Material and Methods
The present study is conducted to evaluate the possible correlation of areca nut and tobacco chewing habits with diseases of oral cavity including lesions of oral soft and hard tissues. The history of habitual usage of various products containing mainly areca nut with or without tobacco, lime, catechu that are prevalent in this part of the world is taken into consideration. The micronuclei in exfoliated buccal mucosa cells were studied as oral mucosa is in close contact with the quid while chewing, which might serve as early biomarker of oral lesions. In addition, copper and zinc levels in saliva and serum were studied to find out any relationship between these metals with the changes in oral cavity induced by the chewing material. The concentrations of these metals were also determined in the chewing material using atomic absorption spectrophotometer. Further, immunoglobulin levels were determined using immunodiffusion method in representative number of samples of chewers and non-chewers.

STUDY DESIGN AND POPULATION:

Selection of Subjects

A cross sectional study was conducted among apparently healthy patients attending Out Patient Department (OPD) of Government Dental College and Hospital, Ahmedabad, Gujarat, India for various dental diseases as well as subjects from the Gynecology and Ophthalmology OPD of Civil Hospital, Ahmedabad (Gujarat), India. The study population comprised of patient group seeking treatment at dental hospital because of some dental
ailment, and Eye and Gynecology OPD for ophthalmic and gynecological ailments, respectively. These patients attended the hospital for complaints other than those related to tobacco chewing. The subjects were enrolled randomly and divided as chewers and non-chewers for comparison. The objectives of the proposed study as well as the benefits of the study to individual, society as a whole and the health planner were explained to them. Further, they were informed about the possible ill effects of chewing tobacco and areca nut. A written consent to participate in study was obtained from each subject. The subjects thus enrolled were either non-chewers or chewers with varying frequency and duration of chewing, smoking and drinking habits. The following criteria were used for inclusion of subjects for the present study:

- No history of drug addiction.
- Not taking any medicines regularly or those that alter the immune response.
- No history of upper respiratory disease of acute, chronic or infectious origin.
- No history of diabetes mellitus, essential hypertension or ischemic heart disease.
- No known history of psychological disorder.

A specific proforma was designed with the assistance of epidemiologist and bio-statistician (Appendix-1). The same was pre-tested before initiation of the study and necessary modifications were made in the proforma accordingly. All the subjects were examined in the Department of Conservative Dentistry, Government Dental College and Hospital, Ahmedabad, India. The following salient information was recorded on proforma by administering the questionnaire to the subjects and by conducting clinical observation of oral cavity.

1. Personal information comprising of name, age, sex, occupation, religion, educational status, annual income and address (whether rural or urban) etc.
2. Oral hygiene measures undertaken.

3. Awareness of possible ill effects of chewing habits.

4. Present relevant complaints associated with chewing habits.

5. Detailed history of chewing habits—product(s) used e.g. areca nut alone (Fig. 1), areca nut with lime and tobacco—locally known as mawa (Fig. 2), tobacco alone (Fig. 3), pan masala plain or pan masala gutkha (Fig. 4), pan (Fig. 5), khaini—a mixture of areca nut with lime (Fig. 6) etc. Duration of habit in years, number of quids per day, duration for keeping quid in mouth, site of placement were also recorded. It was also noted whether the chew was swallowed after chewing or kept it in mouth while sleeping.

6. Details of smoking and drinking habit along with duration and frequency.

7. Information about any difficulty in deglutition, mouth opening, burning sensation of soft tissues, which might be used as indicator of existence and extension of lesions was also recorded.

8. Detailed clinical examination of oral cavity was carried out to note the oral hygiene status, any lesion on cheek, palate, pillars of fauces, lips, retro molar area and tongue and also the site, size, color, contour and texture of the lesions.

9. Gums were examined for periodontal pockets, recession etc.

10. Teeth were examined for staining and attrition; sensitivity towards cold food and beverages was also recorded. In addition, the number of missing teeth and any history of dental treatment were noted.
Representative photographs of various lesions were taken during the course of the study. Following clinical observations were carried out by adopting various standard criteria/methods:

**Oral Hygiene Status:**

The oral hygiene status of the selected subjects was determined by using Simplified Oral Hygiene Index (OHI-S). The OHI-S, introduced by Greene and Vermillion in 1964, comprises of Simplified Debris Index (DI-S) and Simplified Calculus Index (CI-S) (Peter, 1999a). Each of these indexes, in turn, is based on numerical determinations representing the amount of debris or calculus on six pre-selected tooth surfaces. The subjects were examined under artificial light using mouth mirror, explorer and curved probe.

