Discussion
Smokeless tobacco use is continued to be practiced by a large percentage of the population in India ad with the introduction of pan masala it increases continously. The consumption of chewing areca nut with or without tobacco is widespread in South Asia and in the Pacific region and considered to be one of the greatest threats to global health today (Gupta and Warnakulasuriya, 2002). Following migration from South Asia and the western Pacific to both Europe and North America the habit has remained prevalent among new settlers (Prabhu et al., 2001; Avon, 2004). It is estimated that at least 600 million individuals consume areca nut in one form or another worldwide (Warnakulasuriya and Peters, 2002). Although the composition of betel quid varies in different geographic locations, it generally consists of betel nut (Areca catechu, BN), Piper betel (PB) leaf, and slaked lime with or without tobacco. There has been sufficient evidence from epidemiological studies to show that betel quid (BQ) chewing, together with tobacco chewing or smoking is associated with the increased risk of oral cancer (Sanghvi, 1981; IARC, 1985). Approximately one-third of the adult population in the world uses tobacco in some form and half of them dies prematurely. According to an estimate by the World Health Organization, 4.9 million people worldwide died in 2000 as a result of their addiction to nicotine (WHO, 2002). This huge death toll is rising rapidly, especially in low and middle income countries where most of the world’s 1.2 billion tobacco users live. The World Health Organization predicts that tobacco deaths in India may exceed 1.5 million annually by 2020 (Murray and Lopez, 1996).
Thus, the present study was conducted to study the role of various chewing habits, especially areca nut and tobacco on the oral cavity; the genotoxic/ cytotoxic potential of these substances by observing micronuclei in the buccal mucosal and blood cells; chromosomal aberrations, genetic alterations and comet assay in blood; copper and zinc levels in the serum and cotinine levels in the plasma. The habits were correlated with biological endpoints.

In this study, apparently healthy subjects were enrolled and divided as chewers (n=120) and non-chewers (n=99). Further, for analysis purpose, these subjects were also characterized on the basis of chewing habits. The distribution of habits in the present study revealed that various forms of quids such as pan masala gutkha, mawa, khaini, pan, pan masala plain etc. are used by the chewers but majority of the chewers used tobacco along with areca nut. There are clearly many differences in the way areca nut is consumed on its own or often in combination with many other ingredients, including tobacco. In India, there are 38 different combinations of areca nut and tobacco use as reported by Pindborg et al., (1967). Mehta et al., (1971; 1972) had shown that among chewers, only 3% did not include tobacco in the quid. The subject characteristics and demographic data revealed that most of the subjects were from the urban area. This could be explained as the study was conducted at the hospital situated in the urban area. The National Sample Survey of 1999 – 2000 showed that smokeless tobacco is used by at least one member of each household in almost one-third of rural areas and one-sixth of urban areas households.

Male comprised almost 93.33% of chewers group. Higher prevalence of male in the present study corroborates with earlier studies of Pindborg et al., (1967); Yunus and Khan (1997); Chandra and Ganguli (2002). In the whole of India, one-third (38.1%) of the men and around one-tenth (9.9%) of the women use smokeless tobacco, according to the third round of the National Family Health Survey (NFHS–3), conducted in 2005–06. In the NFHS–2 (1998–99), the states with the highest prevalence of smokeless
tobacco use among women of reproductive age were Orissa (34.9%), the North East States (16.5–60.7%), Maharashtra (18.5%), Karnataka (14.9%) and Madhya Pradesh (14.8%), while the national prevalence for women was 12.4%.

In the present study, non-chewers were more educated with respect to chewers indicating the role of education in refraining from the chewing of tobacco and areca nut. Hence there is a need to impart knowledge about the ill effects of chewing habit in the society through electronic and print media, which may help in the eradication/reduction prevention of habit in the society. Also majority of the chewers in this study were belonging to the younger age group (aged 20-39 years). This indicates that the chewing habit starts at an early age. Further, Chandra and Ganguli (2002) also reported that smoking and chewing habits are formed in the age group of 15-25 years. A higher prevalence of ever or current tobacco use in the younger group compared to older group is an indicator of increasing prevalence in the adolescent population, since early use predicts greater likelihood of addiction and lifetime use (Reddy et al., 2006). Thus, there is a need to educate the youth about the adverse health effects of tobacco and areca nut chewing and to prevent them from indulging in such habits. Use of smokeless tobacco was also found to be prevalent among 14.6% (95% CI; 13.1–16.1) of students in 2000–04 in 26 states, according to Global Youth Tobacco Survey, GYTS (conducted by WHO and Centers for Disease control and prevention, CDC) among the Indian youth in grades 8–10.

There may be various reasons to adopt tobacco and areca nut chewing habit. Sometimes the chewing habit might be the result of curiosity, mass media advertisement to attract the youth and sometimes-prevailing social customs or habit prevalent in the family. Chaturvedi et al., (1998) reported that age and occupation had significant association with tobacco use but the influence of educational status on tobacco use was low. In the present study, it was found that 85% of the chewers started chewing due to peer pressure (encouragement by friends). Other cited reasons by the chewers for chewing
betel nut included self-pleasure, social status and curiosity. Chewing and smoking behavior and related attitudes of peers, family, and media could be expected to affect the adolescent’s attitudes, beliefs, values, expectations, and learned behaviors (Wang, 2003).

A significant association of acquiring chewing habit with respect to the physical labourers work was observed in the study. Most of the chewers in the study were physical labourers followed by office workers, industrial workers and students in descending order. Waerhaug (1967) quoted that the habit of betel consumption is particularly prevalent among the lower classes. The higher number of chewers in physical labourer group might be due to the fact that they use these products for feeling relaxation after physical hard work and later uses due to addiction for nicotine. Further, Lu et al., (1993) has also reported that increasing number of areca nut users are found among white-collar workers, especially officers and students, while the number among blue-collar workers, though still higher than the white-collar workers, is declining.

The present study revealed significantly higher prevalence of chewing habit in the family of chewers than non-chewers. This indicates that family environment has a definite role in acquiring the chewing habit. Lu et al., (1993) also stated that the example of the father or other male family figures had some effect on acceptance of chewing habit by the children. Most of the chewers as well as non-chewers were aware that chewing habit might lead to cancer. Also, greater number of chewers felt that chewing was a bad habit, drain economy and most of them were willing to quit the habit. This suggests that Information, Education and Communication (IEC) will play a significant role in reducing/ preventing of tobacco chewing habit in the society.

