5.1. Buccal Cytome Assay in Habituates

The buccal mucosa is a stratified squamous epithelium consisting of four distinct layers (Figure 45). The stratum corneum or keratinised layer lines the oral cavity comprising cells that are constantly being lost as a result of day to day activities such as mastication. Below stratum corneum layer is the stratum granulosum or granular cell layer and the stratum spinosum or prickle cell layer containing populations of both differentiated, apoptotic and necrotic cells. Beneath these layers are the rete pegs or stratum germinativum, containing actively dividing basal cells which produce cells that differentiate and maintain the profile, structure and integrity of the buccal mucosa (Veiro and Cummins, 1994). By comparing the different parameters of the buccal cytome assay among the habituates with different chewing habits, valuable information can be obtained on
markers for genome damage, cellular proliferation and cell death, which may reflect chewing habit related changes in the structural profile of the buccal mucosa.

Buccal mucosa is considered as a suitable target site for monitoring human exposure to occupational and environmental genotoxins. Mucosal cells are not only in the direct route of exposure to ingested pollutants, but also capable of metabolizing chemical agents to reactive species (Zhang et al., 1989; 1994). Analysis of genotoxic parameters like micronucleus and other nuclear abnormalities in the buccal epithelium is simple, non-invasive and time saving also.

Figure 45:
Diagrammatic sketch showing cross sectional area of normal mucosa and different cell types. Adopted from (Thomas et al., 2007)
Betel quid chewing habit with or without tobacco has been reported to alter the cells of oral mucosa (Merchant et al., 2000). Epidemiological studies have revealed that genomic instability markers such as micronuclei are elevated in buccal mucosal cells of habituates as well as following occupational exposure of tobacco such as workers employed in bidi industry (Thomas and MacLennan, 1992; Warnakulasuriya, 1995; Bhisey et al., 1999). It has been reported that chewing of betel quid stimulates mitosis during wound healing and exposes germinal basal cells to genotoxins and infection by HPV (Balaram et al., 1995). It is important to note that the site within the oral cavity, where carcinomas develop, is the one where the quid is placed for a prolonged time (Muir, 1967). A considerable number of reports have been published on the incidence of micronucleus in the buccal epithelial cells following tobacco and betel quid exposure. However, very few studies have considered the degenerative nuclear changes in buccal cells. Degenerative nuclear anomalies other than micronucleus have been reported to play an important role in toxicity assessment studies (Thomas et al., 2007; Celik et al., 2003).

The aim of the present study was to assess the correlation between DNA damage and intake of sadagura, a smokeless tobacco preparation, with other chewing and smoking habits. Besides micronucleus, attempts have been made to analyze other forms of nuclear changes such as binucleated, karyorrhectic, karyolytic, pyknotic and cells with nuclear bud in the buccal epithelial cells. The
present study is the first to examine the correlation between genetic damage and chewing of sadagura.

Betel quid is composed of areca nut flakes wrapped in a leaf of *Piper betle* with a dab of lime paste applied on it. The same may be consumed with or without tobacco. Sadagura is a form of smokeless tobacco often taken with betel quid in the Southern Assam region of India. It is also consumed without betel quid. The details of various constituents of the betel quid is given in Table 3. Sadagura is prepared mainly out of dried tobacco leaf (*Nicotiana tabacum*) with addition of various spices and flavouring agents. During preparation of sadagura the ratio of the spices added may vary. Usually fenugreek and aniseed are added in the proportion of 3g per 100g of tobacco.

In the present study, smokeless tobacco with or without betel quid, induced significant increase in the frequency of micronucleus as compared to the control (Table 5A), which was dependent upon the frequency of consumption of these products (Table 5B). Further, the micronucleus frequency among paan+smokeless tobacco chewers (1.13±0.00) was significantly (P< 0.001) higher as compared to the group consuming smokeless tobacco alone (0.83±0.05). The significant increase in the frequency of micronucleus in the buccal epithelial cells, in the groups where sadagura (smokeless tobacco) was one of the habits, indicates the genotoxic potential of sadagura (Table 5A, Figure 7). Other forms of smokeless tobacco have been reported to induce
genotoxicity (Adhvaryu et al., 1991; Das et al., 1992; Mahimkar et al., 2000). In the present study plain paan induced relatively higher percentage of micronucleus as compared to the control group, but was not statistically different. However, the micronucleus frequency among betel quid with sadagura chewers was significantly higher than the control group (Table 5A). The present findings further clarifies the role of tobacco as a genotoxic agent as reported in other studies (Adhvaryu et al., 1991; Das et al., 1992; Mahimkar et al., 2000). Significant difference in the micronucleus frequency between betel quid with sadagura chewers and only sadagura chewers may indicate an additive effect between sadagura and plain paan chewing.

Significant difference in the percentage of micronucleated cells were observed among habituates having multiple habits as compared to the groups consuming only sadagura or smoking (Table 5A, Figure 7) further indicates the synergistic effect between betel quid and tobacco in causing genotoxicity. However, contrary to expectations, there was no significant difference in the micronucleus frequency between betel quid with sadagura chewers and chewers with multiple habits (Table 5A, Figure 7). This may have resulted due to the fact that total tobacco uptake by multiple habit users may not be practically more than those consume only betel quid with sadagura, which may contribute for the lack of significant difference in the genotoxic parameters observed in the present study between the two groups. In other words, since smokers chewing betel quid with sadagura consume betel quid and smoke
5.1. Buccal cytome assay in habituates

mostly in alternate succession, hence the total number of betel quid cones consumed in a day by them is usually lower than the number of cones consumed by those who consume only betel quid with sadagura only.

In the present study, micronucleus frequency in plain paan chewers was not significantly different from control group (Table 5A, Figure 7). Similar finding was also reported in a cohort study in seven villages in Taiwan (Wu et al., 2004). Areca quid chewing is reported to be associated with micronucleus frequency (Stich et al., 1982); and lower quid intake per day in plain paan chewers in the present study is probably responsible for the low micronucleus frequency in this group. Younger chewers had higher incidence of micronucleus in some cases contrary to the expected "age effect". A similar observation was reported in alcoholic patients (Ramirez and Saldanha, 2002).

The nuclear bud provides a sensitive biomarker of DNA damage with a lower background level than the micronucleus index (Thomas et al., 2003). Nuclear buds probably arise as a result of gene amplification and indicate chromosome breakage/translocation (Thomas et al., 2003; 2007). The study of nuclear bud frequency along with micronucleus frequency increases the versatility of the cytome assay as a genotoxicity test because not only does it allow a measurement of gene amplification that was previously unavailable in micronucleus assay, but it is also possible to reliably distinguish between similar genotoxic agents by their differences in the nuclear bud to micronucleus ratio.
5.1. Buccal cytome assay in habituates

(Kausar, 2010). In the present study, the nuclear bud frequency in buccal epithelial cells exhibited a similar trend as the micronucleus frequency in all the habituate groups (Table 6) indicating the genotoxic properties of sadagura and betel quid along with sadagura (Figure 8).

