CHAPTER 3

MATERIAL AND METHODOLOGY

3.1. Study Area and Sample Population

The District of Cachar is situated in southernmost part of Assam in India. The district lies between latitudes 90.44 °E and longitude 20.04 °N and has an altitude of 36.5 (MSL). It is surrounded by the neighbouring state of Meghalaya in the north, Mizoram in the south and Manipur in east. It shares its western boundary with Bangladesh. There are two Sub Divisions namely, Silchar town and Lakhipur and 895 villages. Silchar town is the headquarter of the district. The district has a total area of 3786 Sq.Km which constitutes 4.83% of the total area of Assam. There are 57 tea gardens in the district with a plantation area 18325 hector [as on 1998].

The total population of the District is 14,42,141 as per 2001 Census. The male population is 7,41,580 and female population is 7,00,561. The population
density is 321 per Sq.Km. The urban population comprises 11% while the rural population constitutes the majority (89%). The population of Cachar is composed of different linguistic and cultural communities viz, Bengali (Hindus and Muslims), Manipuri, Assamese, Tea garden communities and Hill tribes, i.e. H'mar (95% among the hill tribes in Cachar), Jaintia, Riang, Chorai, Hrangkhol, Mizo, Karbi, Naga, Kuki, Vaiphei paite etc.

Approval for this study was obtained from Assam University ethical committee, and ethical committee of Cachar Cancer Hospital, Silchar. A total 952 individuals comprising 128 control (age range 14-85), 579 habituates with different chewing habits and 245 Patients with oral precancerous lesions (age range 31-62 years), oral precancerous conditions (age range 27-44 years) and oral cancer (age range 34-60) were included for the study. Subjects were considered after their diagnosis in Silchar Medical College & Hospital and Cachar Cancer Hospital, Silchar before commencement of their treatment. Volunteers did not receive any remuneration for their participation. The habituate cohort represented groups of different chewing habits practiced in Southern Assam. There were no gender ratio differences between the control and exposed cohorts (P>0.05). After obtaining consent, samples were taken from the buccal mucosa of healthy individuals who were not under any medication or X-ray exposure during past two months. Participants were informed about the study and interviewed to fill in a structured (See Appendix) questionnaire to obtain necessary information on lifestyles and personal factors. Demographic characteristics of
the cohorts are shown in Table 4. The geographical location of the study area is depicted in Figure 2 below.

Figure 2:
Geographical location of study area
3.2. Test Chemicals

Schiff's reagent was obtained from Sigma Chemical Company, U.S.A. Leishman stain was obtained from Hi-Media Laboratories Pvt. Ltd., Mumbai. Giemsa's stain was procured from Glaxo India Ltd., Mumbai, India. All other chemicals used were of analytical grade. The buffer for stain, reagent solutions and stains were always prepared in glass-distilled water. The stains, reagents, buffers and fixatives were prepared freshly prior to use.

3.3. Buccal Cytome Assay

A major goal for genetic toxicological study is to provide precise information on exposure and health risk assessment for effective prevention of health problems. Many approaches and techniques have been developed for monitoring human populations that have been exposed to environmental mutagens (Hulka, 1990). The traditional approach has been to use the readily available blood cells, e.g., lymphocytes and red blood cells, to document biomarker of effects. Although long-term diseases are not expected from the affected blood cells, it is generally accepted that the blood cells can be used as sentinel cell types to provide early warning signals for adverse health outcome. Buccal mucosal cells are excellent for use in monitoring human exposure to occupational and environmental genotoxins because these cells are in the direct route of exposure to ingested pollutants and they are capable of metabolizing proximate carcinogens to reactive chemicals...
3.3 Buccal cytome assay

(Zhang 1994; Salama, 1999). The buccal mucosal cells have been used for biomonitoring of populations exposed to a variety of environmental mutagens, e.g., antineoplastic drugs, arsenic compounds, cigarette smoke, formaldehyde, ozone, and radiation. The buccal mucosal cells have been considered to be more sensitive than lymphocytes to the induction of the cytogenetic damage by agents when the target tissue is epithelial tissue (Salama 1999).