Numerous case-control studies carried out in India have shown high odds ratios for smokeless tobacco use and oral and pharyngeal cancers, and a trend of elevated risk with increasing frequency of use per day (Reddy and Gupta, 2004). Also Trivedy et al., (2002) reported that the degree of attrition is
dependent upon several factors, which include the consistency (hardness) of areca, the frequency of chewing and the duration of the habit.

It has been reported that the health-harming behaviors of both smoking and drinking are frequently associated with betel nut chewing (Wang et al., 2003; Tsai et al., 2003). The present study also showed that both the habits were prevailing with greater frequency in chewers as compared to non-chewers. Alcoholism was significantly higher among chewers as compared to non-chewers. This suggests that person addicted to one habit, may acquire another one easily due to various inter related socio-psychological and environmental factors. The relative risk of oral cancer increases to 11 folds among the chewers of betel quid with tobacco along with the habit of alcohol and smoking (Subapriya et al., 2007).

Leukoplakia is defined as a predominantly white patch or plaque on the oral mucosa that cannot be characterized clinically or pathologically as any other disease. Leukoplakia is considered as one of the precancerous lesions of oral cavity. It is well known for its potential for malignant change and transformation rates between 3-6% which have been quoted in the literature (WHO, 1978). Earlier, Wahi et al., (1970) showed that habit of chewing of mainpuri tobacco (a mixture of mainpuri tobacco, lime and areca nut) was associated with a higher prevalence of oral leucoplakia. Further, Mehta et al., (1969; 1972) also reported that chewing betel quid with tobacco was usually associated with higher prevalence of leukoplakia than the chewing of betel quid alone or no chewing habits. Although there is considerable debate about how to define oral leukoplakia there is little doubt that both tobacco, in any form, and areca nut use are major risk factors for developing this condition (Gupta et al., 1997; Shiu and Chen, 2004). Analysis of the distribution of leukoplakia cases among chewers and non-chewers in the study revealed that 8 cases were found in chewers whereas only 1 case in non-chewers. This finding further corroborates with earlier investigators that areca nut and chewing tobacco plays a significant role in the development of precancerous lesion. Earlier, Mehta et al., (1969) conducted cross-sectional surveys of more
than 50,000 individuals in five districts of India and found that the prevalence of oral leukoplakia ranged from 0.4% to 1.8% among users of smokeless tobacco as compared with almost zero prevalence of leukoplakia in non-users. Gupta et al., (1980) carried out a 10 year follow-up survey of oral lesions in Ernakulam (Kerala), Bhavnagar (Gujarat), and Srikakulam (Andhra Pradesh), in India. The highest incidence was observed in Ernakulam in men who chewed betel quid with tobacco and smoke (6 per 1000), and no new cases were found among those who did not chew or smoked. In the present study, all eight cases of Leukoplakia were found in chewers except one case in non-chewers. However this subjects was a smoker.

The most striking finding of present study was that all the 31 cases of OSMF were found among chewers. Also, among chewers, 29 cases of OSMF were observed among areca nut with tobacco chewing habit, thereby it seems likely that chewing areca nut and tobacco plays an important role in development of OSMF. This may be due to the fact that tobacco and areca nut may act synergistically to the lesion. Also, there were only one case each in the areca nut without tobacco and only tobacco chewers. However, there may be additional co-factors necessary for the development of OSMF. The effects of areca nut, inflorescence piper betel extracts and arecoline on cytotoxicity, total and unscheduled DNA synthesis in cultured gingival keratinocytes has been investigated by Jeng et al., (1999). The results indicated that areca nut, inflorescence piper betel extracts and arecoline take part in the pathogenesis of betel nut chewing-related oral mucosal lesions like leukoplaikia, OSMF and oral cancer, possibly through both genotoxic and non-genotoxic mechanisms. Further evidence of its relationship with areca chewing has come from the increased prevalence of this condition in subjects who suffer from oral submucous fibrosis, which is associated strongly with the habit of areca chewing. Many investigators have described areca nut chewing as the most important factor for development of OSMF (Bhonsle et al., 1987; Lu et al., 1993). Several case-control studies have also shown that there is an increased risk of developing OSMF in subjects consuming areca products (Shiau and Kwan, 1979; Pindborg et al., 1980). The evidence from the
literature coupled with data of the present study, the role of areca nut and tobacco strongly implicates in the etiopathogenesis of OSMF. However, further studies are warranted to uncover the mechanisms of transformation to squamous cell carcinoma.

All areca nut products were associated with OSMF, with the risk being greater for pan masala has been reported by Ranganathan et al., (2004). They further reported that the duration of the habit was more significantly associated with OSMF than the frequency of the chewing habit. In the present study, all the OSMF subjects were found in chewers and none among the non-chewers. Thus, chewing areca nut with tobacco is the most important etiological factor for development of OSMF. Other causes, which have been proposed for causative factors of OSMF in the past but have not been substantiated include excessive consumption of chillies, auto immune reaction and nutritional factors, particularly iron deficiency etc (Murti et al., 1995). Meghji et al., (1982) concluded that tannins from chewed areca nuts might enhance the development of fibrosis in OSMF by reducing the susceptibility of collagen to degradation by collagenase. In vitro studies have shown that extracts of areca nut may stimulate cultured fibroblasts to proliferate and synthesize collagen and this might be involved in the etiology of OSMF (Canniff and Harvey, 1981; Harvey et al., 1986). However, recent studies have shown that arecoline inhibits collagen synthesis and fibroblast proliferation in vitro, suggesting that arecoline may have cytotoxic properties (Chang et al., 1998). The disparity of results on arecoline from in vitro studies suggests that areca may contain other agents that are important in the pathogenesis of OSMF. The disease is clearly multifactorial in origin as only fraction of chewers developed OSMF. Thus there are some other co-factors that are necessary for the development of OSMF such as lack of antifibrotic activity, genetic susceptibility etc. It seems that the disease has a genetic predisposition (HLA-linked), which renders the oral mucosa susceptible to inflammatory changes if they chew betel nut (Canniff et al., 1985).
Complaints of difficulty in mouth opening (which were further corroborated by significantly reduced inter-incisor and inter-molar distances in chewers), difficulty in swallowing and burning sensation in soft tissue of mouth – all symptoms were associated with areca nut and tobacco chewing habit. OSMF patients present themselves with the major complaint of progressive difficulty in adequate mouth opening which might be due to the accumulation of inelastic fibrous tissue in juxta-epithelial region of oral mucosa. The significant lower interincisor and intermolar distance among chewers and OSMF cases was observed as compared to non-chewers in the study. The fibrosis may lead to difficulty in mastication, speech and swallowing (if esophagus gets involved). Further, the inelastic fibrotic mucosa is forced against the edges of teeth causing ulceration, which may become secondarily infected (Canniff et al., 1986).