Combustion of organic matters produces polycyclic aromatic hydrocarbons, some of which may be metabolize to form carcinogenic epoxides. Combustion of flavoring agents used in sadagura at the time of roasting may also produce hazards pyrolysis products. Thus, alternative tobacco products, which many believe to be safer, may in fact produce carcinogens other than those commonly found in tobacco (Hashibe et al., 2000). Some chemicals, which are not mutagenic based on Ames assay, become mutagenic when treated with nitrite. Nitrite is abundant in tobacco (Hashibe et al., 2000) and hence the nitrite present in sadagura may react with otherwise non-mutagenic dietary components in spices and covert them to mutagenic species.

In the present study, basal cell frequency among plain paan chewers (1.25 ± 0.05) was relatively higher than the control group (1.07 ± 0.05), but was not statistically significant (Table 7, Figure 9). However, among the groups using either smokeless tobacco alone or paan with smokeless tobacco, the frequency of basal cells was significantly higher as compared to the control group. Thomas et. al. (2007) studying with Alzheimer’s patients reported an increase in the frequency of basal cells in the buccal epithelia. They concluded that since
Alzheimer’s disease is characteristic of mainly aged people, the observed increase in the basal cell frequency is due to the thinning of the buccal mucosa thus bringing the basal cell layer to more superficial level and making these cells vulnerable to be peeled off at the time of cells sampling by scapping with swab (Thomas et. al., 2007). The increased frequency of basal cells in the present study among the tobacco chewers with or without betel quid (Figure 9) may indicate a thinning of the buccal mucosa as a result of physical damage due to repeated mastication, chemical damage to the integrity of the mucosal membrane, increased proliferation of basal cells or a combination of all these factors. In a recent report, Giri et. al. (2010) demonstrated that arecoline caused disruption of tightjunctional protein zona occluding-1 (ZO-1). Betel quid has been reported to stimulate mitosis during wound healing and exposes germinal basal cells to genotoxins (Balaram et al., 1995). It is important to note that the site within the oral cavity, where carcinomas develop, is the one where quid is habitually placed for a prolonged time (Muir, 1967). On the other hand, the lack of significant increase in the frequency of basal cells among the smokers as compared to the control group (Table 7, Figure 9), futher emphasizes the harmful effect of the chewing form of tobacco (smokeless tobacco) and/or areca nut to the buccal mucosa. The potential of smokeless tobacco to cause profound effect to the buccal mucosa as compared to other habits has also been reported (Reichart et al., 1996).
The significant increase in the frequency of binucleated cells among the betel quid chewers (Table 8, Figure 10) indicates cytokinesis defects in the exposed cells. Betel quid contains various alkaloids mainly arecoline, arecaidine, guvacoline, guvacine among others. Arecoline, the major alkaloid present in betel nut, have been reported to produces 11 metabolites in mouse during the process of metabolism (Giri et al., 2006). Areca nut ingredients and metabolites may cause cytotoxicity by deregulation of cell cycle control, GSH homeostasis, mitochondrial function or reactive oxygen species production (Chang et al., 2001). Tobacco specific alkaloids along with areca nut alkaloids may further potentiate the cytotoxic effects in betel quid chewers with or without tobacco.

The combination of karyorrhectic, karyolytic and pyknotic cells is considered to be potential cell death biomarkers within the buccal mucosa, in addition to micronucleus. This may be useful as possible diagnostic tool to identify individuals with a high risk of tobacco related tissue damage. Analyses of cell death parameters were significantly higher (P< 0.001) among the betel quid with sadagura chewers compared to the control group (Tables 9 – 11). Karyorrhectic cells have fragmented nuclear material and may represent the late stage of apoptosis. Percentage of karyorrhectic cells was significantly higher in paan with smokeless tobacco chewers (0.99 ± 0.01), smokeless tobacco (sadagura) chewers (0.86 ± 0.07) and chewers with multiple habits (1.33 ± 0.04) compared to control (0.10 ± 0.07) as well as plain paan chewers (0.10 ± 0.01) and smokers (0.26 ± 0.03) (Table 9, Figure 11).
Karyolytic cell percentage (Table 10, Figure 12) was also significantly elevated in these groups and multiple habit users had the highest frequency (3.37 ± 0.04) followed by paan+smokeless tobacco chewers (2.56 ± 0.02). The precise mechanism of pyknotic cell formation is not clear. However, it is considered to be an indication of cell death (Thomas et al., 2007).

Condensed chromatin cells probably represent an early stage in apoptosis and might give rise to pyknotic cells or condensed chromatin cells (Thomas et al., 2007). Incidence of pyknotic cells (Table 11, Figure 13) and condensed chromatin cells (Table 12, Figure 14) also showed an elevated trend compared to control group as the other cell death parameters. Interestingly, the incidence of karyorrhectic cells in smokers (Table 9) was significantly lower than the other groups of smokeless tobacco chewers which could be because of a possible difference in the mechanism of induction of cell death in smokers and smokeless tobacco (sadagura) users along with or without betel quid. This is a matter of interest since tumor growth depends on a balance between cell death via apoptosis and cell proliferation (Wyllie, 1985). Greater susceptibility to apoptosis may be a common feature of an increased risk to various tumor types (Kumar et al., 1998). Thus, the present findings provide further support to the findings that betel nut and tobacco chewers constitute high-risk group for development of oral malignancies (Stich et al., 1991).
It was observed that apart from the various markers discussed above, a considerable percentage of cells exhibited atypical nuclear morphology in the exfoliated buccal epithelial cells (Table 13, Figure 15). The percentage of such cells among smokeless tobacco chewers (0.62 ± 0.05), betel quid with smokeless tobacco users (1.00 ± 0.00) and users with multiple chewing habits (1.09 ± 0.01) was significantly different from control (0.20 ± 0.03) at p<0.001 and smokers (0.56 ± 0.10) at p<0.05. The nuclear shape abnormality percentage was relatively higher in plain paan chewers (0.32 ± 0.05) but was not statistically significant as compared to the control group. Further, the percentage of cells with abnormal nuclear shape among paan with smokeless tobacco chewers was higher and significantly different from the plain paan chewers (p<0.001) as well as from smokeless tobacco chewers (p<0.01) (Table 13, Figure 15). The percentage of cells with abnormal nuclear shape among smokeless tobacco chewers was also significantly higher from the plain paan chewers (p<0.001).

There are several studies on cell morphological abnormalities in other cell types such as germ cells which are regarded as an important endpoint for biomonitoring studies (Bonde et al., 1996). Aberrations in gross nuclear morphology, such as increase in nuclear size, changes in nuclear shape, and loss of nuclear domains, are often used to identify cancerous tissue (Zink et al., 2004). Therefore, an increase in the incidence of cells with altered nuclear shape may indicate predisposition to increased cancer risk. The exact mechanism of the formation of altered nuclear shape in human buccal epithelial cells is yet to be clearly
understood. However, in many other cell types, altered nuclear shape is due to changes in the nuclear lamina and in some cases by forces that act from the cytoplasm has been reported (Webster et al., 2009). The functional relationship between altered nuclear shape and cellular transformation, or even the underlying cause of altered nuclear morphology, is often not known; although it has been speculated that changes in nuclear shape lead to changes in chromosome organization, which in turn can affect gene expression (He et al., 2008). Higher incidence of cells with abnormal nuclear morphology in groups where tobacco is a constituent as observed in the present study indicates a probable role of tobacco constituents in causing atypical nuclear changes.

