APPENDIX - I

NATIONAL INSTITUTE OF OCCUPATIONAL HEALTH
Meghaninagar, Ahmedabad 380016

EVALUATION OF CYTOGENETIC DAMAGE AMONG CHEWERS OF ARECA NUT AND TOBACCO

Dental OPD No.: □□□□□□□□□□ Date: □□□□□□□□

A. Personal information:

Name ________________________________

Address _______________________________________

Area: 1) Rural □ 2) Urban □

State to which belong ________________________________

Age: _____(Years)  Sex: 1) Male □ 2) Female □

Education: 1) Illiterate □ 2) Primary □ 3) Secondary □ 4) Graduate □

Marital Status 1) Married □ 2) Unmarried □

Occupation (Present)  Occupation (Past)

1) Physical Labour □ 1) Physical Labour □
2) Office Worker □ 2) Office Worker □
3) Industrial worker (Particularly exposed to dust or Chemicals) □
3) Industrial worker (Particularly exposed to dust or Chemicals) □
4) Student □ 4) Student □
5) Housewife □ 5) Housewife □
6) Tobacco related □ 6) Tobacco related □
7) Others____ □ 7) Others_______ □
8) Duration in Years □□ 8) Duration in Years □□
Knowledge & Belief

a) Do you know that:
   1. Areca nut or tobacco chewing can cause cancer
   2. Chewing drains your economy
   3. Chewing is a social problem
   4. Would you like to quit the habit? 1) No 2) Yes

b) Habit of chewing areca nut / Tobacco in family 1) No 2) Yes

c) Incidence of cancer in family 1) No 2) Yes

Personal Habits:
A. Chewing habit
   Have you ever chewed tobacco, areca-nut, pan-masala or any other mixture containing either or applied any of the these substances?
   1) No 2) Yes 3) Not known
   If yes, Chewing any mixture without Tobacco 1) No 2) Yes
   If yes, 1. Are you chewing Pan-masala (Plain-without tobacco)?
      1) No 2) Yes
   2. Are you chewing Areca nut (without tobacco) 1) No 2) Yes
   3. Are you chewing Pan (without tobacco)? 1) No 2) Yes

Chewing any mixture with Tobacco (Are you chewing tobacco in any form?) 1) No 2) Yes
   If yes, 1. Pan masala (Gutkha) 1) NO 2) Yes
   2. Tobacco with betel nut/areca-nut/lime (Mawa): 1) No 2) Yes
   3. Tobacco with lime (Khaini): 1) No 2) Yes
   4. Pan with Tobacco (Pan): 1) No 2) Yes
   5. Any other form of tobacco chewing :1) No 2) Yes
      If Yes, Please specify_______________________________
Site of placement in mouth

Where do you place any of these materials (Please specify)

1) Buccal left
2) Buccal right
3) Labial front
4) Do not place constantly at one place

If Chewers or Past Chewers, please specify

1) Frequency Nos./ day
2) Duration (Years)
3) What was your age when you started chewing? Yrs.
4) What made you to start chewing?
   1) Encourages by the friends
   2) Curiosity
   3) Feel self-pleasure
   4) Social status
   5) it relive from tension
   6) Other reasons (specify) ______________________

If past Chewers, since how long have given up Years

1) What made you to stop chewing?
   1) Due to bad habit
   2) Due to illness of throat or mouth
   3) Relative & friends does not like chewing
   4) Due to fear of major disease such as cancer
   5) Any other reasons
2) Do you (did you) swallow the chewed material when you chew?
   1) No   2) Yes   3) Occasionally

3) Do you keep the chew material in your mouth when you sleep?
   1) No   2) Yes   3) Occasionally

4) How many Minutes you keep/ chew the material in mouth? 

Any Other Tobacco habit (Snuff etc) ______________

Smoking Habit: 1) Non-smoker   2) Smoker   3) Ex-Smoker

If Smoker / Ex-Smoker, Please specify
  a) Frequency Nos./day  b) Duration (Yrs)
  c) If Ex-Smoker, since how long have you given up smoking (Yrs)

Alcoholism: 1) Non-alcoholic   2) Alcoholic   3) Past alcoholic

If alcoholic or past alcoholic, please specify
  1. Frequency (times/month)  2. Duration (Yrs)
  If past alcoholic, since how long have you given up (Yrs)

