients
> 24	6	8	9
25–40	13	12	13
> 40	6	5	3
Total	25	25	25

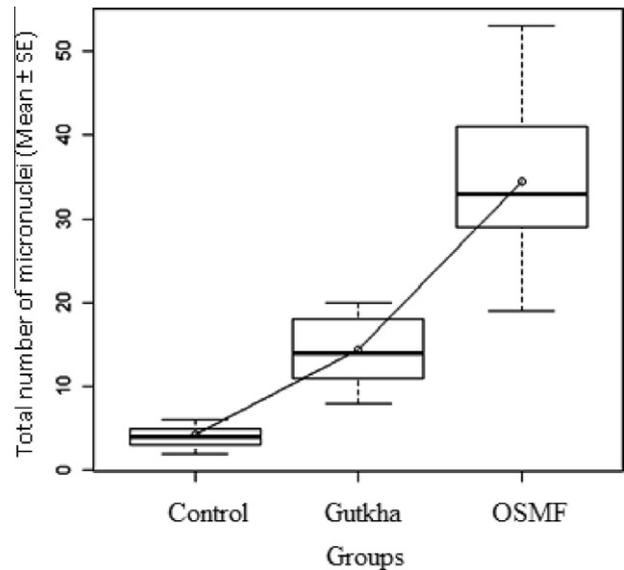


Figure 2 Total number of micronuclei distribution among various groups.

oxygen species produced by arecanut in the betel quid chewers is responsible for the initiation of OSMF [21]. Development of OSMF, due to arecanut has not been fully understood. In vitro

studies with cultured cells showed that the alkaloids of arecanut such as arecoline, guvacoiline etc stimulate fibroblast cell and collagen synthesis [22,23]. Arecanut components such as flavonoids, catechins and tannins cause collagen fibers to crosslink and induce collagen metabolism ultimately leading to fibrosis [24]. In OSMF patients due to chewing arecanut, increase in total serum protein was observed and the levels of ascorbate and iron were lower. The collagen content increased significantly in OSMF patients in the third stage of the disease [25]. When betel quid is taken along with smoking, the chances of OSMF increasing are more due to other reactive agents of cigarettes [26]. These carcinogenic agents are responsible for the DNA damage and to work out the intensity of DNA damage chromosomal aberrations and micronuclei are excellent markers [27]. A micronucleus is a small extranucleus which is separated from the main nucleus and formed during cellular division due to lack of proper division of chromosome fragments. To observe the genotoxic and mutagenic damage micronucleus assay can be performed [27]. It is important that OSMF should be diagnosed early, to avoid its complications or the conversion of this precancerous stage to squamous cell carcinoma [28].

In the present study, oral submucous fibrosis (OSMF) disease was found in gutkha chewers with a smoking habit, but not observed in the individuals of gutkha chewers alone. This shows that local habits increase the risk of this dreadful disease. Smoking habit alone is not responsible for the development of OSMF, but when the addiction of the areca nut is involved, the risk of OSMF increases [29]. Regular use of the arecanut is the major cause of OSMF [12,13,30]. The frequency of micronuclei was found to be significantly higher in smokers having the habit of chewing a mixture of betel leaf, areca nut and tobacco than that in chewers without a smoking habit [31,32]. Higher frequency of micronucleated cells was found in the buccal cells of patients with precancerous oral lesions like lichen planus, leukoplakia and OSMF [33,34]. In India, the majority of OSMF cases were seen in low socioeconomic groups [35]. The reasons for OSMF in this group were malnutrition, poor consumption of vitamins and minerals, poor quality of food, and the use of more spices and chillies [36]. The frequency and duration of chewing and smoking habits are also an important factor for the development of OSMF [29]. OSMF was seen within 1 year of exposure to the use of local products. It may be due to the differences in the preparation of the areca nut consumed [37,38]. Micronucleus is the predictive indicator of DNA damage, so it can easily highlight the severity of the disease, as early as possible. It is an excellent genotoxic biomarker and when the cells are stained with acridine orange it gives a bright stain to the nuclear material, leading to the clear identification of MN if any. The chances of errors during scoring are less when the cells are stained with a fluorescent stain such as acridine orange.

4. Conclusion

MN is found to be the most sensitive, non invasive and a very economical assay. Hence micronucleus is an "internal dosimeter" to estimate exposure to genotoxic agents. This study brings to light that the consumption of gutkha along with the smoking habit is highly hazardous for the individuals and responsible for OSMF. It is important to increase aware-

ness programs to guide and educate the public regarding the adverse effects of the disease.

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