Therefore, on the basis of the present analysis and those reported earlier (IARC, 2004; Garewal et al., 2002) it is likely that tobacco constituents play a major role in inducing the various cytogenetic changes in the buccal epithelial cells of habituates and betel quid ingredients may work synergistically. The level of damage was highest in users with multiple habits and betel quid with smokeless tobacco users (Table 13, Figure 15).

The risk of oral cancer from long-term smokeless tobacco use has been attributed to the presence of tobacco specific nitrosamines. Higher concentrations occur during curing when amine alkaloids in the tobacco leaf react with nitrous oxides, which are combustion byproducts of fire-curing (Peele et al., 2001). Pulverized products are processed with little control over fermentation and
curing, which may increases the production of tobacco specific nitrosamines (Brunnemann et al., 1985). Fire curing is a part of sadagura preparation, which may also increase its nitrosamine concentration. Although studies done so far on American and Swedish snuff show the presence of tobacco specific nitrosamines in trace amounts (Brunnemann et al., 1985), no information is available on sadagura, which needs further biochemical investigation. Most tobacco preparations do not involve any refining steps during manufacturing process; hence the probability of presence of contaminants is high in these products. No information exists regarding the nature, absorption and metabolism of such contaminants contaminants of smokeless tobacco. Further, in case of other smokeless tobacco use, higher exposure of a very limited mucosal surface occurs as the users habitually place these products repeatedly in the same location each time they consume and the process continues for years (Rodu and Jansson, 2004). However, in case of sadagura, which is a chewing form of tobacco, the entire oral cavity is exposed. Lime taken with the tobacco may stimulate the clastogenic process by creating an alkaline environment in the target site and is likely to generate free radicals in combination with the polyphenols (Nair et al., 1992). The present findings indicate that sadagura is as an important risk factor for induction of genotoxicity as well as cytotoxicity in the exposed individuals. Apart from smokeless tobacco, smoking has been implicated in oral and lung cancer risk and also induction of micronucleus in the buccal mucosal cells (Celik et al., 2006).
However, others have shown lack of correlation between smoking and micronucleus frequency (Nersesyan et al., 2006). In the present study a positive correlation between smoking and micronucleus as well as incidence of nuclear bud in the buccal epithelial cells was observed. Smokeless tobacco is suggested to be a safer version of tobacco intake (Rodu and Jansson, 2004). In India, studies have shown association between use of smokeless tobacco and increased risk of oral cancer (Critchley and Unal, 2003). Results of the present study indicate that sadagura, which is a unique smokeless tobacco preparation, is as harmful in inducing genotoxicity as any other smokeless tobacco preparation, and most harmful when taken along with betel quid. Further, a cytome approach using degenerative nuclear changes in buccal cells and micronucleus can provide valuable information on the cytogenetic damages in buccal epithelial cells while evaluating potential genotoxic agents.

**5.2. Buccal Cytome Assay in Precancerous Lesions and Conditions**

The four layers of the buccal mucosa such as the stratum corneum or keratinised layer, the stratum granulosum or granular cell layer, the stratum spinosum or prickle cell layer containing populations of both differentiated, apoptotic and necrotic cells, and the rete pegs or stratum germinativum, containing actively dividing basal cells which produce cells that differentiate and maintain the profile, structure and integrity of the buccal mucosa (Veiro and
In preneoplastic stages, major mucosal alteration takes place in the epithelium in varying degree according to the severity of dysplasia (Mehrotra et al., 2006).

The oral mucosa is exposed to chronic or recurrent, mechanical, thermal, and chemical trauma. These lead to various reactive mucosal hyperplasias which vary in clinical appearance depending on location (Krahl et al., 2008). Most human cancers are epithelial in origin, about 92% being derived from the external and internal epithelium, i.e., the skin, the bronchial epithelium and the epithelia lining the alimentary canal (Picker and Fox., 1996). Effective techniques have not yet been developed for making direct chromosome preparations from epithelial tissues. However, unstable chromosome aberrations can be studied in epithelial cells by the detection of micronucleus and other nuclear aberrations in exfoliated cells (Picker and Fox., 1996).

The importance of cytological investigations has been recently emphasized in a multicenter study where nearly 5% of clinically benign-appearing mucosal lesions were sampled by this technique and later confirmed by typical scalpel biopsy to represent dysplastic epithelial changes or invasive cancer (Ahmed et al., 2003).

One of the objectives of the present study was to assess the correlation between DNA damage and premalignant disorders of the oral cavity. The precancerous conditions studied in the present investigation are erythroplakia,
leukoplakia, erythroleukoplakia and oral submucous fibrosis. The details of these conditions have been provided in section 2.4.4 at page 39. The photographs and histological details are given in Figure 5 at page 84.

The micronucleus percentage was found to be significantly (P< 0.001) elevated in leukoplakia (2.67±0.13), erythroplakia (2.71±0.18) and erythroleukoplakia (3.38 ±0.18) groups as compared to the control (0.17±0.01) group (Table 15 and Figure 17). This is an indication that subjects with precancerous lesions have increased levels of DNA damage. Elevated micronucleus formation has also been observed in precancerous lesions in the oral cavity of chewers in other studies (Stitch et al., 1991; Hashibe et al. 2000). Malignancy is considered as a process caused by the accumulation of multiple genetic alterations, which affect the cell cycle as well as normal cell differentiation process. These genetic alterations which occur during carcinogenesis are mainly acquired (somatic) although some of them may be inherited. Thus, an increase frequency in DNA damage markers like micronucleus indicates a greater risk for malignant transformation. It has been suggested that DNA damage markers can be used as tools for detecting tumour cells in clinical samples (Ogden et al., 1991). Significant differences in micronucleus frequency between erythroleukoplakia and leukoplakia patients (P< 0.001) observed in the present study (Table 15) could be due to difference in the pathways and target DNA lesions. Similar suggestions have been made by other workers (Barnes et al., 2005; Reichart and Philipsen, 2005). Further, micronucleus frequency in patients with oral
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Submucous fibrosis was significantly ($P<0.001$) higher from control (Table 15). Similar observation was reported in other studies also (Yang et al., 2001; Lee et al., 2003). Significant difference in the micronucleus percentage between patients with oral submucous fibrosis and patients with leukoplakia and erythroleukoplakia was observed in the present study. Although all these precancerous lesions and condition have high risk of malignancy, there might be a probable disparity in the extent of DNA damage load and differences in the mechanism which bring about changes in the buccal mucosa in subjects with these conditions. Epigenetic changes and chemical alterations in homeostasis that may be mediated via tissue necrosis, apoptosis, or cellular turnover have been found to indirectly lead to the expression of mutations in DNA involved in the process of carcinogenesis (Barrett, 1992).