The use of the micronuclei test (MNT) to detect and quantify the genotoxic action of carcinogens is well established, its sensitivity being compared to the analysis of chromatid breaks and exchanges (Kliesch and Adler, 1980). This test presents great advantages over other techniques, not requiring cell culture nor metaphase preparations, is a good indicator of chromosome mutations (Majer et al., 2001), is not invasive and has a low cost (Titenko-Holland et al., 1994; Broschinski et al., 1998; Calvert et al., 1998; Keshava et al., 1998; Maluf and Erdtmann, 2000). The assay can be used to detect chromosome breakage or mitotic interference, events thought to be relevant to carcinogenesis.

The use of the micronucleus test on epithelial cells was first proposed by Stitch and Rosin as a method of estimating the efficacy of chemoprevention regimens (Rosin 1983). The buccal cytome assay as a biomonitoring tool for cytogenetic endpoints in buccal epithelial cells was proposed by Thomas et al. (2007) based on a modified version of the assay proposed by Tolbert et al. (1997). The micronucleus assay has been incorporated in the buccal cytome assay in which other parameters in addition to micronucleus are studied. The
various distinct populations used in the buccal cytome assay were determined based on criteria outlined by Tolbert et al (1997). These criteria are intended to classify buccal cells into categories that distinguish between "normal" cells and cells that are considered "abnormal", based on nuclear morphology. The abnormal nuclear morphologies are thought to be indicative of DNA damage, cell proliferation or cell death. Basal cells are also scored along with differentiated cells. The buccal cytome assay provides a wider window to study buccal cell dynamics and alterations in the profile of the buccal mucosa. Detailed descriptions of the various cell types present in the buccal mucosa and scored in the buccal cytome assay are given below.

3.3.1. Basal Cells

These are the cells from the basal layer. The nuclear to cytoplasm ratio is larger than that in differentiated buccal cells which are derived from basal cells (Figure 3 & 4a). Basal cells have a uniformly stained nucleus and they are smaller in size when compared to differentiated buccal cells. Basal cells can contain micronuclei and were scored in the assay.

3.3.2. Normal Differentiated Cells

These cells have a uniformly stained nucleus which is usually oval or round in shape. They are distinguished from basal cells by their larger size and by a smaller nuclear to cytoplasmic ratio (Figure 3 & 4a). No other DNA containing structures apart from the nucleus are observed in these cells. These cells are
considered to be terminally differentiated relative to basal cells because no mitotic cells are observed in this population.

### 3.3.3. Abnormal "Differentiated" Cells

#### 3.3.3.1. Cells with micronuclei

These cells are characterised by the presence of both a main nucleus and one or more smaller nuclei called micronuclei. The micronuclei have smooth oval or round shape (Figure 3 & 4a). The diameter of micronucleus may range from 1/3 to 1/16 of the diameter of the main nucleus. The micronuclei are not attached to the main nuclei. The micronuclei take the same staining colour, intensity, texture and refraction as the main nucleus. The micronuclei must lie be on the same plane of focus with the main nucleus. Cells with micronuclei usually contain only one micronucleus. It is possible but rare to find cells with more than 6 micronuclei. The nuclei in micronucleated cells may have the morphology of normal cells or that of dying cells (i.e. condensed chromatin cells). The micronuclei must be located within the cytoplasm of the cells. The presence of micronuclei is indicative of chromosome loss or fragmentation occurring during previous nuclear division (Fenech, 1986). Micronuclei were scored only in basal and differentiated cells with uniformly stained nuclei. Cells with condensed chromatin or karyorrhectic cells were not scored for micronuclei.
3.3.3.2. Cells with nuclear buds

These cells have nuclei with an apparent sharp constriction at one end of the nucleus suggestive of a budding process, i.e. elimination of nuclear material by budding. The nuclear bud and the nucleus are usually in very close proximity and are apparently attached to each other (Figure 3 & 4a). The nuclear bud has the same morphology and staining properties as the nucleus, however its diameter may range from a half to quarter of that of the main nucleus. The mechanism leading to this morphology is not known but it may be due to elimination of amplified DNA or DNA repair complexes (Fenech 2002; Shimizu 1998, 2005).

