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Cytomorphological changes in buccal epithelial cells of khaini chewers in different age groups

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Abstract
The present study was carried out to assess the cytoplasmic diameter and nuclear diameters of normal buccal mucous membrane in different age groups of khaini chewers. The study group consisted of 105 khaini chewers and 105 age and sex matched controls with no history of tobacco chewing/smoking. They were sub divided into 3 groups each, less than 25 years of age (Group – I), 25 -50 years of age (Group – II) and more than 50 years of age (Group – III). The buccal epithelial cells of these individuals were collected with moistened wooden spatula and the cells were measured cytomorphometrically using soft ware. Students't' test was carried out to find the significance of each sample. A significant increase was seen in the normal nuclear diameter and nuclear cytoplasmic ratio of the Khaini chewers when compared to the controls ($p<0.05$). A clear proportional increase in nuclear diameter was observed in khaini chewers aged 25-50 years and more than 50 years with a decrease in cellular area. Oral exfoliative cytological techniques could possibly be a noninvasive alternative prognostic marker for detecting early oral malignancy.

Keywords: Buccal epithelial cells, smokeless tobacco, nuclear diameter, abnormal cytoplasm.

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INTRODUCTION

In India, there is a clear relationship between chewing tobacco and oral cancer (Peter and Jan, 2009). There are various brands of chewing tobacco including Gutkha, Pan masala, Khaini, Panparag and other branded sachets are commonly available in India (Chitta et al., 2009). Especially, khaini is a popular variety, particularly used by tobacco consumers lived in rural areas (Saman et al., 2010). Homemade khaini are usually composed of sun dried boiled tobacco leaf mixed with wet slaked lime and areca nut. The users usually become addicted due to the nicotinic effect of the product and many chew khaini 10-15 times every day for 15 -20 years (Chitta et al., 2009).

The side effects of smokeless tobacco arise in peoples of all ages like young and old. Routine usage of khaini and other smokeless tobacco has numerous adverse effects on oral tissues (Saman et al., 2010; Rohatgi et al., 2006; Proia et al., 2006). Numerous studies showed that khaini and other smokeless tobacco chewing is associated with the increased risk of oral cancer (Peter and Jan, 2009; Scheifele et al., 2007; Epstein et al., 2002; Nidhi et al., 2005; Khan, 2012). Mutagenicity screening showed high mutagenicity of khaini product, suggestive of carcinogenic effects (Saranya and Sudha, 2013). Studies on khaini associated oral cancer and its adverse effects on health, in terms of carcinogenesis, were previously investigated at cellular level (Rohatgi et al., 2006).

Buccal epithelial cells form the first barrier in the oral mucosa exposed to chewing tobacco. Consequently, it could be argued that oral epithelial cells signify an ideal target site for early genotoxic events induced by carcinogenic agents entering the body via inhalation and ingestion (Bina and Padala, 2012; Saranya and Sudha, 2013; Shiv et al., 2012; Spivack et al., 2004). In healthy individuals, epithelial cells of the buccal mucosa in the oral cavity are naturally exfoliated every day (Mustafa et al., 2011). Exfoliated buccal cells are at the final stage of cell differentiation, and rarely display mitotic features (Mustafa et al., 2011; Scheifele et al., 2007). Cytomorphometric examination of these cells is one such priceless quantitative method to assess the influence of tobacco chewing on buccal mucosa (Einstein and Sivapathasundharam, 2005).

Taking this into consideration the present study has been carried out to assess the effect of khaini chewing on buccal mucosa and compare the cytomorphology of cells collected from buccal mucosa of chewers with those of non-users in different age groups.

MATERIALS AND METHODS

The present study was conducted in rural areas of Tamil Nadu state, South India in accordance with Helsinki declaration. The written consent from the subjects involved in the study was obtained. The study population included 210 subjects (132 male and 78 female), comprising of 105 khaini users and 105 nonusers aged between 15-70 years. Both control and experimental subjects were from same socio economic status and were involved in same type of occupation. Individuals were clinically healthy and had not been exposed to known genotoxic agents and were matched by age and sex. Exclusion criteria for the subjects included presence of any self reported acute illness, chronic diseases, heart failure, malignant, liver or kidney failure, diabetes mellitus, history of alcohol or drug use and smoking habit for at least last three years.

Chewers and controls were sub divided into 3 groups; Group I – individuals less than 25 years of age, Group II – individuals between 25 -50 years of age and Group III – individuals more than 50 years of age. Before sampling, each subjects rinsed their mouth thoroughly with tap water. Exfoliated buccal cells were obtained by gently scraping the inside of both cheeks with moistened wooden spatula, smeared on to a clear glass slide and immediately fixed with 95% ethanol for a minimum of 15 minutes.

A minimum of two smears were taken from each subjects to give 100 cells per subject (50 cells per smears). Collected smears were immediately fixed using 3:1 methanol: acetic acid for 15 min. The cells were stained using the Feulgen plus fast-green method, following the procedure of Moraes et al., (2005), with a minor modification: fast-green 0.5% solution in ethyl alcohol was used for 30 sec.

Two hundred cells per subject that were unfolded with clear outline were selected for the study. Only smears with unclamped, monolayered and consistent squamous cells were used for analysis. Cells were analyzed for cellular diameter (CD), nuclear diameter (ND), and nucleo cellular (N/C) ratio using a microscope equipped with a 100X objective (Olympus 20i, Japan) and a 2.25X video projection lens (Nikon CCTV/Microscope Adapter, Yokohama, Japan). The received images were transmitted to a video camera for display on a video monitor (Sony, Tokyo, Japan). A screen shot of each slides were captured, saved, and transferred to the computer for image analysis. Analysis was done using Magnus Pro Software. The statistical significance was determined by student's t test. P value less than 0.05 was accepted as statistically significant. The relationship between ages with duration of khaini use was evaluated via Pearson correlation test.