Nuclear buds percentage was found to be significantly higher compared to control in all the groups representing different precancerous lesions (Table 16, Figure 18). In a study with alcoholics, Ramirez and Saldanha (2002), observed higher frequency of cells with nuclear buds in the oral epithelial cells and suggested that this could be due to a lower repair index and high cytogenetic damage. The present findings may also arise out of similar phenomena. It was interesting to note that nuclear bud percentage in subjects did not vary significantly when compared between the groups unlike micronucleus percentage which was significantly different between patients with leukoplakia, erythroplakia, erythroleukoplakia and oral submucous fibrosis. This observation suggests that
nuclear buds and micronuclei have partly different mechanistic origin. The present findings are supported by the work on nuclear buds using fluorescence in situ hybridization with pancentromeric and pantelomeric DNA probes where it was reported that interstitial DNA without centromere or telomere label was clearly more prevalent in nuclear buds (43%) than in micronuclei (13%). DNA with only telomere label or with both centromere and telomere label was more frequent in micronuclei (62% and 22%, respectively) than in nuclear buds (44% and 10%, respectively (Lindberg et al., 2007). Most nuclear buds are suggested to originate from interstitial or terminal acentric fragments, possibly representing nuclear membrane entrapment of DNA that has been left in cytoplasm after nuclear division or excess DNA that is being extruded from the nucleus unlike micronuclei which are believed to be primarily derived from lagging chromosomes and terminal acentric fragments during mitosis (Thomas et al., 2007; Lindberg et al., 2007).

It was observed that basal cell frequency significantly (P< 0.001) increased in subjects with leukoplakia (2.94±0.59) as compared to the control (1.07±0.05) group (Table 17, Figure 19). Percentage of basal cells was significantly (P< 0.001) elevated in erythroplakia (2.10±0.07), erythroleukoplakia (2.66±0.13) and oral submucous fibrosis (3.01±0.32) conditions also. Basal cells differentiate to form mature buccal mucosal cells and an alteration in the cell turn over rate indicates an altered condition in cell proliferation kinetics and cell cycle, which is hall mark of carcinogenesis (Perrotte et al., 1999). Thus, a
higher incidence of basal cells presently observed indicates a higher risk for malignancy (Table 17, Figure 19). Increase in incidence of basal cells in oral submucous fibrosis may also be due to reduction in the thickness of the epithelial mucosa as a result of atrophy as seen in other diseases (Thomas et al., 2007). Since a very limited number of reports have so far been published on basal cell frequency in exfoliated buccal epithelial cells in patients with precancerous lesions and conditions, the present findings are important for further research in the field of identification of biomarkers of precancer lesions and conditions and possibly cancerous lesions also.

Binucleated cells are regarded as biomarkers of disturbances or damage in cell cycle checkpoint kinetics (Fenech et al., 1999). In the present study a higher incidence of binucleated cells is seen in subjects with erythroleukoplakia leukoplakia and erythroplakia as well as oral submucous fibrosis was observed as compared to the control group (Table 18, Figure 20). Similar findings have also been reported in a study by Garewal et al. (2004). Increase of binucleated cells increases the probability of possible presence of dysplasia and has a high risk of malignant transformation (Pindborg, 1980; Ramirez and Saldanha, 2002).

There was a very high increase in karyorrhetic (Table 19, Figure 21), karolytic (Table 20, Figure 22); pyknotic (Table 21, Figure 23) and condensed chromatin cells (Table 22, Figure 24) among the groups with various precancerous lesions and conditions as compared to control group. Although the precise
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The mechanism of formation of these cells is yet to be elucidated, studies have revealed that they represent early and late stages of cell death via apoptotic and necrotic pathways (Thomas et al., 2007; Fenech et al., 1999). The present findings suggest that the genotoxic change in buccal cells in these preneoplastic stages induce cytotoxic effects by increasing cell death. Inhibition of apoptosis may allow cells that have undergone a significant level of DNA damage to proceed through the cell cycle and survive as mutated cell (Fenech et al., 1999). Accumulation of mutations in genes related to cell cycle checkpoint control and apoptosis could lead to transformation from premalignancy to malignancy in patients (Woodburn, 1999). Moreover, it is interesting to observe that the incidence of all the four cell death markers showed a distinct pattern in each disorder (leukoplakia, erythroplakia, erythroleukoplakia and oral submucous fibrosis) (Figure 19-22) and in most cases were significantly different indicating mechanistic differences in the pathogenesis and probable pathway to carcinogenesis. It also justifies the status of erythroleukoplakia as a separate identity from leukoplakia and erythroplakia (Wamakulasuriya et al., 2007).

It was observed that the percentage of cells with abnormal nucleus shape increased significantly (P< 0.001) in buccal epithelial cells of study subjects and the incidence was highest (2.03±0.14) in subjects with erythroleukoplakia (Table 23, Figure 25). Increase in abnormal shaped nucleus was also reported in patients with leukoplakia by Garewal et al. (2002). However, details of the different shapes observed was not mentioned in that study. In the present
study the various shapes observed included beak shaped, elongated kidney-bean shaped, triangular, lobed and amorphous (Figure 4c). Alteration in nuclear shape in buccal cells of oral cancer patients has been reported in several studies although shapes were not discussed in detail (Mehrotra et al., 2006). Various morphological abnormalities were demonstrated in normal and malignant oral cells before and after radiation and it was found that a consistent significant increase with radiation dose (Mehrotra et al., 2004). Presence of large number of keratohyalin granules in human oral leukoplakia have also been observed in other studies (Singh et al., 2008; Nersesyan, 2002).

The risk factor is an agent, attribute or behavior which forms a part of the casual chain of the disease. Analysis of chewing habits, socioeconomic background and lifestyle habits of subjects with precancerous lesions and conditions in the present study were done to deduce probable etiology and risk factors. Incidence of tobacco related cancer is very high in the North-East region of India (ICMR Cancer Registry, 2006-2007; 2007-2008) in which the study area is a part (Figure 2). In a numerical comparison among males and females, it was found that male subjects were more vulnerable to oral precancerous lesions than the female subjects the ratio being 154:91. This could be due to the fact that males are more habituated to smoking, chewing betel quid and tobacco. In fact, it is found that all the subjects in the present study cohort were either habituates of chewing paan with smokeless tobacco,
smokers or multiple habit users (Figure 16A-D). Majority of the patients had multiple chewing habits.

Sadagura is a form of smokeless tobacco which is homemade and also sold in small paan shops and widely consumed in southern Assam. It is chewed with or without betel quid with a dab of lime paste. Thus, tobacco chewed or smoked or both along with betel quid chewing may act synergistically. Synergistic effects of betel chewing/ tobacco use have been reported by Lee et al. (2003). In case of oral submucous fibrosis, betel quid chewing along with sadagura (smokeless tobacco) was found to be practiced by a large number of patients. Betel quid chewing has been reported to be the principal etiological factor for oral leukoplakia, erythroplakia and oral submucous fibrosis in a cohort study in Vietnam (Reichart and Nguyen, 2008). Incidence of precancerous lesions among habituates chewing plain paan in the present study population is lower compared to South-East Asian countries. This is probably because most habituates with higher quid intake per day chew betel quid along with sadagura in this region unlike these countries and frequency of in take per day is very low in plain pan chewers.