CLINICAL INTRA ORAL EXAMINATION:
1. Oral hygiene status: i) Good    ii) Fair    iv) Poor
2. Oral mucosal lesions:
   1) No   2) Yes
   i) Site  1) Buccal left   2) Buccal right   3) Upper Labial
   4) Lower Labial   5) Others
   ii) Size
   iii) Color  1) White   2) Pale   3) Pink
   iv) Contour
   v) Texture  1) Stiff   2) Rough
   vi) Degree of severity 1) Mild   2) Moderate   3) Severe
3. Specific lesions:-

1) Lichen planus  
   1) No  2) Yes

2) Leucoplakia  
   1) No  2) Yes

If Yes: 1---Color texture change but no thickness
   2—Color & texture change with moderate thickening
   3---No normal color severe texture changes heavy
       thickening/Creating

3. Sub mucous Fibrosis:  
   1) No  2) Yes

If yes 1) Mild  2) Moderate  3) Severe

4. Ulcer  
   1) No  2) Yes

5. Any other lesions (Please specify_______________________

Mouth Opening

   i) Inter-incisor distance in cm.  
   ii) Inter-molar distance in cm.

♦ If Oral Disease is present, patient is referred to
   _________________ Department

BIOLOGICAL SAMPLES COLLECTED:

1) Blood sample

2) Buccal sample
List of Publications/ Presentations


Joshi MS, Lakkad BC, Parmar G, Kumar S. International conference of Biomedical and genomic research organized by Human genetic Centre (HGC), Gujarat University, Ahmedabad, January 2009.
Cytogenetic alterations in buccal mucosa cells of chewers of areca nut and tobacco

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ABSTRACT

Objective: The rationale of the study was to evaluate the cytological alterations especially micronucleus (MN) and other nuclear anomalies in buccal mucosa cells of chewers to understand the genotoxic and clastogenic potential of chewing mixture (containing areca nut and tobacco as main ingredients).

Methods: The buccal cytome assay involves the examination of epithelial smear to determine micronucleated cell and other nuclear anomalies after the Feulgen plus light green staining. The assay was applied to exfoliated buccal mucosa cells of 262 subjects [non-chewers – 161 and chewers – 101 (includes 20 subjects with OSMF)] and 1000 cells per individual were examined microscopically. Nuclear anomalies were compared among chewers, non-chewers and OSMF subjects and correlated with consumption of quids per day and duration of chewing in years.

Results: MN cells were found significantly (p < 0.0001) higher among chewers and OSMF subjects as compared to non-chewers. Further analysis indicated that MN was significantly higher in OSMF subjects with respect to even chewers. Nuclear buds were significantly higher (p < 0.0001) in OSMF subjects as compared to chewers as well as non-chewers. Nuclear anomalies viz. binucleated, karyorrhexis and karyolysis were also considerably higher in OSMF subjects as compared to non-chewers.

Conclusion: The MN and other nuclear anomalies reflected genetic damage and cytotoxicity, associated with tobacco and areca nut consumption. Further, these data reveal a risk for development of OSMF among chewers of mixture containing areca nut and/or tobacco, as all the OSMF subjects were chewers.

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1. Introduction

Betel quid/areca nut use is common in Southeast Asia and the Asia Pacific region and also among migrated communities in Africa, Europe and North America. Use of tobacco and tobacco-related products are as old as the earliest records of oral cancer. In India, one-third of men and about 1.6% women aged 15–49 years smoke, while more than one-third (38.1%) of the men and around one-tenth (9.9%) of the women use smokeless tobacco [National Family Health Survey (NFHS-3) conducted in 2005–06]. Areca nut and betel quid chewing leads to oral submucous fibrosis (OSMF), a potentially precancerous condition of the oral mucosa. Betel quid chewing is a major risk factor for cancer of the buccal mucosa and gingival. The habit of tobacco and areca nut chewing has been reported to be associated with cancers of the mouth, pharyngeal cavity and upper part of the digestive tract. Further, chewing and smoking habit have been reported to act synergistically in these cancers. Epidemiological studies also showed that betel quid chew-
ing, together with tobacco chewing or smoking is associated with the increased risk of oral cancer. Based on the studies available on pan masala (mixture of areca nut, catechu, lime, cardamom, spices, and unspecified flavouring agents, etc.), with tobacco (gutkha) or without tobacco (plain) suggests that it has the potential in causation of various oral diseases. Studies reviewed on these chewing mixtures also revealed that it is likely to be carcinogenic, as tobacco and areca nut have carcinogenic potential and both have encompassing addictive potential leading to dependence on chewing mixture containing areca nut and tobacco.8

The micronucleus (MN) assay has been used as a biomarker of genetic damage in buccal mucosa cells9,10 which are in direct contact with the chewing material. The increased use of the MN assay in buccal cells, has led to a wide diversity in refining the techniques, timing for cell collection relative to exposure period, cellular and nuclear staining procedures, scoring criteria for MN and other nuclear anomalies.11,12 Owing to these cytogenetics changes in target tissue, i.e. buccal mucosa cells were studied among chewers using chewing mixture containing mainly areca nut and tobacco.