An international working group of scientific experts convened by the Monographs Programme of the International Agency for Research on Cancer (IARC), has reviewed the published studies related to cancer and chewing betel quid and areca nut. A previous evaluation in 1985 had found that chewing betel quid with tobacco is carcinogenic to humans. The new evaluation goes further to conclude that chewing betel quid without tobacco is also carcinogenic to humans. The working group also suggested that the areca nut, a common component of many different chewing habits, is carcinogenic to humans (IARC, 2000). Trivedy et al., (2002) suggested that areca nut might have potentially harmful effect on the oral cavity. It is a risk factor for oral cancer, oral cancer recurrence, adult periodontal diseases, suppresses the immune system’s response to oral infection, retards healing following oral surgical and accidental wounding.

A close association has been reported between areca nut and tobacco chewing with oral lesions and that is necessary to find out the early biomarkers of the oral diseases. The epithelial surface continuously shed cellular material and the microscopic analysis of such cells provides useful information regarding the status of the tissue from which they originate. It has
been known that inflammatory, infectious, premalignant and malignant cellular features are often identifiable through such microscopic analysis. It has recently been recognized that similar studies can be used to monitor and detect early biological changes in target tissues due to exposure to potential mutagenic and carcinogenic chemicals. The buccal cytome assay is one such sensitive measure that has received attention as a sensitive biological marker of genotoxic exposure. Oral exfoliative cytology might reveal various cellular alterations such as karyorrhexis, karyolysis, micronucleus formation, binucleation, broken-egg nucleus etc. in the buccal mucosa cells of snuff users by Tolbert et al., (1992). To study the biomarkers of the genetic damage, particular interest is given to target tissue because they are primarily affected by the toxicants (Fenech, 2003). A significant increase in the micronuclei of buccal mucosa cells was observed among chewers and OSMF subjects with respect to non-chewers indicating the genotoxic/mutagenic potential of these chewing materials and also their possible role in the causation of OSMF. Recently, an elevation in micronuclei in buccal mucosa cells was observed among chewers as well as chewers suffering from OSMF as compared to non-chewers (Joshi et al., 2011). This elevation in MN is due to the fact that these chewing material have adverse effects on chromosome. The presence of MNs is indicative of chromosome loss or fragmentation occurring during previous nuclear division (Fenech and Morley, 1986). Earlier, a very high frequency of MN has been observed among tobacco users (Bohrer et al., 2005; Saatci et al., 2008). Similarly, increase in the frequency of MN in pan masala consumers was reported by Gandhi and Kaur (2000). A recent finding indicated that micronuclei frequencies in oral exfoliated cell were higher in squamous cell carcinoma patients than in control subjects (Devendra and Jagdish, 2008). Chatterjee et al., (2009) revealed that MN frequencies in cancer and pre-cancerous cases were 4-fold (p<0.001) and 3.87-fold (p<0.002) elevated than other non-malignant pathologies. In addition, significant associations between use of tobacco in various forms and development of oral pathologies has also been established in this study. Further, frequency of micronuclei (MN) were elevated in the buccal mucosa of areca nut without tobacco, only tobacco and areca nut with tobacco chewers.
as compared to non-chewers indicating that chewing of areca nut alone also induces MN in buccal mucosa cells. It has also been shown that a mixture of betel leaf, areca nut and tobacco is unsafe for oral health (Sellappa et al., 2009). Saatci et al., (2008) found that the MN frequency was significantly higher in maras powder (MP= smokeless tobacco) users and smokers than in healthy volunteers. Evidence indicated that MP usage induces DNA hypomethylation and increases frequency of MN formation.

The combination of cell death parameters within the buccal mucosa, in addition to MN, may be useful as possible diagnostic to identify individuals with a high risk of tobacco related tissue damage (Kausar et al., 2009). In buccal mucosa cells of chewers an increased frequency of nuclear anomalies was observed that in turn might indicate adverse cellular effect to eliminate cells with genetic damage. Hence, the increase in the karyorrhectic and karyolytic cells among the chewers of areca nut and tobacco as well as OSMF subjects may represent the cytotoxicity caused by these chewing materials. The increase in binucleated cells may be due to the defects in cytokinesis whereas higher nuclear buds may represent the elimination of amplified DNA or DNA repair complexes. It is suggested that apoptosis also act as a surveillance mechanism, eliminating the cells with genetic damage (Tolbert et al., 1991). Thus, apoptosis in excess of normal levels may serve as an indicator of genotoxic insult. Occurrence of increased percentage frequencies of KL and KR anomaly takes place in the pre-keratinization process (Pindborg et al., 1980). This anomaly represents cytotoxicity, which is also evident in necrotic cells (Wyllie, 1981; Tolbert et al., 1991). Also this anomaly is intrinsic to the squamous epithelium, especially because of the chronic effect of the masticatory process on the oral mucosa and the constant action of mutagenic agents such as tobacco and areca nut increases the rate of cellular deaths, as indicated by the significant elevation of this anomaly (Ramirez and Saldanha, 2002). Karyolysis is associated with cytotoxicity, and karyorrhexis accompany apoptosis (Ramirez and Saldanha, 2002), a process under genetic control. Recently, a finding has shown that the frequencies of karyorrhexis, karyolysis, N buds and binucleated were increased significantly only in smokers of
medium filter and non-filtered types of cigarettes while only MN levels were elevated (p<0.0001) in the group that smoked non-filtered cigarettes, which suggests that these endpoints, both reflecting DNA damage, are more sensitive than MN, which is the only parameter scored in most earlier studies (Nersesyan et al., 2010). In the present study, a significant elevation in N buds, binucleated cells and karyorrhexis was observed in the subjects having areca nut with tobacco chewing habit as compared to other chewing groups.