Subjects whoever chewed areca nut experienced a upto 11-fold risk of these precancerous conditions. The risks increased with the duration and frequency of the habit, as previously shown in Pakistan, India, Taiwan and Mainland China (Mehta et al, 1981; Maher et al, 1994; Tang et al, 1997; Shiu et
al, 2000). In Southern Assam regular betel quid chewers with a higher number of quid intake per day usually use smokeless tobacco (sadagura) as an ingredient for areca nut products (called 'paan'). The relative risk of oral cancer for betel quid chewing with tobacco has been reported to be notably higher than that for betel quid chewing without tobacco, and the evidence for leukoplakia was also in the same direction (Gupta et al, 1982). Studies in betel quid chewers from Taiwan showed that nonsmokers and nondrinkers who chewed betel quid, had respectively, a 10.0–15.6- and 26.5–39.3-fold significant risk of leukoplakia and oral submucous fibrosis respectively and both risks were lower than that reported for tobacco contained areca nut products (OR 17.4 and 44.1 for leukoplakia and oral submucous fibrosis, respectively) (IARC, 1985; Hashibe et al, 2000). Cessation of areca nut chewing has been associated with a regression in the incidence of leukoplakia (Gupta et al, 1995). The difference in risks between areca nut with and without tobacco implies that tobacco could have an additional effect on occurrence of leukoplakia and oral submucous fibrosis. Large scale case control studies showed that the risk of oral submucous fibrosis at each exposure level of betel quid chewing was stronger than those of leukoplakia, although the difference was not large enough to reject the null. (Hashibe et al, 2000, 2002). In the present study, it was also found that mainly younger patients had oral submucous fibrosis compared with comparatively older patients with leukoplakia and erythroplakia. The fact that most oral submucous fibrosis patients started betel quid chewing at a younger age than
leukoplakia and erythroplakia patients may partly explain the age differences between the two diseases.

While the associations between tobacco smoking (bidi and cigarette) and oral premalignant diseases have not been definitely established, comparable findings were observed in India and Europe (Banoczy et al, 2001; Hashibe et al, 2000, 2002). In the present study a significant increase in precancer lesions of smoking among subjects who did chewed even lower numbers of betel quid has been noted.

The areca nut, which contains alkaloids, such as arecoline, and other chemicals, such as catechin and tannin, plays a major role by stimulating production of collagen fibres and making them less susceptible to the action of collagenase. It is suggested that components of the areca nut also affect gene expression in the fibroblasts leading to the production of greater amounts of normal collagen. Areca nut has been shown to have a high copper content, and chewing areca nuts for 5-30 minutes significantly increases soluble copper levels in oral fluids. This increased level of soluble copper supports the hypothesis that copper acts as an initiating factor in oral submucous fibrosis by stimulating fibrogenesis through up regulation of lysyl oxidase activity. Malnutrition is implicated in the pathogenesis of oral submucous fibrosis leading to deranged repair processes of the inflamed oral mucosa, contributing to defective healing and scarring. The resulting atrophic oral mucosa is more
susceptible to the effects of areca nut. An immunologic process and a genetic component are assumed to be involved because of reported cases in non-Areca users (Sinor et al., 1990). However, all patients with oral submucous fibrosis in our study were betel quid users.

Tobacco, whether chewed or smoked, causes both promotion and initiation of cancer in the oral cavity. Atleast 300 carcinogens have been identified in tobacco that may leach into saliva. Thus, gravity dependent regions account for cancers of the floor of mouth, ventral and lateral surface of tongue. The major carcinogens are are hydrocarbon benza (a) pyrene and tobacco specific nitrosamines (TSN), N-nitrosonor nicotine,N'-nitrosopoyrrolidine (NPYR), N'nitrosodimethlamine (NDMA) and 4′-methyl nitrosamo-1-(3-pyridyl)-1butanone (NNK). The exact mechanism of the carcinogenic agents is unclear. However, the carcinogenic agents act locally on keratinocyte stem cells and act on other tissues of the body. They produce DNA adducts, principally 0-6-methyl guanine and these interfere with accuracy of DNA replication, leading to mutations which thus contribute to the molecular chain of events leading to preneoplastic, and ultimately, malignant transformation of a cell and its clonal derivatives. There is damage to all replicating cells, including those involved in immune response. In users with multiple habits the buccal epithelial cells remain exposed to both Areca alkaloids, tannins as well as tobacco derived nitrosamines, thereby, causing genotoxic and cytotoxic damage to structural profile and integrity of buccal epithelial mucosa which might get manifested in the form of premalignant
disorders (Liu, 1997). However, it is reported that inherited differences and genetically determined host factors such as impact of GSTM1-null genotype play a crucial role in the effectiveness of detoxification/activation of carcinogens (Alexandrov, 2002).

5.3. Buccal Cytome Assay in Oral Squamous Cell Carcinoma

Major mucosal alteration takes place in the structural profile of buccal epithelium in varying degree in patients with oral squamous cell carcinoma. Squamous cell carcinoma manifests islands or cords of malignant epithelial cells in the underlying connective tissue with loss of desmosomal attachment. The aberrant malignant cells disrupt the basement membrane zone. In the verrucous form, the epithelium has prominent bulous elongated rete pegs that have a pushing front rather than an infiltrating quality. The rete pegs may be long, pointed, blunted or short. The epithelial cells are large with abundant cytoplasm and show frequent keratinisation (Shear and Pindborg, 1980; Mehta et al., 1969).

Although there is considerable debate regarding how a cell progresses to malignancy, it has been suggested that, besides normal mutation rates and possible clonal expansion of these mutations, three main sources of error can affect genomic stability in human cancers are nucleotide excision repair
instability, microsatellite instability, and chromosomal instability. Loeb and colleagues refer to these terms collectively as a 'mutator phenotype', in which a mutation in any of the genes responsible for maintaining DNA fidelity through replication, repair, chromosome segregation, damage surveillance, or apoptosis may be responsible for human tumor formation and progression (Loeb et al., 2003).

The cytological study of oral cavity cells is simple and rapid, non-invasive and hence well accepted by patients and suitable for routine application in population screening programmes, for early analysis of a wide range of suspect lesions (Ahmed et al., 2003). It also aids to uncover similar type lesions that were not clinically suspicious for carcinoma or preinvasive disease. Yet controversies exist related to the real value of traditionally used cytological technique in the early detection of oral squamous cell carcinoma. The existence of false positives has been pointed out showing high sensitivity and low specificity (Rick and Slater, 2003). Hence, new methods are being developed to improvise over the existing limitations and various comparative studies have shown that it can offer significant advantages over conventional exfoliative cytology (Mehrotra et al., 2004). The use of exfoliated cells for micronucleus assays has become well established in epidemiological studies aimed at defining genotoxic effects on target tissue following chronic exposure to genotoxic and cytotoxic agents (Chakraborty et al. 2006; Smith et al. 1993). Micronucleus assay is more rapid, economic, and at least as sensitive an
The cytome assay is a comprehensive study of the buccal mucosa that aims to assess micronucleus and other parameters of genome damage, cell proliferation and cell death in the buccal mucosa and to be used to identify potential biomarkers that are associated with individuals who have just been clinically diagnosed with Alzheimer’s disease and Down’s syndrome (Thomas et al., 2007). In the present study the buccal cytome assay was used on exfoliated buccal epithelial cells of patients who been clinically diagnosed with oral squamous cell carcinoma. The cytome assay is a prime candidate for use as an intermediate marker of chemoprevention because this assay measures events highly relevant to carcinogenesis and in the target tissue in a non-invasive method.