2. Materials and methods

The subjects were enrolled randomly among apparently healthy subjects attending OPD of Government Dental College and Hospital, Ahmedabad, India and categorised as chewers (101) and non-chewers (161). Among chewers, 20 subjects were diagnosed as OSMF clinically. The chewers were chewing mainly areca nut, pan masala (plain and gutkha), mawa (a mixture of sun-cured unflavoured tobacco, dried pieces of areca nut, and lime) and tobacco with lime (kheni), etc. This study is a part of a major study for which ethical approval was obtained from Institutional Ethics Committee. The mean age of the subjects was 36.5 years for chewers, non-chewers and OSMF subjects, respectively. Lifetime chewing exposure was taken into account.

Before the collection of buccal mucosa cells, subjects were asked to rinse the mouth thoroughly with tap water. The smears were then stained with Schiff’s reagent for about 90 min and transferred to tap water for 10 min. Slides were dipped 3 times in 0.5% sodium metabisulphite solution and then rinsed in tap water. The slides were counter stained with 1% light green, cleared by xylene and mounted with DPX. Cytome analysis was carried out at 63 and 100× using light microscope (Leica Germany, DMLA) as per the criteria of Thomas et al.14 Slides were placed for 10 min in 1 N HCl at 60 °C and rinsed in distilled water for 3 min. The smears were allowed to air dry at room temperature. The coded slides were then stained with Schiff’s reagent for about 90 min and transferred to tap water for 10 min. Slides were multiplied by the duration of habit and it was termed as lifetime chewing exposure (LCE) as calculated for tobacco consumption.13

Before the collection of buccal mucosa cells, subjects were instructed to wash the mouth thoroughly with tap water. The exfoliated buccal mucosa cells were scraped using wooden spatula with cotton plug. These cells were spread on to the clean micro glass slides and fixed in Carnoy’s fixative (methanol and glacial acetic acid in the ratio of 3:1) for 30–35 min. The slides were allowed to air dry at room temperature. The coded slides were stained with Feulgen reaction for micronuclei following the modified method of Thomas et al.14 Slides were placed for 10 min in 1 N HCl at 60 °C and rinsed in distilled water for 3 min. The smears were then then stained with Schiff’s reagent for about 90 min and transferred to tap water for 10 min. Slides were dipped 3 times in 0.5% sodium metabisulphite solution and then rinsed in tap water. The slides were counter stained with 1% light green, cleared by xylene and mounted with DPX. Cytome analysis was carried out at 63 and 100× using light microscope (Leica Germany, DMLA) as per the criteria of Thomas et al.14 and 1000 cells per subjects were examined. The frequency of MN was calculated as total number of MN, next to the main nucleus, as well as those in binucleated cells. Furthermore, metanucleated anomalies other than MN, such as binucleated cells (BN), karyorrhexis (KR), karyolysis (KL) and N Buds (NB) were also taken into account.

Data were subjected to statistical analysis using SPSS software (version 16.1). One-way analysis of variance (ANOVA), Tukey’s test was performed to compare MN, BN, KL and NB between chewers, OSMF and non-chewers. Pearson’s correlation analysis was performed to correlate chewers and MN, BN and NB. Frequencies of MN, BN and NB among chewers were correlated with LCE using linear regression analysis.

3. Results

The frequency (%) of micronuclei in buccal mucosa cells, nuclear buds, proliferation markers (normal differentiated cells, binucleated cells) and cell death parameters (karyorrhexis cells and karyolytic cells) are summarised in Table 1. Cells with MN were significantly (p < 0.0001) higher in chewers and OSMF subjects, while nuclear buds were significantly higher (p < 0.0001) only in OSMF subjects as compared to non-chewers. Further, binucleated cell frequency increased in OSMF subjects with respect to non-chewers. Karyolytic cells were also significantly higher in OSMF subjects (p < 0.05) with respect to non-chewers. Further, an elevation in karyorrhexis cells was also found among chewers and OSMF subjects as compared to the non-chewers (Table 1) but these alterations were statistically non-significant. In addition,