Further, dose and duration dependent increase in MN was also found. Similarly other nuclear anomalies were elevated with the elevation in chewing frequency (quids/ day) and duration (in years) groups. Earlier, a significant positive trend was demonstrated between MN frequency and either daily cigarette consumption or cumulative smoking pack-years. However, by contrast, negative trends were demonstrated for the analogous relationships with areca quid chewing. These results indicate that heavy smoking, but not areca quid chewing, increases MN formation. These findings suggest that the carcinogenesis of the oral cancers induced by areca quid chewing in Taiwan may be through a pathway other than genotoxicity (Wu et al., 2004). However, a significantly higher frequency of micronucleus was found among the chewers as compared to non-chewers in the present study. Increased percentile frequency of micronucleus cells was found in duration dependent regular chewing (a mixture of betel leaf, areca nut and tobacco) and snuffing habit groups (Sudha et al., 2009). In the present study, a positive significant correlation was found between the duration in years with MN, N buds, BN, KL and KR. This corroborates with the earlier finding of Yadav and Chadha (2002). They found an elevation of MN in dose (frequency per day) and duration (in years) dependent among pan masala chewers and positive linear regression analysis was found between MN verses pouches consumed per day and duration in years.

Classic cytogenetic analysis of chromosome aberrations in metaphases has been the gold standard of biological dosimetry for many years, but the complexity and tediousness of this technique has led to the
development of much faster and simpler cytokinesis-blocked micronucleus (CBMN) assay (Fenech and Morley, 1985). An increased frequency of micronuclei in the cell may be considered as a biomarker of permanent genotoxic damage, reflecting either clastogenic or aneugenic modes of action (Albertini et al., 1999).

Among the different cytogenetic approaches, the MN assay in human lymphocytes using the cytokinesis-block method (Fenech and Morley, 1985) has increasingly been accepted as a reliable biomarker of cytogenetic damage induced by genotoxic agents, both physical and chemical (Bauchinger, 1990; Fenech, 1993). A prospective study had shown that MN in peripheral blood lymphocytes is a valid biomarker for predicting an increased cancer risk in humans (Bonassi et al., 2007). Milic et al., (2008) investigated chromosomal damage in workers occupationally exposed to tobacco dust and showed that the micronucleus frequency noted by CBMN and sister chromatid exchange were more reliable indicators of genome damage in monitoring chronically exposed subjects. In the present study, micronucleus in binucleated as well as multinucleated cells and nucleoplasmic bridges were found to be elevated among chewers and OSMF subjects as compared to non-chewers. Also the frequency of BN MN, N buds and NPB were also positively correlated with lifetime chewing exposure. The frequency of nucleoplasmic bridges was significantly higher in the chewers as compared to non-chewers whereas micronuclei were significantly higher in both chewers and OSMF subjects as compared to non-chewers, suggesting a higher level of genetic instability among the chewers. Earlier it has been also reported that frequencies of sister-chromatid exchanges and chromosome aberrations in peripheral blood lymphocytes and micronucleated cells in exfoliated buccal mucosa were significantly elevated in tobacco-areca nut chewers and OSMF subjects (Adhvaryu et al., 1991). Also, in the present study, a significant increase in the binucleated cells with micronucleus, total micronucleus and nucleoplasmic bridges were observed in the areca nut with tobacco chewers as compared to areca nut without tobacco, only tobacco as well as non-chewers. Further, an experimental study from this laboratory also reported an
increased frequency of MN in bone marrow cells of mice exposed to pan masala plain and pan masala gutkha (Mojidra et al., 2009). Earlier, Bonassi et al., (2003) also observed that heavy smokers group showed a significant increase in genotoxic damage as measured by the micronucleus assay (CBMN) in lymphocytes. These results coupled with the data available affirms that this multi-endpoint CBMN assay can be useful to evaluate the genotoxic potential of areca nut and tobacco and can act as sensitive biomarkers of genotoxic damage.

Chromosomal aberrations are the changes in chromosome structure resulting from the break or an exchange of chromosomal material. Most of the chromosomal aberrations are lethal, but there are many corresponding aberrations that are viable and can cause genetic effects either somatic or inherited. Usually, lymphocytes are obtained from exposed populations and examined for chromosomal damage. This methodology has been applied to numerous occupational and environmental exposures to chemicals and radiation (Burgaz et al., 2002). In the present study, a significant increase in the frequency of chromosomal and chromatid aberrations was observed in cultured lymphocytes. These data are in accordance with the earlier report on tobacco chewers (Beena et al., 2009). Moreover, the gaps and breaks were the most common type of aberrations observed. It is known that the aberrations induced in the G1 and early S phase are chromosome-type, whereas those induced in the late S and G2 phase are chromatid type. The inhibitors of DNA synthesis also induce a very high frequency of gaps when the cells are treated in late S or G2 phase (Natarajan and Obe, 1982).

The detection using the multiple cytogenetic biomarkers to identify the damage causing genotoxic agents enhance the importance of such method in studying the biomarkers of the genetic damage. Further, lymphocytes have a half-life of 3–6 months and travel throughout the body, integrating genotoxic events across body tissues while in comparison, buccal cells turn over every 21 days (Fenech, 2003). The advantages of peripheral blood lymphocytes as an ideal test system for analyzing the effects of chronic exposures to low
doses of mutagens has been detailed by Natarajan and Obe (1982). The cytogenetic endpoint, namely CA assay was used to monitor the individuals addicted to one/both of the areca nut/tobacco chewing products (Pinto et al., 2000; Lucero et al., 2000) which can be very well expressed in this study.