The present study is the first to identify potential biomarkers that are associated with individuals who have just been clinically diagnosed with oral squamous cell carcinoma and could be used to identify those at risk of developing oral squamous cell carcinoma.

The results from the buccal cytome assay suggest significant changes within the buccal cytome profile of oral squamous cell carcinoma patients when compared to the control cohort (Table 25, Figure 30). Oral squamous cell carcinoma patients were histopathologically confirmed by endoscopy and tissue
biopsy (Figure 5D). A high incidence of micronucleated cells (2.9±0.10) was observed in exfoliated buccal epithelial cells of patients with oral squamous cell carcinoma (Table 25, Figure 30) as compared to control (0.17±0.01). Similar finding have also been reported (Ramirez and Saldanha, 2002). In studies with micronucleus analysis in exfoliated buccal epithelial cells of primary cancer patients with breast cancer, uterine and cervix cancers, higher frequency of micronucleus have been observed (Nersesyan, 2002). In other words, in cases of cancers in sites other than the oral cavity, genetic instability in somatic cells may be observed in buccal epithelial cells reflected in terms of increased micronucleus frequency. Therefore, analysis of micronucleus and other cytome assay parameters in exfoliated buccal epithelial may likely to serve as possible markers of precancerous lesions and conditions even in sites other than the oral cavity.

**Micronuclei** are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Majer et al., 2001). There was a significantly higher incidence of micronucleated cells in the buccal epithelial cells from patients with oral squamous cell carcinoma compared to control (Table 25). Genetic instability is thought to play a key role in the multistep process of tumorigenesis and micronucleus serves as an internal dosimeter of the process. Chromosomal
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instability often leads to aberrant cellular phenotype with aberrant chromosome number (aneuploidy). Premature anaphase promotion result in mitotic abnormalities in oral squamous cell carcinoma cell line. Defects in spindle assembly check point are believed to be responsible for the high rate of aneuploidization during tumourigenesis (Mondal et al., 2007). A higher incidence of micronucleated cells observed in patients with oral squamous cell carcinoma directly reflects genomic damage caused in the target cells.

A higher incidence of cells with nuclear bud (1.10±0.11) was observed (Table 25) compared to control (0.04±0.01). Nuclear bud formation arises as a result of gene amplification (Thomas et al., 2007) and are suggested to originate from interstitial or terminal acentric fragments (Lindberg et al., 2007). Gene amplification is a common event in tumors and has been observed both in vivo and in vitro (Tisty et al., 1995). Moreover, the differences in individual micronucleus and nuclear bud frequency can be partially attributed to oral squamous cell carcinoma stage and chewing and tobacco smoking habit of patients (Ramirez and Saldanha, 2002; Rapidis et al., 1976).

There was a significant (P< 0.001) increase in binucleated cells (1.84±0.20) as compared to the control (0.17±0.02), which is a characteristic of oral squamous cell carcinoma (Table 25). Cells with multiple nuclei were also observed but were not accounted as they were not observed in the other study groups in our investigation. Binucleated cells arise due to alterations in genes
governing cell-cycle control which provide a green light for continued proliferation of defective cells (Thomas et al., 2003). Lengauer and colleagues (1998) described four main types of genes which, when altered, promote tumor progression: oncogenes, tumor suppressor genes, DNA repair genes, and those regulating programmed cell deaths (apoptosis). Tumors displaying complex chromosomal aberrations often contain increased copy numbers of oncogenes known to promote cell differentiation and proliferation, while simultaneously accumulating deletions or loss of genes responsible for detecting DNA damage, halting cell-cycle progression, and/or mediating DNA repair (tumor suppressor genes) in cells prior to replication and/or cell division (Knuutila et al., 1999).

The basal cell frequency (4.22 ± 0.11) in oral precancerous subjects was markedly higher than the control (1.07 ± 0.04) group and was significant at P<0.001 (Table 25, Figure 30). Several studies have reported an increase in basal cell and basal cell nuclear size as the disorder progresses from normal, to dysplasia, to neoplasia (Rich et al., 1991). Basal cells are the target for agents that cause alterations in epithelial cell differentiation and changes in these cells may alter future cell behaviour, including the development of neoplasia (Potten and Morris, 1988). The increase in the number of basal cells could be due to loss of epithelial integrity as well as malfunction of cell cycle checkpoint (Brandwein-Gensler et al., 2005). Recent investigations have described the presence of mitotic malfunction in tumor cells (Gisselsson, 2003). Karyotypic heterogeneity visualized by interphase cytogenetic analyses suggests that
cytoskeletal defects may promote both clonal and non-clonal structural rearrangements in solid tumors, resulting in daughter cells that do not resemble either each other or their mother cell (reviewed in Pihan and Doxsey, 1999; Reshmi et al., 2004). The mitotic machinery includes microtubules, centrosomes, kinetochores, and molecular motors. Properly choreographed coordination of these structures is essential for accurate chromosome segregation during mitosis and gets affected after onset of carcinogenesis process (Reshmi and Gollin, 2005).

The percentage of karyolytic cells (5.67 ± 1.26) was very high (Table 25, Figure 30) as compared to the control group (0.37 ± 0.19). Similar observations have been made in oral cancer patients (Ramirez and Saldanha, 2002). Increased frequencies of karyolytic cells occur in the pre-keratinisation process, which represents an adaptive response to cellular injuries (Pindborg et al., 1980). This anomaly is also evident in necrotic cells (Wyllie, 1985) and is related to cytotoxicity (Tolbert et al., 1991). Karyolytic cell frequency is also increased due to constant action of mutagens such as tobacco and betel quid (Tolbert et al., 1991).

There was a very high increase in karyorrhectic cells (3.97 ± 0.33) and extensive karyorrhectic debris was observed in the buccal epithelial cells (Table 25, Figure 30). A significant increase (P< 0.001) in incidence of pyknotic cells and condensed chromatin cells in patients as compared to control were also observed (Table 25, Figure 30) indicating a high incidence of cell death and
apoptosis. Although the precise mechanism of formation of cells with condensed chromatin is yet to be elucidated, studies have suggested that they represent early and late stages of apoptosis (Thomas et al., 2007; Fenech et al., 1999). Analysis of cell death parameters among oral squamous cell carcinoma patients is very important as tumor cells undergo apoptosis when cytokines become limited and tumor progression depends on the balance between apoptosis and proliferation which is mediated by different families of proteins (Korsmeyer et al., 1993).