**Debris Index (DI-S):**

It was used for the evaluation of the extent of debris present on the six pre-selected tooth surfaces i.e. buccal surfaces of the selected upper molars, lingual surfaces of the selected lower molars, labial surfaces of the upper right and lower left central incisors. The surface area covered by debris was estimated by running explorer on each tooth surface.

**Debris Index – Simplified – Scoring System**

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Absence of debris</td>
</tr>
<tr>
<td>1</td>
<td>Soft debris covering less than cervical one-third of tooth surface.</td>
</tr>
<tr>
<td>2</td>
<td>Soft debris covering more than cervical one-third of the exposed tooth surface but less than cervical two-third.</td>
</tr>
<tr>
<td>3</td>
<td>Soft debris covering more than cervical two-third of the exposed tooth surface.</td>
</tr>
</tbody>
</table>
Calculus Index – Simplified (CI-S):

An explorer was used for scoring of calculus. The same teeth were examined as for debris index. The surface area covered by calculus was detected supragingivally and subgingival calculus was explored for randomly selected quadrant of teeth.

Calculus Index – Simplified – Scoring System:

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Absence of calculus</td>
</tr>
<tr>
<td>1</td>
<td>Calculus covering less than cervical one-third of the exposed tooth surface.</td>
</tr>
<tr>
<td>2</td>
<td>Supragingival calculus covering more than cervical one-third, but not more than cervical two-third of the exposed tooth surface, or presence of individual flecks of subgingival calculus around the cervical portion of tooth.</td>
</tr>
<tr>
<td>3</td>
<td>Supragingival calculus covering more than cervical two-third of the exposed tooth surface, or a continuous heavy band of subgingival calculus around the cervical portion of tooth.</td>
</tr>
</tbody>
</table>

The Simplified Oral Hygiene Index score for the individual is obtained by combining the Simplified Debris Index and Calculus Index. The Simplified Debris Index (DI-S) score of an individual is obtained by totaling the debris score per tooth surface and dividing by the number of the surfaces examined. The same method is used to obtain CI-S

Thus,

\[ OHI - S = DI - S + CI - S \]
The Simplified Oral Hygiene Index (OHI-S) values ranges from 0 to 6. The clinical levels of oral hygiene that can be associated with group OHI-S scores are as follows:

- **Good** – 0.0 to 1.2
- **Fair** – 1.3 to 3.0
- **Poor** – 3.1 to 6.0

**Examination of teeth**

The examination was earned out by doing following observations:

(a) **Attrition (Tooth Wear)**
(b) **Caries**
(c) **Extrinsic tobacco stains**

**Attrition index:**

The data on attrition i.e. wear patterns whether present or absent was collected from all the subjects. The subject was considered positive for attrition, if destruction of cusp and presence of small facet on occlusal surface was seen clinically and presented data is in the form of attrition present or absent. However, criteria of Scott (1979) and Smith (1984) were considered for the attrition present or absent.

**Caries Index (DMF- Decayed-Missing-Filled Index):**

DMF index is the most universally employed index for measuring dental caries. The DMF Index is applied only to permanent teeth. The Decayed–Missing–Filled Index (DMF Index) was introduced by Klein *et al.* 1938 as quoted by Peter, 1999b and described as follows:

- **D** – used to describe decayed teeth.
- **M** – used to describe missing teeth due to caries.
- **F** – used to describe teeth that have been previously filled.
The following principles and criteria were adopted in recording DMF:

- Tooth was not counted more than once. It was recorded either as decayed, missing, filled or sound.
- Decayed, missing and filled teeth were recorded separately since the components of DMF are of great interest.
- While counting the number of decayed teeth, those teeth were also included which had restorations with recurrent decay.
- Care was taken to list as missing only those teeth, which were lost due to decay. Also included were those teeth, which were so badly decayed that they were indicated for extraction.