3.3.3.3. Binucleated cells

These cells have two nuclei instead of one. The nuclei are usually very close to each other and may be touching. The nuclei usually have the same morphology as that observed in normal cells (Figure 3 & 4a). The significance of these cells is unknown but they may be indicative of failed cytokinesis following the last nuclear division.

3.3.3.4. Karyorrhectic cells

These cells are characterised by the more extensive appearance of nuclear chromatin aggregation (relative to condensed chromatin cells) leading to fragmentation and eventual disintegration of the nucleus (Figure 3 & 4b). These cells may be
undergoing a late stage of apoptosis but this has not been conclusively proven. These cells should not be scored for micronuclei in the assay.

3.3.3.5. Karyolytic cells

In these cells the nucleus is completely depleted of DNA and apparent as a ghost-like image that has no Feulgen staining. These cells thus appear to have no nucleus (Figure 3 & 4b). It is probable that they represent a very late stage in the cell death process but this has not been conclusively proven. These cells should not be scored for micronuclei in the assay.

3.3.3.6. Pyknotic cells

These cells are characterised by a small shrunken nucleus, with a high density of nuclear material that is uniformly but intensely stained. The nuclear diameter is usually one to two-thirds of a nucleus in normal differentiated cells (Figure 3 & 4b). The precise biological significance of pyknotic cells is unknown but it is thought that these cells may be undergoing a form of cell death however the precise mechanism is unknown. These cells should not be scored for micronuclei in the assay.

3.3.3.7. Condensed chromatin cells

These cells have nuclei with regions of condensed or aggregated chromatin exhibiting a speckled or striated nuclear pattern. In these cells it is apparent that chromatin is aggregating in some regions of the nucleus while being lost in other areas (Figure 3 & 4b). When chromatin aggregation is extensive the nucleus
may appear to be fragmenting. These cells may be undergoing early stages of apoptosis although this has not been conclusively proven. These cells may appear to contain micronuclei but should not be scored for micronuclei in the assay.

Figure 3:
Schematic diagram of the exfoliated buccal cells used as endpoints in the present study (Cytome assay) originating from basal cells.
3.3.4. Protocol

3.3.4.1. Cell sampling and preparation

Buccal cells originate from a multilayered epithelium that lines the oral cavity. Buccal cells were collected from consented volunteers using an established procedure based on a modified version of the method used by Beliën et al [Beliën 1995] Prior to buccal cell collection the mouth was rinsed thoroughly with water to remove any unwanted debris. Pre-moistened cotton swab (Johnson & Johnson, India) were rotated 20 times in a circular motion against the inside of the cheek, starting from a central point and gradually increasing in circumference to produce an outward spiral effect. Both cheeks were sampled using separate brushes. The heads of the swabs were individually placed into separate 2ml appendorff microcentrifuge tubes containing 0.9% M sodium chloride solution (Qualigens) at pH 7.0 and agitated to dislodge the cells. Cells from both right and left cheeks were transferred into separate centrifuge tubes (Appendorf, code 60.9921.829) and spun for 10 minutes at 2000rpm (SpinWin 2000). Supernatant was removed and replaced with 10mls of fresh normal saline solution. Left and right cell populations were pooled and drawn into a syringe with a 21G gauge needle and expelled to encourage cellular separation. Cells were further spun at 1500rpm for 10mins and the supernatant removed. Slides containing two spots of cells were air dried for 10 mins and then fixed in ethanol:acetic acid (3:1) for 10 minutes.
Smears were prepared in pre-cleaned slides and air-dried for 10 minutes prior to staining either with Schiff's reagent or Leishman stain.

### 3.3.4.2. Feulgen staining

Fixed slides were treated for 1 min each in 50% and 20% ethanol and then washed for 2 minutes in water. Slides were treated in 5M hydrochloric acid for 30 minutes and then washed in running tap water for 3 minutes. Slides were drained but not allowed to dry out before being treated in room temperature Schiff's reagent (Sigma 3259016) in the dark for 60 minutes. Slides were washed in running tap water for 5 minutes and rinsed well in deionised water for 1 minute. Nuclei and micronuclei are stained magenta. Slides were scored using a light-microscope (Leica DMLS) at 1000X magnification.