There was a significant increase (P< 0.001) in cells with abnormal nuclear shape (2.63 ± 0.14) compared to control (0.20 ± 0.03) group (Table25, Figure 30). There are characteristic differences in the nuclear architectures of cancer cells, compared with normal cells, and some anticancer treatments restore normal nuclear structure and function. In the present study a higher occurrence of lobed and amorphous shaped nucleus with increasing degree of dysplasia was observed. The exact molecular mechanism for the formation of different nuclear shape in the buccal epithelial cells remains an area to be explored. Development of extremely lobulated nuclei in neutrophils is associated with loss of lamin A/C141 and expression of lamin B receptor (Hoffmann et al., 2007). Nuclear shape can be affected by lipid synthesis (Golden et al., 2009). In the Drosophila embryo a change in nuclear shape from spherical to ellipsoid has been found to be dependent on both cytoplasmic microtubules and an inner nuclear membrane protein called kugelkern or charleston (Brandt et al., 2006).
Cells with abnormally shaped nuclei are often seen in diseases in which lamina proteins are mutated (collectively called laminopathies) (Capell and Collins, 2006). The different altered shape of nucleus observed in the present study (Figure 4c) could be formed by a mechanism similar to one of the mentioned above mechanism or each shape could be formed by specific mechanistic pathway different from one another. Moreover, nuclear:cytoplasmic ratio and nuclear size is important for cell function. Disturbance of this ratio is associated with certain types of cancers (Slater et al., 2005; Zink et al., 2004), suggests that the ratio between nuclear and cytoplasmic volumes is very critical for maintaining cell integrity. Multiple nuclei within single cells are also observed in the present study. Cancer cells have been known to contain multiple nuclei (Norppa and Falck, 2003). Multiple nuclei formation might be triggered by environmental factors, exposure to genotoxic chemicals (Norppa and Falck, 2003) or depletion of factors required for chromosome segregation and congression to the metaphase plate (Salina et al., 2003). Increase in the incidence of nuclear shape abnormality in buccal cells might also be associated with the severity of dysplasia. Advances in understanding nuclear structure have revealed insights into the process of malignant transformation and hence the nuclear shape abnormality as an end point provide a basis for the development of new diagnostic tool (Zink et al., 2004).

Oral squamous cell carcinoma arise through an accumulation of genetic alterations, including chromosomal alterations, DNA changes (e.g., mutations,
amplifications, or deletions), and/or epigenetic alterations, such as changes in methylation that affect genetic regulation. These events are further influenced by exposure to environmental agents, including tobacco smoke, alcoholic beverages, and viruses, such as human papillomavirus (Forastiere et al., 2001). In the present study all the individuals with oral squamous cell carcinoma were addicted to betel quid chewing or were smokeless tobacco users and majority chewed betel quid with smokeless tobacco (chiefly the locally made sadagura) (Figure 16). In some of the cases in the study, lesions developed at the site in the oral groove where the betel quid or tobacco quid was placed habitually. It is hypothesize that the true effects of tobacco chewing along with betel quid were confounded by smokers increasing the risk of occurrence of oral squamous cell carcinoma and habituates with multiple habits have the highest risk of developing oral squamous cell carcinoma. Multiplicative interaction was observed between chewing and poor oral hygiene in oral cancer patients (Balaram et al., 1995).

5.4. Buccal Cytome Assay in Occupational Exposure Conditions

In the present study, genotoxic and cytotoxic damage in two major occupational group of people in this region were investigated. It is important to conduct such study because different type of occupational exposure have been reported to induce genotoxic damage and pose a potential risk factor to exposed
individuals inducing different health hazards including initiation of different types of cancer (Celik et al., 2003; Viel and Challier, 1995). Therefore, in the present study the frequency of micronuclei and other nuclear parameters in exfoliated buccal epithelial cells among the tea garden workers and farmers cultivating mainly rice and Ravi crops during winter with betel quid and tobacco chewing habit was investigated.

The frequency of micronucleus and other cytome parameters analyzed in the present study have been summerised in Table 26-34 and Figure 31-39. Significant increase in the frequency of all the cytome assy parameters as compared to the respective control values in the female workers groups of tea industry indicate that this population is more vulnerable towards chromosomal damages, cell proliferation damages as well as towards events leading to apoptosis. Higher prevalence of underweight individuals was recorded among the tea plantation workers and in farmers.

Micronuclei are chromosomal fragments or the whole chromosomes that are not included in to the daughter nuclei during cell division and are incorporated as a much smaller nucleus. The formation of micronucleus is therefore induced by substances that cause breakage of chromosomes (clastogens) as well as by agents which affect the spindle apparatus (aneugens) (Ghosh et al., 2008). The present findings show increased trend of micronucleated cells among tea garden workers and farmers (Table 26) which
was significantly different from control (p<0.001) and micronucleus frequency was significantly higher in tea plantation workers compared to farmers (Figure 31). Cytogenetic biomonitoring studies among agricultural workers, forestry workers, floriculturists, vineyard cultivators, cotton field workers show significant incidence of cytogenetic damage such as chromosome aberrations, sister chromatid exchanges and micronuclei. Some of these studies have reported much higher frequencies than observed in the present study (Pastor et al., 2002). This could be due to different exposure conditions, crop types and environmental factors (Bolognesi et al., 1997) and lower usage usage of pesticides in this region.

The nuclear bud provides a sensitive biomarker of DNA damage with a lower background level than the micronucleus index (Thomas et al., 2003). The nuclear bud frequency observed in the present study (Table 27, Figure 32) exhibited similar trend as that of micronucleus frequency in both the groups indicating the genotoxic damage. Since farmers in this region are not extensively exposed to pesticides all the year round and hence nuclear bud incidence is lower in our study group than reported in other studies (Falck et al., 1999; Carbonell et al., 1993; Carbonell et al., 1995).

The basal cell percentage of tea plantation workers (Table 28, Figure 33) was higher from control but not significant. The basal cell percentage of farmers was significantly higher from control (p<0.001) and tea plantation workers
The basal cell frequency is however more likely due to chewing habit practiced by the farmers which affect the mucosal lining.

Binucleated cell percentage (Table 29, Figure 34) of tea plantation workers and farmers was significantly higher from control (p<0.001). There are very few reports on studies of binucleated cells in farmers (Padmavathi et al., 2000) and scarce if any on tea plantation workers. However the trend is similar to that observed in habitual chewers of betel quid with smokeless tobacco. Areca nut ingredients and metabolites may cause cytotoxicity by deregulation of cell cycle control as binucleated cells are believed to be formed due to defects in cell cycle arrest checkpoints (IARC, 2004; Fenech et al., 1999).

The karyorrhectic cells in buccal epithelial cells (Table 30, Figure 35) of tea plantation workers and farmers was significantly higher from control (p<0.001 and p<0.01 respectively. Karyorrhectic cells have fragmented nucleus and may represent the late stage of apoptosis (Thomas et al., 2007). This is probably due to chewing habit as we found similar result in our study on habituates with different chewing habits.