Chromosomal aberrations were found to be significantly higher in the chewers of areca nut with tobacco as compared to other chewing groups, which suggest that when areca nut and tobacco are taken together, they might have the potential of causing more genetic damage. The enhancing effect of areca nut in tobacco carcinogenesis had also been documented by Ranadive and Gothoskar (1978). Adhvaryu et al., (1989) studied cytogenetic end points like SCE and CA in in vitro short term mammalian test systems and found genotoxic potential of an aqueous extract of pan masala. They found CA to be significantly increased in pan masala extract exposed sample. Similarly, the cytogenetic end points like SCE and CA demonstrated a statistically significant increase among the pan masala consumers as compared with the non-consuming controls (Dave et al., 1991). Brogger et al., (1990) have reported that persons with high frequency of CA develop cancer twice as often as others. The present investigation showed that areca nut with tobacco consumers had significant increased CA as compared with non-chewers. Stich and Stich (1982) have reported induction of CA frequency in CHO cells by the saliva of Indian tobacco chewers. Similarly, aqueous extracts of snuff, zarada, khaini, etc. also have been shown to elevate the frequency of CAs (Stich and Stich, 1982). Most of the in vivo and in vitro studies showed the genotoxic potential of various chewing materials containing areca nut and tobacco.

The frequency of aberrant metaphases and the proportion of individuals with CA were significantly higher in tobacco processors than in controls, indicating that occupational exposure to tobacco imposes considerable genotoxicity among tobacco processors (Manoj et al., 1995). Increased chromatid gap (significant) and chromosomal gap (non-significant) were observed in the present study among chewers as well as OSMF
subjects. Tobacco particulate matter has also been shown to induce structural and numerical chromosome aberrations (Lafi et al., 1988) as well as micronuclei (Jones et al., 1991) in cultured mammalian cells. Dönbak et al., (2007) showed significant elevation of CA and MN frequencies in peripheral lymphocytes of smokeless tobacco consumers demonstrating a potential cytogenetic hazard associated with Maras powder exposure. Aqueous extracts of both pan masala and gutkha induced chromosomal aberrations, sister chromatid exchange and micronucleated cells in Chinese hamster ovary cells in the presence or absence of an exogenous metabolic system, although metabolic activation markedly inhibited the chromosome damaging effect, implicating the presence of direct-acting mutagens (Dave et al., 1991).

Induction of DNA damage and the resulting adverse sequel such as mutations and chromosomal rearrangements are the primary mechanisms for induction of cancer. Studies have shown that how these events are involved in activation of dominant oncogenes and the inactivation of tumor-suppressor genes (Cooper, 1990). Chewing tobacco has been causally associated with oral cancers, however, looking to the elevated frequencies of CA in peripheral blood lymphocytes, the possibility of damage caused to other systems/tissues should also be considered, as is the case with smoking which causes lung cancer but also elevates the risk of cancer at other sites like bladder, pancreas etc. Here, CA frequencies were higher in OSMF subjects as compared to non-chewers. Our study showed elevated CA in all the three groups of areca nut and/or tobacco chewers as well as higher CA was found with increase in the frequency of chewing (quids/day) as well as duration (years). This increase can also be explained on the basis of the clastogenic potential of tobacco and areca nut alkaloids. Earlier, increases in SCE and CA frequencies have also been recorded in different premalignant and malignant conditions (Adhvaryu et al., 1986; Murty et al., 1986).

Comet assay can sensitively detect DNA single stranded break(s) and alkali-labile site (Hecht et al., 1978; Oesch et al., 1994; Tice, 1995). In the present study, comet assay was used to examine DNA damage in lymphocyte
of areca nut and tobacco chewers. The comet assay just measures strand breaks and alkali-labile sites, i.e. primary DNA lesions which are short-lived and rapidly repaired in mammalian cells. Bulky DNA adducts which are formed from many mutagens/carcinogens (which are also present in areca nut and tobacco), do not directly cause comet assay effects but only increase DNA migration when they persist and are repaired by excision repair in cells under investigation (Speit and Hartmann, 1995). It has been shown that the comet assay can be used as a cost-effective biomonitoring tool to assess damage and identify the individuals at risk for a progressive pathology and even to malignancy (Vasavi et al., 2010).

In the present study, areca nut and tobacco chewers were found to possess higher DNA damage in blood lymphocytes. Our finding is in accordance with earlier findings (Betti et al., 1995; Sardas et al., 1995) which observed that smoking with chewing tobacco use can increase DNA damage. T-lymphocytes are the cells that circulate through different organs before they return to peripheral blood. On average, their circulation takes 3 min. Thus, the lymphocytes exposed to tobacco and areca nut nitrosamine in any body organ or tissue. Lymphocytes circulating for years or even decades, accumulating mutations in their DNA produced by exposures throughout their existence (Buckton et al., 1978, Natarajan and Obe, 1980, Carrano and Natarajan, 1988).

The comet assay further revealed a statistically significant increase in the level of DNA damage among chewers as compared to non-chewers. Sardas et al., (2009) studied the genotoxicity in the peripheral lymphocytes of MP (Maras Powder, smokeless tobacco) users, cigarette smokers and non-smokers using comet assay. They found that the oral use of smokeless tobacco has the potential to cause genotoxic hazard, which is even higher than the DNA damage observed in cigarette smokers. In the present study OTM was found to have significant positive correlation with chewing frequency per day and also a positive significant correlation was observed between the DNA damage parameters. This shows that chewing areca nut
and tobacco induces DNA damage in the lymphocytes. It has also been suggested that cyto-genotoxicity of cigarette smoke condensates from different brands of cigarettes varied greatly and comet assay might be a sensitive assay in assessing the genotoxicity induced by cigarette smoke condensates (Lou et al., 2010).