| Table 1 – Frequency of normal differentiated cell, micronuclei in buccal mucosal cells (MN), karyorrhexis (KR), binucleated cell (BN), karyolysis (KL), nuclear buds (NB) in chewers, OSMF subjects and non-chewers (NC). |
|-----------------|--------|--------|---------|--------|--------|--------|
| Normal differentiated cells | MN | N Buds | BN | KL | KR |
| NC (n = 161) | 88.24 ± 0.53 | 0.35 ± 0.03 | 0.39 ± 0.03 | 1.00 ± 0.05 | 8.65 ± 0.46 | 1.34 ± 0.11 |
| Chewers (n = 81) | 85.81 ± 0.98 | 0.68 ± 0.09 | 0.57 ± 0.08 | 0.89 ± 0.07 | 10.59 ± 0.88 | 1.43 ± 0.16 |
| OSMF (n = 20) | 81.93 ± 1.23 | 1.18 ± 0.18 | 1.41 ± 0.19 | 1.43 ± 0.28 | 12.83 ± 1.98 | 1.88 ± 0.26 |

Values are mean ± SE; n = number of subjects.

* p < 0.05 compared with NC.
* b p < 0.0001 compared with NC.
* c p < 0.05 compared with chewers.
* d p < 0.0001 compared with chewers.
normal differentiated cells were significantly lower in OSMF subjects \( (p < 0.05) \) and marginally decreased among chewers with respect to non-chewers. Further MN, N Buds and BN cells were significantly higher among OSMF subjects as compared to chewers.

The data pertaining to MN, N Buds, BN, KR and KL with respect to quids chewed/day and duration of chewing in years is shown in Table 2. The representative photographs of micronucleated cell, nuclear buds and binucleated cell in buccal epithelial cell have been shown in Fig. 1. Dose and duration dependant elevation in MN and N Buds were observed. The linear regression analysis reflected fairly linear relationship between LCE verses MN, N Buds and BN. The linear coefficient correlation and regression equation is shown in Fig. 2. The frequency of BN, MN and N Buds correlated positively with LCE having correlation coefficient \( R^2 = 0.94, 0.71 \) and 0.25, respectively.

The Pearson correlation coefficients were analysed between different variables MN, N Buds and BN of the buccal cytome assay. The positive correlation was found between MN with N Buds \( (r = 0.578, p < 0.0001) \) and MN with BN \( (r = 0.276, p < 0.0001) \). Further, analysis indicated that all the OSMF cases were chewers of mixture containing mainly areca nut and tobacco indicating the role of areca nut and tobacco in development of OSMF.

### Table 2 – Frequency of micronuclei (MN) in buccal mucosal cells, karyorrhexis (KR), binucleated (BN), karyolysis (KL), ‘broken egg’/nuclear buds (N Buds) with respect to frequency/day and duration in years of chewing.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Normal differentiated cells</th>
<th>MN</th>
<th>N Buds</th>
<th>BN</th>
<th>KL</th>
<th>KR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>88.24±0.53</td>
<td>0.35±0.03</td>
<td>0.39±0.03</td>
<td>1.00±0.05</td>
<td>8.65±0.46</td>
</tr>
<tr>
<td>Chewing frequency (quids/day)</td>
<td>≤5</td>
<td>85.87±0.97</td>
<td>0.70±0.09*</td>
<td>0.66±0.85*</td>
<td>0.90±0.07</td>
<td>10.50±0.86</td>
</tr>
<tr>
<td></td>
<td>6–9</td>
<td>83.16±2.26</td>
<td>1.04±0.15**</td>
<td>0.90±0.14**</td>
<td>1.18±0.21</td>
<td>12.25±2.25</td>
</tr>
<tr>
<td></td>
<td>≥10</td>
<td>83.00±3.88</td>
<td>0.90±0.34*</td>
<td>0.95±0.17*</td>
<td>1.31±0.45</td>
<td>12.37±3.19</td>
</tr>
<tr>
<td>Chewing duration (years)</td>
<td></td>
<td>85.50±1.00</td>
<td>0.75±0.10**</td>
<td>0.71±0.08**</td>
<td>0.94±0.09</td>
<td>10.69±0.86</td>
</tr>
<tr>
<td></td>
<td>≤10</td>
<td>82.52±3.2</td>
<td>0.80±0.19</td>
<td>0.55±0.10</td>
<td>0.99±0.15</td>
<td>13.44±2.85</td>
</tr>
<tr>
<td></td>
<td>11–19</td>
<td>86.05±2.00</td>
<td>1.06±0.37**</td>
<td>1.30±0.53***</td>
<td>1.50±0.44</td>
<td>9.19±1.11</td>
</tr>
<tr>
<td></td>
<td>≥20</td>
<td></td>
<td></td>
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</tbody>
</table>

Values are mean ± SE.