**Calculation of the Index:**

a. Individual DMFT:
   Total each component, i.e. D, M, & F separately, then, total D+M+F = DMF

b. Group Average:
   Total the D, M and F for each individual. Then, divide the total 'DMF' by the number of individuals in the group.

\[
i.e. \text{ Average DMF} = \frac{\text{Total DMF}}{\text{Total number of the subjects examined}}\]

**Stains Index:**

The following criteria were evolved in a pilot study for scoring of stains on randomly selected quadrant of teeth and the same was used in the study (Shah, 1996).
<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Absence of stains</td>
</tr>
<tr>
<td>1</td>
<td>Stains covering one-third of exposed tooth surface.</td>
</tr>
<tr>
<td>2</td>
<td>Stains covering the tooth surface more than cervical one-third, but not more than cervical two-third portion of the tooth.</td>
</tr>
<tr>
<td>3</td>
<td>Stains covering more than cervical two-third of exposed tooth surface.</td>
</tr>
</tbody>
</table>

**Sensitivity of teeth towards cold food and beverages:**
Each subject was asked about the sensitivity towards cold food and beverages especially with cold water and the verbal response was noted accordingly.

**Gingival Recession:**
This was done to specifically determine the extent of gingival recession, i.e. displacement of the gingival margin at least 1 mm apical to the cemento-enamel junction in chewers. The same was also studied in control group for the comparison.

**Measurement of mouth opening:**
The subjects were asked to open the mouth to their maximum and measurement of distance between upper and lower incisors and molars were carried out using Vernier caliper at mm level and the subjects were sub grouped according to the chewing habits.

**Oral soft tissue lesions:**
Clinical examination of oral cavity was carried out using curved probe, mouth mirror and torch light to observe the clinical changes, if any, in oral cavity of all the subjects. The oral cavity was checked for submucous fibrosis, leukoplakia, lichen planus, and pigmentation in oral cavity etc. The salient
clinical features of these lesions, which are used to record the lesions in this study, are described below.

Oral submucous fibrosis is characterized by early symptoms of burning sensation in mouth especially on eating spicy food. Vesicle formation, ulceration, excessive salivation/xerostomia and defective gustatory sensation may accompany the disease. Ultimately, the subject develops stiffening of certain areas of the oral mucosa with difficulty in opening the mouth and swallowing the solid food. The mucosa eventually becomes blanched and opaque and fibrotic bands appear usually involving buccal mucosa, soft palate, lips and tongue (Shafer et al. 1997a).

Oral submucous fibrosis has been graded into three stages according to clinical signs and symptoms as follows (Bhatt and Dholakia, 1977):

- **Mild** - Very slight fibrous banding and little narrowing of mouth opening.
- **Moderate** - Moderately pronounced symptoms, fibrous bands extending from cheek to palate.
- **Severe** - Excessive amount of fibrous banding involving cheeks, palate, uvula, tongue and lips and marked narrowing of mouth opening.

WHO (1978) criteria were adopted for the diagnosis of leukoplakia. Leukoplakia is a raised white patch of the oral mucosa measuring 5 mm or more which cannot be scrapped off and which cannot be attributed clinically or pathologically to any other diagnosable disease. On clinical examination, patches of leukoplakia may vary from a non-palpable, faintly translucent white area to thick, fissured, papillomatous, indurated lesions. An intermediate stage consists of localized or diffuse, slightly elevated plaques of irregular outline, which are opaque, white and may have a fine granular texture. The
surface of the lesion is often finely wrinkled or shriveled in appearance and may feel rough on palpation. The lesions are white, gray or yellowish-white but, with heavy use of tobacco, may assume a brownish-yellow colour.

In the oral cavity, lichen planus appears as lesions consisting of radiating white or gray, velvety, threadlike papules in a linear, annular or retiform arrangement forming typical lacy, reticular patches, rings and streaks over the buccal mucosa and to a lesser extent on the lips, tongue and palate. A tiny white elevated dot is often found at the intersection of white lines, known as striae of Whickham. When plaque like lesions occur, radiating striae may often be seen on their periphery. These lesions usually produce no symptoms, although occasionally patients will complain of a burning sensation in the involved areas (Shafer et al. 1997b).

In addition, other oral mucosal lesions such as nicotine stomatitis (Smoker's palate), tobacco quid lesion, leukoedema and ulceration on soft tissues were also diagnosed (Neville et al. 2004). Nicotine stomatitis is a white keratotic change associated with tobacco smoking. The palatal mucosa becomes diffusely gray or white; numerous slightly elevated on the teeth. Leukoedema is characterized by a diffuse, grayish-white, milky, opalescent papules are noted with punctate red centers. A heavy brown or black tobacco stain may be present appearance of the mucosa. The surface appears folded, resulting in wrinkles or whitish streaks. The white appearance disappears when the cheek is everted and stretched.