Chewing of tobacco led to an early sign of damage to the oral mucosa and often develop clinically visible whitish lesions and stiffening of the oral mucosa and oral submucous fibrosis (OSMF). Almost every tobacco chewing-related oral malignancy is preceded by a clinically distinct premalignant stage at the site of cancer development (WHO, 1984; Gupta et al., 1989; Murti et al., 1995). Results of comet assay in the present study indicate an increase in the DNA damage in peripheral blood cells. Comet results showed the differences between DNA damage in areca nut and/or tobacco chewers and OSMF subjects than non-chewers. Also, our results for DNA damage using the comet assay are in agreement with Ceylan et al., (2006) who detected a significant increase in DNA damage using the same technique in patients with chronic obstructive pulmonary disease due to smoking, as well as in patients with chronic obstructive pulmonary disease due to the use of biomass fuel, such as wood for cooking. The high standard deviations for comet values found in our study reflect the large inter-individual variation, a frequent finding when this technique is used (DeM´eo et al., 1991; Tice, 1995). Jayakumar and Sasikala (2008) reported cigarette smoking has a synergistic effect on inducing DNA damage among the jewellery workers occupationally exposed to nitric oxide. Recently, association has also been showed between smoking and induction of maximum amount of DNA damage (Manikantan et al., 2010). Chang et al., (1999) showed that occupational exposure and smoking had a significant and independent effect on tail moment.

In the Indian oral cancer patients, the prevalence of chewing tobacco is high, with the attributable risk of tobacco varying from 61% to 70% for cancer (Notani, 2000). However, not all tobacco habituates develop oral cancer. Genetic susceptibility might be one of the reasons for such differences.
Hence, such studies at specific loci suspected to increase genetic susceptibility to human cancers are necessary. Thus, p53 genotype might be associated with the increased susceptibility to OSMF and/or oral cancer development. Genetic instability is putatively involved in the multistep process of carcinogenesis of most cancers. Current evidence suggests that this genomic instability occurs at two levels: the nucleotide level and the chromosome level (Papavasileiou et al., 2009). Gains or losses of whole or large parts of human chromosomes in tumor cells are found in most cancers (Charlotte et al., 2002). This has been proposed as a major driving force for determining the rate of accumulation of specific genetic hits in several human cancers (Jin et al., 1995). A significant number of deletion and gain were detected in most of the cells, and this was frequently represented by polysomy, rather than monosomy of chromosome 17. Chromosome 17 abnormality has been shown to have a strong correlation with neoplastic development and progression (Hopman et al., 1994). The p53 gene is a tumor suppressor gene, which induces a G1 arrest and is involved in the DNA repair and apoptosis. Abnormalities in the p53 gene cause an inefficient checkpoint system for the repair and destruction of mutant cells, which results in an increased genomic instability. Point mutations are the most frequent mutations that affect wild-type p53, which thus becomes inactivated in human tumors. Earlier, a significant higher risk for p53 mutations has been observed in smokers compared with non-smokers (Sankaranarayanan et al., 2005).

Considering that 17p allelic deletions are the hallmark of many human tumours, including colon, breast, bladder, lung and liver cancer (Yokota et al., 1987; Vogelstein et al., 1988; Mackay et al., 1988) and that >90% of human tumors arise from epithelial tissues, the proposed methodology offers the possibility of detecting DNA lesions with special relevance to carcinogenesis. From a mechanistic point of view, 17p gains probably arise from areca nut and tobacco induced chromatid translocations formed in the G2 phase of the continuously proliferating blood cells. In the present study, chromosome breakage in the areca nut and tobacco chewers resulted in a significant increase in 17p gains and 17p deletions involving the p53 gene. A multivariate
analysis confirmed p53 deletion as an independent prognostic factor predicting shortened progression-free (P=0.0009) or overall survival (P=0.0002) in patients with multiple myeloma after high-dose chemotherapy and autologous stem cell transplantation (Chang et al., 2005). Earlier, Sasano et al. (1992) observed no significant accumulation of p53 hybridization signals in carcinoma cells.

Mutation in p53 allele has been reported in 22% of precancers and 20% of oral cancers. The prognostic significance of p53 in oral cancer is yet to be established, although there are multiple studies comparing the expression of p53 in premalignant lesions and subsequent malignancies (Murti et al., 1998; Shahnavaz et al., 2000). Combining other parameters to assess either precancer or cancer may provide more significant correlation (Lee et al., 2000). A few genes related to p53 and cell cycle regulation such as P16, P27, P63, and P73 were also altered in varying degrees in oral cancer (Gao et al., 2005). Our study demonstrates that the genomic loss of the 17p13.1 region (p53 gene) occurs in the chewers and OSMF subjects. Total aberrations of 17 chromosomes were significantly higher in OSMF as well as in areca nut with tobacco chewers. Mutations of p53 and 17p13.1 loss of heterozygosity have been reported in upto 92 and 100% of esophageal adenocarcinomas (Krishnadath et al., 1995; Wu et al., 1998; Riegman et al., 2001). Recent study had shown that p53 is overexpressed in 63% of oral carcinomas, with p53 mutations in 36% of individuals and altered p53 expression in premalignant lesions is associated with increased chromosomal polysomy (Acha-Sagredo et al., 2009). Similar results were obtained in our study in which significant p53 gain (over expression) was found in chewers as well as in OSMF subjects. Earlier studies also revealed that p53 alterations can occur early in carcinogenesis and the alterations are maintained upon progression to evident malignancy (Fadl-Elmula, 2005). Further, it has also been reported that all the patients who presented recurrence, metastasis, and death with tumor dissemination had p53 overexpression (Boyle et al., 1993). James et al., (1999) found clinicopathologic variables in endometrial cancer using fluorescence in situ hybridization (FISH) cytogenetic analysis and found
deletion of p53 in 31/47 (66%) cases and was associated with poor histologic grade (P = 0.008).

The present results coupled with previously reported findings suggest that loss and gain of genes mapped to chromosome 17 play a major role in the etiopathogeny of OSMF or oral cancer. The degree of numerical abnormality of chromosome 17 varied from subject to subject. This finding suggests that numerical chromosome 17 abnormality is involved in the process of carcinogenesis and development of oral malignant neoplasm. Such genotypic parameters may provide a genetic basis for the development of an early recurrence or second primary tumors after therapeutic treatment of oral squamous cell carcinomas. Deletion of 17p or p53 gene was found significantly higher among chewers and OSMF subjects as compared to non-chewers. Similar results have been shown in Acute Myeloid Leukemia and Myelodysplastic Syndrome patients by Jean Luc and Pierre, (1998). They studied p53 mutation analysis in one of the subject was negative whereas in the 14 other cases, FISH showed a 17p deletion of variable extent but that always included deletion of the p53 gene. All 14 patients had typical dysgranulopoiesis, and all but one had p53 mutation and/or overexpression. These findings reinforce the morphologic, cytogenetic, and molecular correlation found in the 17p- syndrome and suggest a pathogenetic role for inactivation of tumor suppressor gene(s) located in 17p, especially the p53 gene.