RESULTS

Table 1 presents the gender, age and years of khaini use of the study population. Table 2 represents the results of cytomorphometric analysis. The buccal mucosa of healthy volunteers with khaini chewing habit showed a significant variation in the size of mean ND and CD when compared with the respective controls. The results showed that mean ND in buccal mucosa was noticeably elevated ($p<0.05$) in users group (7.49µm) than in the control group (8.44 µm), and mean CD in buccal mucosa was markedly lower ($p<0.05$) in user group (51.03 µm) than in the control group (65.13 µm). In addition, mean N/C ratio in users group was apparently higher than in the control group ($p<0.05$).

Characteristics		*Controls*	Users
No. of subjects		105	105
Mean Age (years)		42.78±12.28	41.02±14.63
Age range (years)	<25	17.65±4.61	19.6±4.18
	25-50yr	39.02±8.80	42.34±6.68
	>50yr	60.17±5.95	62.97±4.93
Gender (male/female)		66/39	66/39
Years of khaini use		-	9.90±3.75

Table. 1: Characteristics of the study population

The ND results showed (Table 2) an age dependent progressive increase in the mean nuclear diameter of users from controls. When mean CD and ND was compared among different age groups, the mean difference of group I, II and group III was found to be statistically significant. There was a significant difference ($P<0.05$) between khaini users in terms of N/C ratio irrespective of age.

Parameter	Groups	Group I	Group II	Group III
Nuclear diameter (µm)	Controls	7.1±0.02	8.07±0.05	7.32±0.07
	Users	7.87±0.03*	8.75±0.06*	8.70±0.11*
Cytoplasmic diameter (µm)	Controls	67.13±0.18	62.56±0.12	65.71±0.16
	Users	59.10±0.14*	46.59±0.10	48.2±0.09
Nuclear: Cytoplasmic ratio	Controls	0.11±0.01	0.12±0.01*	0.14±0.04
	Users	0.16±0.02*	0.19±0.01*	0.22±0.09*

$p<0.05$*

Table 2. Comparison of mean values of cellular diameter, nuclear diameter and nuclear cytoplasmic ratio in buccal epithelial cells of Controls and Khaini users with respect to age.

DISCUSSION

Most of human cancers are derived from the external and internal epithelium, thus morphological characteristics of epithelial cells in the cytological prognosis of cancer and other diseases is essential. The mouth is the only body site that permits viewing with the naked eye the damages of smoked and smokeless tobacco (Nicole et al., 2006). There are several mechanisms by which smokeless tobacco may have an influence on the oral mucosa, through injury to cells and increased carcinogen penetration across the oral mucosa, by raising carcinogen solubility by increasing mucosa permeability (Sílvia et al., 2006). The constant use of tobacco has long been recognized as prominent risk factor in the development of oral cancer (Hashibe et al., 2009). Oral exfoliative cytology is a simple, non-invasive, and painless method that involves microscopic analysis of cells collected from the surface of the oral mucosa (Diniz et al., 2004; Proia et al., 2006).

The adverse effects on the buccal mucosa of smokeless tobacco, betel quid, and diverse admixtures have been reported by our previous studies (Sudha et al., 2009a, Sudha et al., 2009b; Sudha et al., 2009c; Sudha et al., 2010; Bindhya et al., 2010; Ramya et al., 2011; Rafiq Khan and Sudha, 2012) and several articles were addressed the tobacco associated buccal cell changes (Proia et al., 2006; Sharma et al., 2013; Mustafa et al., 2011; Komali et al., 2012; Palaskar S and Jindal C, 2010; Kashyap and Reddy, 2012; Rim et al., 2013; Kamath et al., 2014; Duanjun et al., 2008). For all articles, the diverse types of smoking and smokeless tobacco were addressed; there were no articles that addressed the smokeless tobacco in the form of khaini along with age as measurement.

In the present study, increased occurrence of abnormal nucleus and cytoplasm ratio was noticed in the exfoliated cells of the khaini users. Increase in age and extent of khaini use showed a significantly higher frequency of every investigated cytological changes. This finding is positively associated with oral carcinogenesis and support earlier investigations that disclose statistically significant decline in mean cytoplasmic area of cells taken from normal buccal mucosa of tobacco chewers (Mustafa et al., 2011; Alka and Minal, 2010; Goregen et al., 2011; Acharya et al., 2013). The buccal mucosa collected from below 25 years of age group khaini users also showed a significant result when compared to that of controls.

Increase in nuclear size is an indicator of cellular damage in tobacco users. Decreased cellular turnover as a result of prolonged khaini use following ageing would result in more number of mature cells with large nuclei in the smear (Prasad et al., 2010). This also accords with our earlier observations, which showed that a reduction in the size of CD and increase in the size of ND in khaini chewers with smoking habit than those with the habit of using khaini alone (Gemitha et al., 2013).

The results observed in the present study were, a clear proportional increase in ND was shown in those aged

25-50, a decrease in CD Khaini users and a steady increase in N/C ratio from control individuals to khaini users. In addition to this age dependent increase in abnormal nucleus and cytoplasm ratio was observed in khaini users.

CONCLUSION

From the present study, we conclude that long time khaini use produces definite morphological and morphometric changes in the exfoliated buccal mucosal cells.

This study reports that khaini use was high among younger individuals (43.3%), followed by subjects aged between 25-50 years (23.3%). These young individuals are subjected to greater risks of developing oral pathological changes, which may lead to cancer with prolonged exposure. However, it is essential to find ways to tackle this condition, therefore help to reduce the rate of chewing-tobacco induced oral malignancy in younger people.

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