The appearance of tobacco-quid lesion varies from superficial lesion with a color similar to the mucosa with slight wrinkling and no obvious thickening to a marked white or yellowish to brown and heavily wrinkled lesion with intervening deep and reddened furrows and or heavy thickening (Zain, 1999). In this study, all the oral lesions are diagnosed using the above mentioned criteria among chewers and non-chewers.
Collection of the buccal mucosa cells for micronuclei (MN) study:

The buccal mucosa cells were scrapped with wooden spatula containing wet cotton plug at the top from the representative number of chewers and non-chewers. These cells were spread over on to the clean micro glass slides. These smears were fixed in a solution of ethanol and glacial acetic acid at the ratio of 3:1 for 30-35 minutes. The slides were kept at room temperature and allowed to air dry. The slides were stained with Feulgan reaction (Culling, 1974) for micronuclei with some modification in order to obtain best possible staining. The reaction is based upon cleavage of the purine-deoxyribose bond by mild acid hydrolysis to expose a reactive aldehyde group. The aldehydes then react with Schiff reagent to restore the latter's quinoid structure, which in turn imparts red color to the DNA. The detailed method for the preparation of Schiff reagent (de Thomasi Schiff reagent) is given in Appendix-2. After the preparation of the reagent, the slides were stained as described below:

1. Slides were placed in coupling jar for 6-8 minutes in 1N HCl at 60 °C.
2. Slides were rinsed in distilled water for few minutes (2-3 minutes)
3. Slides were placed in the Schiff's reagents for about 90 minutes.
4. Slides were transferred to tap water for 10 minutes.
5. Slides were dipped five – six times in 0.5% sodium metabisulfite solution.
6. These were then rinsed in tap water.
7. After thorough rinsing, the slides were immersed for 1 minute in light green solution (counter-stain, 1gm light green dissolved in 100ml of 95% alcohol).
8. Slides were then dipped for a few seconds or two dips in absolute alcohol for dehydration. The same was repeated to ensure dehydration.
9. Cleared in xylene for 6-8 minutes.
10. Dehydrated smears were mounted with DPX.
The micronuclei were observed at 40X, 63X and 100X magnifications using light microscope (Leica, Germany). The criteria used for identification of MN were: round to oval shape and size less than one-third of the diameter of the main nucleus. Further, it was ensured that the micronucleus be in the same focal plane as the main nucleus, yet clearly separated from it and exhibiting similar staining intensity (Schmid, 1976). Coded slides were mostly observed at 400 X magnifications to detect MN and confirmation was done at 1000X. The slides were decoded and they were grouped as chewers, non-chewers.

**Collection of saliva and measurement of pH:**

The subjects were asked to rinse their oral cavity with tap water. Thereafter about 50 ml of distilled water was given to the subjects to gargle for 20-30 seconds in oral cavity with normal force and spit out. After about 5 minutes the subjects were asked to collect saliva in sterilized tubes. Saliva samples were collected between 10 to 12 a.m. Four to six milliliters of saliva was collected over a 10-12 minutes period in plastic tubes. The samples were then immediately transported to the National Institute of Occupational Health (NIOH), Ahmedabad (Gujarat), India, within one hour of collection. The sample was filtered using good quality filter paper and pH was measured using the digital pH meter (Systronic, India). The saliva sample was stored at -20 °C in deep freeze (REMI, India).

**Collection of blood sample:**

Five–six milliliters of blood was collected from medial cubital vein using vaccutainer syringe. Serum was separated by centrifuge machine (REMI, India) at 3,000 rpm for 10 minutes if necessary and stored at -20 °C in deep freezer for the analysis of metals i.e. copper and zinc and immunoglobulins.
Determination of Immunoglobulins in the serum:

Serum IgG, IgA, and IgM were estimated according to the method of Mancini et al. (1965). He introduced a novel technique involving single diffusion for accurate quantitative determination of antigens. Radial diffusion is based on the principle that a quantitative relationship exists between the amount of antigen placed in a well cut in the agar-antibody plate and resulting ring of precipitation (Fig. 7, 8 and 9). The area circumscribed by the precipitation ring is proportionate to the antigen concentration. The standard kits for the estimation of immunoglobulins were procured from M/s Biocientifica S.A. Buerous Aires, Argentina. The immunodiffusion plates were stored in a flat surface in the refrigerator. The serum samples as well as immunodiffusion plates were brought to room temperature before use. The first well was used for the control sera while other wells were filled with 5 µl of the sera of the subjects using micropipette as per the instruction provided with the kit. The plates were kept at room temperature for incubation for about 20 hrs. After incubation the precipitated ring was measured with 0.1mm precision level. The concentration of immunoglobulins was determined according to the diameter from the reference table supplied by the manufacturer.