### 3.3.4.3. Leishman staining

Leishman staining was done when number of cells in samples collected in the field was less and Schiff's reagent was not readily available. Fixed microscopic slides were stained with Leishman stain and diluted after 10 seconds with distilled water in the ratio of 1:2 and kept for 15 minutes. Slides were washed and rinsed well in distilled water for 1 minute to remove excess stain. Nuclei and micronuclei are stained purple. Slides were scored using a light-microscope (Leica DMLS) at 1000X magnification.
3.4. Nuclear shape abnormality

3.3.4.4. Scoring method

1000 – 1500 cells were scored (500 per microscopic slide) per subject for all the various cell types outlined in the buccal cytome assay. These consisted of cells containing micronuclei, nuclear buds, basal cells, binucleates and the cell death parameters condensed chromatin, karyorrhectic, pyknotic and karyolytic cells. A total of 1000 differentiated and basal cells were scored in order to determine the frequency of micronuclei in a total of 1000 – 1500 cells.

3.4. Nuclear Shape Abnormality

The Nuclear shape abnormality is a parameter which is not included in the Cytome Assay by Thomas et al (2007). However, keeping in mind their prevalence in the exposed group, we have included it as a separate parameter. The major cause may be membrane destabilization leading to apoptosis or cell cycle dysregulation leading to death or other abnormality (Figure 4c).

3.5. Identification of Precancerous lesions, Conditions and Oral Squamous Cell Carcinoma

After obtaining consent, samples were taken from the buccal mucosa of patients attending the outpatient department. The Cases were properly diagnosed by a practicing doctor from the Department of ENT, Silchar Medical College and Hospital and Cachar Cancer Hospital, Silchar. The photograph of the different types of precancerous lesions were taken (Figure 5) and some of
the cases were identified histologically from the biopsy in the pathology
Department of Silchar Medical College for confirmation of cellular changes
typical for each type of precancerous lesions (Figure 5).

3.6. Preparation of Histological Sections

Biopsy specimen from patients with oral lesions were processed for
histology. The paraffin sections were cleared in Xylol, hydrated in different
grades of alcohol (100%, 90%, 70% and 30% respectively) and washed in
running tap water. The sections were then stained in Haematoxyline and
washed in running tap water. The sections were then dehydrated in graded
alcohol (30%, 50%, 70%, 90% and absolute alcohol respectively for 5 minutes
each and counterstained in 1% Eosin solution, were washed in 2 changes of
Xylol, dried a mounted in DPX. The slides were studied using a light-microscope
(LeicaDMLS) at 1000X magnification.

3.7. Statistical Analysis

One way ANOVA analysis was used to determine the significance of the
 cellular parameters measured between the control, different habituates and
 patient cohorts. Pair wise comparison of significance between these groups
 was determined using Tukey’s test. ANOVA analysis values were calculated
 using Graphpad PRISM (Graphpad incSan Diego, CA). Correlation and Chi
 Square test were done to analyse demographic parameters. Significance was
 accepted at P<0.05.
Figure 4a:
Photographs showing cytogenetic damage in exfoliated buccal epithelial cells. A–C = normal differentiated cell; D = basal cell; E–F = cell with nuclear bud; G–I = cell with micronucleus; J–L = binucleated cells.
Figure 4b:
Photographs showing cytogenetic damage in exfoliated buccal epithelial cells; A–C = pyknotic cell; D–F = condensed chromatin cell; G–I = karyorrhectic cell; J–L = karyolytic cell.
Figure 4c:
Photographs showing cytogenetic damage in exfoliated buccal epithelial cells in the form of nuclear shape abnormalities other than those described in Figure 4a & 4b as observed in the present study. A = normal cell; B–I = cells with atypical nucleus.
Figure 5:
Dysplastic changes along with histology of the disease. A = leukoplakia. B = erythroplakia; C = erythroleukoplakia; D = oral squamous cell carcinoma.