* \( p < 0.05 \).
** \( p < 0.001 \).
*** \( p < 0.0001 \).

### 4. Discussion

The higher frequency of oral cancer in Southeast Asia as compared to other part of the world is believed to be associated with higher prevalence of tobacco and areca nut chewing in this part of the world. It was eminent that all the OSMF subjects were chewers of mainly areca nut and tobacco indicating their role in the causation of oral diseases. Further, Ariyawardana et al. had shown a strong association of betel quid chewing (including tobacco as an ingredient) with the causation of OSMF.16 Results from studies conducted in 1996 indicated 2.5 million people globally whereas in 2002 showed more than 5 million people having OSMF among Indian population.17 However, these data might be underestimation of the disease as habit increased alarmingly in recent years. Gupta et al. concluded an increase in the prevalence of OSMF, especially in the lower age group, directly attributable to the use of areca nut products.18 OSMF is an excellent model for the study of molecular events occurring in a pre-malignant condition. The transformation rates of pre-cancers to cancers vary from 0.5 to 15 percent.19 Arecoline, safrole and nicotine which are released in saliva during betel quid chewing plus cigarette smoking, inhibit collagen phagocytosis by fibroblasts in a dose-dependent manner and may induce OSMF.

![Fig. 1 – Nuclear anomalies in buccal epithelial cells: (a) micronuclei, (b) nuclear buds and (c) binucleated cell.](archives_of_oral_biology_56_2011_63-67)
formation. Areca nut alkaloid, arecoline and its hydrolysed product arecaidine stimulate the cultured fibroblast in a dose-dependent manner shown in an in vitro study by Harvey et al. Thus it can be inferred that areca nut along with tobacco had major role in development of OSMF.

A significant increase in micronuclei in buccal mucosa cells was observed among chewers and OSMF subjects with respect to non-chewers. The elevation was more prominent among OSMF subjects than chewers and non-chewers in descending fashion indicating the genotoxic potential of these chewing materials and also their role in OSMF. Further, dose and duration dependent increase in MN was also noted. Earlier experimental study also indicated an increased frequency of MN in bone marrow cells of mice exposed to panmasala plain and panmasala with tobacco (Gutkha). A high frequency of MN has been observed among tobacco users and similarly an increased frequency of MN has also been reported in panmasala consumers by Gandhi and Kaur. In buccal mucosa cells of chewers an increased frequency of nuclear anomalies was observed that in turn may indicate adverse cellular effect to eliminate cells with genetic damage. A recent finding indicated that micronuclei frequencies in oral exfoliated cell were found to be higher in squamous cell carcinoma patients than in control subjects.

It is suggested that apoptosis also act as a surveillance mechanism, eliminating the cells with genetic damage. Thus, apoptosis in excess of normal levels may serve as an indicator of genotoxic insult. Occurrence of increased percentage frequencies of KL anomaly has significance as these occur in the pre-keratinization process. This anomaly represents cytotoxicity, which is also evident in necrotic cells. 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. A study carried out among firefighters indicated that in addition to elevated MN frequency, raised prevalence of several other nuclear anomalies like N Buds, binuclei, karyorrhexis and karyolysis were also exhibited. Karyolysis is associated with cytotoxicity, and karyorrhexis accompany apoptosis, a process under genetic control.

Areca nut contains not only genotoxic and cytotoxic agents but also have the ability to stimulate human buccal mucosal fibroblast proliferation, and which might act synergistically in the pathogenesis of oral submucous fibrosis and oral cancer. Areca nut is also reported to possess cytotoxic, mutagenic and genotoxic properties. In addition to genotoxic effects, these chewing mixtures may have other adverse effects on periodontal tissues and oral hygiene status of the chewers.

Results of the present study affirms that any mixture containing areca nut and tobacco have genotoxic and cytotoxic potential that induces the nuclear anomalies in the buccal mucosa cells indicating higher oral diseases among chewers as indicated by all the OSMF subjects which were chewers.

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**Competing interests**: None declared.

**Ethical approval**: Not required.

**References**


2. Chang YC, Hu CC, Tai KW, Liao PH, Yang SH, Chou LS, Chou MY. Cytotoxicity and genotoxicity of areca nut related