Estimation of Copper and Zinc level in various chewing materials:

Pan masala (plain) of five different brands and dried pieces of commercially available different types of areca nut were purchased from the local market for the estimation of copper and zinc. The copper and zinc were estimated after wet digestion of these materials using the mixture of nitric acid (HNO₃) and perchloric acid (HClO₄).

Wet Digestion Procedure:

About 2-3 gm of pan masala and areca nut each (at least five samples of each chewing materials) was digested with 15 ml of acid digestion mixture
(nitric acid: perchloric acid) in the proportion of 2:1 in 150 ml conical flask. The solution was heated gently in the beginning on sand bath on the hotplate for 5-6 hrs. After this, the samples were digested directly on hotplate until they became colorless. If required the mixture of HNO₃ + HClO₄ was added drop by drop till solution became colorless. Finally, 10 ml volume of the solution was made using 1M HNO₃. Blank sample was run parallel during the digestion procedure.

**Pretreatment or dilution of serum and saliva for copper and zinc estimation:**

The serum and saliva samples were brought to the room temperature and diluted with deionised water as follows for metal estimation:

For the determination of copper in serum, the sample was diluted with an equal volume of deionised water (1:1) and for the estimation of zinc, the sample was diluted with deionised water in the proportion of 1:3. The same dilution was used throughout the study.

**Analytical Procedure for estimation of copper and zinc:**

The digested samples of chewing materials as well as diluted serum and saliva samples were brought to trace metal laboratory for copper and zinc analysis. These samples were analyzed using Atomic Absorption Spectrophotometer (AAS), Perkin Elmer Double beam Model No.3100, USA.

**Principle of Atomic Absorption Spectrophotometer (AAS):**

Every element has a specific number of electrons associated with its nucleus. The normal and most stable orbital configuration of an atom is known as the "ground state". The "ground state" atom absorbs light energy of a specific wavelength (from hollow cathode lamp) as it enters the "excited state". As the number of atoms in the light path increases, the amount of light absorbed also increases. By measuring the amount of light absorbed, a
quantitative determination of the amount of analyte can be made. The use of special light sources and careful selection of wavelengths allow the specific determination of individual elements (Perkin Elmer, 2000).

\[ \text{Light source} \rightarrow \text{Ground state atom} \rightarrow \text{Excited state atom} \]

**Fig:** Atomic Absorption Process (Adopted from “Analytical Methods for Atomic Absorption Spectrometry, Perkin Elmer, 2000”)

The lamp and wavelength used for analysis of Cu and Zn are as follows:
- **Lamp used for Cu:** Hollow Cathode Lamp, Lumina.
- **Wavelength for Cu:** 324.7 nm.
- **Lamp used for Zn:** Electrodless Discharge Lamp.
- **Wavelength for Zn:** 213.9 nm.

The standards used for the above analysis were of E Merck (ICP Grade). The sample readings were taken and multiplied with appropriate correction factor.

The data from the predesigned proforma was entered and analyzed using Epi Info-6 software program. The statistical significance between various groups was determined using various tests such as Chi square test, Student ‘t’ test, ANOVA and non-parametric tests as required.
Fig. 1 – Photograph showing sliced areca nut, which may be chewed alone or with other chewing products

Fig. 2 – Photograph showing contents of mawa - areca nut, tobacco and slaked lime
Fig. 3 – Photograph showing plain tobacco

Fig. 4 – Photograph showing contents of Gutkha.
Fig. 5 – Photograph showing leaf of betel (plain) and with contents of pan containing tobacco

Fig. 6 – Photograph showing contents of khaini – tobacco and slaked lime
Fig. 7 - Photograph showing agar-antibody plate (along with cover) with precipitation rings for determination of IgG.

Fig. 8 - Photograph showing agar-antibody plate (along with cover) with precipitation rings for determination of IgA.
Fig. 9 - Photograph showing agar-antibody plate (along with cover) with precipitation rings for determination of IgM.