The karyolytic cell incidence (Table 31, Figure 36) of tea plantation workers and farmers was significantly higher from control (p<0.001 and p<0.01 respectively) and karyolytic cell incidence of tea plantation workers was significantly different from that observed in farmers (p<0.05). Karyolytic cells have a ghost-like image of the nucleus and is a biomarker of cell death.

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The increase in incidence of karyolytic cells is probably due to chewing habit as similar result was found in the study on habituates with different chewing habits and pesticide exposure in this population is not sufficient to induce cell death as has been observed by Gomez-Arroyo et al. (1993) where the level of pesticide exposure was much higher.

The pyknotic cell incidence (Table 32, Figure 37) of tea plantation workers and farmers was significantly higher from control (p<0.001) and (p<0.001) respectively and percentage of tea plantation workers was significantly different from of farmers (p<0.01). Pyknotic cells are considered to be indicators of cell death. Thus, genotoxic damage in buccal cells induced a cytotoxic effect by increasing cell death. This is a protective mechanism to repair DNA damage. There is good evidence, in particular, that the trends in cell death can be linked to changes in DNA damage (Mitra and Kaina, 1993).

The condensed chromatin cells percentage (Table 33, Figure 38) of tea plantation workers and farmers was significantly higher from control (p<0.001) and (p<0.001) respectively. Recent studies suggest that open euchromatin structures are more deformable than tightly packed heterochromatin structures and so external or intracellular forces could reorganize gene rich areas relatively easily (Loden and van Steensel, 2005). Several specific proteins and characteristic histone modifications present in heterochromatin are responsible
for silencing genes (Pajerowski et al., 2007). Thus, the elevated incidence of condensed chromatin cells might lead to genome instability and high rate of toxicity (Fenech et al., 1999).

We observed a higher incidence of altered nuclear shape morphology in exfoliated buccal epithelial cells (Table 34, Figure 39). Nuclear architecture - the spatial arrangement of chromosomes and other nuclear components - provides a framework for organizing and regulating the diverse functional processes within the nucleus (Zink et al., 2004) and hence it is useful as a parameter to study in addition to those mentioned in the cytome assay (Thomas et al., 2007). Studies on nuclear shape morphology in buccal cells in subjects with different occupation are few (Garewal et al., 2002) and hardly any in tea plantation workers. Altered nuclear phenotype might affect the transcription profile of cells, thereby interfering with their functional ability (Misteli, 2008). In fact, altered nuclear shape is one of the key diagnostic tools used in identifying cancerous cells (Webstar et al., 2009) and an increase in this parameter in our study group as compared to control is an issue of serious concern.

The participants in the study population had either betel quid (areca nut, piper betel leaf and lime) with sadagura or without tobacco chewing habit. Their chewing habit and the analysis of the different cytome parameters revealed comparable trend with the different habituates which indicate the chewing habit as the major risk factor for the genotoxic and cytotoxic damage. Betel nut
contains an important alkaloid arecoline, which shows cytotoxic as well as genotoxic effects (Giri et al., 2006). An additive effect of smoking in inducing a chromosomal damage was observed in smokers compared with non-smokers in agricultural workers (Rupa et al., 1989; Padmavathi et al., 2000). Genetic polymorphisms of metabolising enzymes have been demonstrated as an important risk factor in development of cancer when combined with harmful exposures of betel quid and tobacco. Fewer studies are available on other occupational and environmental exposures and the information about such exposure levels is scarce (Knudsen et al., 2001).

Although the significance of increased genotoxic effects is difficult to predict for individual subjects the positive findings ensuing from this study suggest a genotoxic hazard at the group level.

5.5. Baseline Micronucleus Frequency

The buccal cytome assay was carried out in different sections of the population in our study area i.e. the district of Cachar in Assam, India, and cytome parameters were analyzed in people with all the chewing habits practiced in this region. The cytome parameters were also analyzed in two major occupations practiced by the residents in the study area as well as residents with premalignat disorders and those with oral squamous cell carcinoma. The micronucleus in exfoliated buccal epithelial cells is widely regarded as an
important endpoint for DNA damage instability (Fenech et al., 1999) and baseline micronucleus frequency evaluation in population is therefore important.

The micronucleus test in exfoliated buccal epithelial cells is one of the most popular assays of genetic damage in human biomonitoring (Neri et al., 2009). There is growing interest in this test mostly due to the ease at which the test is performed, the non-invasiveness as well as the accumulating evidence that the frequency of micronucleus in healthy subjects may be considered a marker of risk for cancer (Davis, 2003). The estimates of the baseline micronucleus frequency provide reference values of a population for genotoxic studies and therefore, it is necessary to conduct such studies in population while conducting studies on genetic damage.

In the present study (Table 35) the average micronucleus percentage ranged from 0 – 0.79 in individuals without any known exposure to genotoxic agents. The average reported healthy population micronucleus frequency is 1–3 per 1000 cells, with no significant variation between different types of exfoliated cells (Alexandrescu et al., 2006). The individual micronucleus frequency in our study population is higher but the mean micronucleus percentage is similar to micronucleus incidence reported in other studies (Basu et al., 2002; Stitch et al., 1982). Females have significantly higher incidence of micronucleated cells (P<0.05) when compared to males. It is hypothesized that X-chromosomes play an important role in the occurrence of micronuclei by interaction of their
products with receptors located on the nuclear membrane and with proteins of
the spindle apparatus (Fenech et al., 1994). However, there is also report of
higher incidence in males than females (Rajkokila et al., 2010). Age was
positively correlated with micronucleus incidence in control and there was a
significant increase in incidence of micronucleus in both males and females with
age (p<0.001) (Table 36 ) as the cell renewal process becomes less efficient
with increasing age (Squier and Kremer, 2001) and micronuclei has been reported
to spontaneously accumulate in an age dependent manner (Norppa and Falck,
2003). The micronucleus incidence is high in the habituates with different chewing
habits. There was an increase in incidence of micronucleus with age in
habituates which was statistically significant in females when comparisons
between different age groups were done. There was no significant changes
micronucleus incidence in males and this is probably due to variation in chewing
habit with age (Pastor et al., 2001). The largest fraction of the population with
different chewing habits had mean micronucleus percentage in the range of
0.31-1.50 while a sizeable number had mean micronucleus percentage in the
range of 1.51-3.90 (Figure 40) which is comparable to the frequency reported in
subjects from arsenic contaminated areas (Basu et al., 2002). No significant
difference in gender was observed in habituates as chewing frequency per day
has been found to be a major factor affecting the micronucleus frequency in
habituates irrespective of gender. An interesting finding is the baseline frequency
of incidence of micronucleus in the population from Barak Valley irrespective of
age or habit is higher. The habit of betel quid chewing with smokeless tobacco among the general population is confirmed as an independent risk factor, and the extremely strong relation between being exposed to both tobacco smoke and betel quid and having preneoplastic disease and condition exist. This probably also at least in part explains the reason behind the observation that the present study area records one of the highest incidence of oral squamous cell carcinoma in both males and females in the entire country (Merchant et al., 2000).