Considering the implications of 17p alterations among the areca nut and tobacco chewers as well as OSMF subjects, it can be suggested that the fluorescent in situ hybridization could be useful to assess the genotoxic potential of mutagenic/carcinogenic agents and, thereby could be used as a valid biomarker to assess the people at risk of developing the precancerous lesions.

Cotinine, the major proximate metabolite of nicotine, has been widely used as a biomarker of tobacco exposure (Benowitz, 1983; Etzel, 1990).
Plasma cotinine concentrations correlate better to various measures of biologic effects of cigarette smoking than does self-reported cigarettes smoking (Perez-Stable et al., 1995; Benowitz and Sharp, 1989).

Blood cotinine level can be measured in order to find whether a person is a tobacco chewers or smoker, or a non-smoker/non-chewers or being exposed to environmental tobacco smoke (ETS). However, compared with nicotine, the half-life of urinary cotinine is long (10 to 20 hours) so it is more commonly determined (Haufroid and Lison, 1998). Nicotine is converted to cotinine by cytochrome P450 (CYP) 2A6 (Messina et al., 1997). According to one of the previous study, individuals who had genetic homozygous CYP2A6 deletion had decreased urinary cotinine excretion despite smoking (Kitagawa et al., 1999). This genetic factor may also be involved in the misclassification rate according to subject habits. So, in the present study non detectable level of cotinine was more among non-chewers as compared to other chewers groups and minimum range was also lower in non-chewers than chewers and OSMF subjects. The mean level of those showing a cotinine level that exceeded the detectable threshold was higher among the chewers and smokers, showing a difference in the distribution of the cotinine level. About 55% of non-chewers were found to have cotinine level because; it is probable that non-chewers are being exposed to environmental tobacco. Similar results have been shown in one of the US study by Pirkle et al., (1996). The high proportion of the population with detectable serum cotinine levels indicates widespread exposure to environmental tobacco smoke in the US population. Both the home and workplace environments significantly contribute to environmental tobacco smoke exposure (Klesges et al., 1995; Gonzalez et al., 1996).

In the present study non-chewers and non-smokers were found to have slightly high cotinine levels as these subjects might have exposure to environment tobacco smoke but the level was lower with respect to either smokers or tobacco chewers. The highest serum cotinine concentration that would be expected in a nonsmoker exposed to environment tobacco smoke is
~10–13 µg/L (Jarvis et al., 1987; Benowitz et al., 1983). In the present study positive correlation was found between lifetime chewing exposure and cotinine level (R sq linear= 0.021). These findings were in agreement with those reported in smokers by Bergstrom (1989), Preber and Kant, (1973 and Preber et al., (1980).

In recent years, copper has been implicated in pathogenesis of OSMF. High level of copper is found in the areca nut as compared to other eatable nuts (Trivedy et al., 1997). In the present study, marginally higher level of copper was noted in the serum of chewers as compared to non-chewers. However, these changes were statistically non-significant. Jayadeep et al., (1997) found that serum copper was significantly increased in oral leukoplakia and cancer. They further reported that the level of zinc decreased significantly only in male patients with leukoplakia and cancer. Trivedy et al., (1999) proposed that the high copper content of the areca nut might play an important role in the etiopathogenesis of OSMF. Later they reported raised copper levels in oral biopsies from patients with OSMF (Trivedy et al., 2000). In the present study a slightly higher level of copper was observed in the serum of the OSMF subjects than non-chewers. Recently Trivedy et al., (2001) also reported that the addition of copper to fibroblasts at concentrations compatible with that found in saliva after chewing areca nut could cause a significant increase in the synthesis of collagen. This suggests a possible role of copper in the OSMF. Lysyl oxidase, an extracellular enzyme, is responsible for cross-linkage of collagen that may become more resistant to digestion by collagenase. When compared with fibroblasts from normal mucosa, elevation of lysyl oxidase in OSMF fibroblasts is noted that contribute to collagen deposition (Ma et al., 1995). Presence of high levels of copper may also up-regulate the lysyl oxidase activity, leading to excessive cross-linkage and deposition of collagen (Trivedy et al., 1997).

The role of copper cannot be segregated from that of zinc. The transport of copper in to the oral epithelium might be due to the composition of the quid rather than the time of exposure. Zinc is implicated in the modulation
of mucosal metallothionein, thereby interfering with copper absorption. The bioavailability of zinc in its turn depends on elements like calcium and iron present in oral fluid. The usage of slaked lime (calcium hydroxide) as an ingredient of betel quid, thereby causing an interference with zinc bioavailability is a matter of concern (Rajendran and Karunakaran, 2002). The presence of high concentration of copper (as is seen in areca) may also diminish zinc transport (ATSDR, 1994). Although direct data pertaining to this type of interaction is scanty, there is at least one study by Scott and Turnlund (1994) that compared zinc kinetics in subjects with normal, high or low copper intake. They also investigated sites of interaction between copper and zinc and the degree of interaction at each site. They reported that zinc absorption was lower on high copper intake (28% versus 34%, respectively). Varghese et al., (1987) reported a significant reduction in the serum zinc levels in both oral submucous fibrosis and oral cancer. The copper/zinc ratio was also found to be elevated in oral submucous fibrosis and depressed in oral cancer. Kumar et al., (1991) conducted a clinical study to evaluate the possible therapeutic role of zinc in the treatment of OSMF and found significant decrease in the severity of the disease following oral supplementation of zinc. The study signifies the role of deficiency of zinc in OSMF patients. The present study showed higher zinc level in non-chewers followed by chewers and OSMF patients.

A positive correlation in MN frequency was observed between target tissue (Buccal mucosa) and peripheral tissue (Blood). Similar cytogenetic endpoints obtained by Haveric et al., (2010) with peripheral blood and exfoliated buccal mucosa cells of smokers. They found significant positive correlation, indicating complementarities of those analyses. Lewińska et al., (2007) showed a significant increase in MN frequency in peripheral blood lymphocytes and in buccal epithelial cells of smelter workers as compared to the controls.

A significant positive correlation was observed between buccal MN and peripheral blood cells chromosomal aberration per cells in the present study.
Beena et al., (2009) also found significant positive correlation between buccal MN and CA. They found that buccal MN can better discriminate tobacco exposure index than CA analysis which might be due to the fact that buccal MN is analyzed from target tissue while CA was analyzed from peripheral blood. MN in buccal cells originate from genome damage events in the basal layer of the oral mucosa while CA analysis from lymphocyte culture allows a measure of genome damage that accumulated while lymphocytes circulate around the body in the quiescent phase. Dave et al., (1992) showed statistically significant increase in the frequencies of sister chromatid exchanges and chromosome aberrations in peripheral blood lymphocytes and the percentage of micronucleated cells in exfoliated cells of buccal mucosa among chewers. In the present study, micronucleus in buccal mucosa cells and chromosomal aberrations were found to have significantly positive correlation with the chewing frequency per day and duration in years. Earlier, Yadav and Chadha (2002) also found increased frequency of CA and MN end points to be significantly correlated with the duration of consumption and number of pouches consumed per day. Donbak et al., (2007) observed significant elevation of CA and MN frequencies in peripheral lymphocytes from smokeless tobacco consumers which demonstrated a potential cytogenetic hazard associated with Maras powder exposure.

In the present study, a positive correlation was observed between micronucleus in binucleated cells and olive tail moment by comet assay. This indicate the correlation between DNA damage with MN. Earlier, a study conducted by Danadevi et al., (2004) to study the genotoxic evaluation on welders showed a larger mean comet tail length (23.05 ± 3.86 versus 8.94 ± 3.16; p<0.001) as well as significant increase in the micronucleated cells (1.30 versus 0.32; p<0.001) with respect to controls. A significant stepwise increase in the DNA damage was found in buccal epithelial cells and peripheral blood leucocytes from control to pre-cancer patients and from pre-cancer to cancer patients (Rashmi et al., 2007). Micronucleus frequency was also observed to increase in the same way.
A significant positive correlation between chromosomal aberration per cells and olive tail moment by comet assay was also observed in the study. One of the study by Paz-y-Miño et al., (2002) evaluated DNA damage in human lymphocytes due to occupational exposure to low levels of ionizing radiation using two assays: the comet assay and chromosomal aberration (CA) analysis including and excluding gaps. The results obtained reveal a higher correlation between both methods when chromatid and chromosome gaps were included in the correlation analysis ($r=0.78$ versus $r=0.50$). They further hypothesized that the gaps constitute a type of chromosome aberrations.

A positive correlation has been found between CA/cell and plasma cotinine level in the study. Similar results had been shown by Balachandar et al., (2008) from Tamilnadu, India. They showed that CA increased with an increase in environmental tobacco smoke (ETS) and active smoke exposure period in passive smokers quantified on the basis of serum cotinine levels. In earlier study, investigated the consumption of cigarettes by measuring nicotine and cotinine with gas chromatography (GC) and it was correlated with the CYP2A6 genotype and chromosomal aberration. Results showed that, there was a significant increase in CA frequency in smokers compared to non-smoker subjects (Neslihan et al., 2005).

It has been reported that a tumour suppressor gene like p53 is effective in protecting cells against DNA damage induced by various agents including ionizing radiation (Hall et al., 1993; Zhan et al., 1994). In this study, p53 gene deletions has been correlated positively with the DNA damage marker i.e. olive tail moment assessed by comet assay whereas negatively with apoptosis in the buccal cells. It has been reported earlier that upon high or irreparable DNA damage, p53 promotes the cells towards apoptosis (Yopnish-Rouach et al., 1991; Bates and Vousden, 1996) whereas the loss of the normal p53 gene function is known to contribute to genomic instability (Lee et al., 1994; Schwartz et al., 1997). Similar results have been obtained in our study which shows that DNA damage (olive tail moment) correlated positively
with p53 gene deletion which further be responsible for apoptosis. The wild-type p53 gene is a tumour suppressor gene coding for a nuclear phosphoprotein that plays a crucial role in the cellular response to DNA damage by inducing cells to arrest in G1 or enter into apoptotic cell death (Kastan et al., 1991; Kuerbitz et al., 1992). Thus failure to express functional p53 protein, after loss or mutation of the p53 gene, leads to an increased incidence of spontaneous tumours, as a result of instability to undergo cell-cycle arrest or to induce the apoptotic pathway of the cell death in response to a damage induced by an environmental agent. Inactivation of p53, with resulting inappropriate entry into S phase, leads to genetic instability (Lee, 1994) and aneuploidy in vitro (Livingstone et al., 1992; Yin et al., 1992) and in vivo (Chen et al., 1991; Blount et al., 1994).

Positive correlation between buccal apoptosis cells and OTM (comet assay) has been observed in the present study. The comet assay is very sensitive and detects DNA fragmentation occurring in the apoptotic process as early as exposure of phosphatidylserine residues on the outer leaflet. Thus the comet assay can be used for the recognition of apoptosis that follows the death signal caused, for example, by genotoxic stress as well as lack of survival signal as in growth factor deprivation (Florent et al., 1999). Present study also showed positive correlation between serum copper levels and buccal MN. Earlier, Lewinska et al., (2007) showed a significant increase in MN frequency in peripheral blood lymphocytes and in buccal epithelial cells of copper smelter workers, compared to the controls.

The results of the present study indicated that chewing of areca nut and tobacco have adverse effects on oral soft tissues in the form of higher oral mucosal lesions and also on mouth opening. The genotoxic and cytotoxic damage observed in the target tissue by buccal cytome assay and non-target tissue by CBMN and chromosomal aberration assay showed that toxicity induced by chewing materials may lead to genetic damage. The comet assay in the peripheral blood lymphocytes showed increased DNA damage among chewers. Alterations in the p53 gene has been observed among chewers and
OSMF subjects. The present study clearly indicates that areca nut and/or tobacco chewing have deleterious cytogenetic effects on the oral as well as peripheral tissues and might be responsible for the development of oral submucous fibrosis.