3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Subjects

Individuals suffering from different types of cancer were studied in the present investigation. Healthy individuals, matched with the diseased with respect to age, sex, smoking and alcohol drinking habits, drug intake, if any, and social class, were taken as normal subjects for the study. A total of 200 individuals were studied, of which, 120 were randomly selected cancer patients and 80 were healthy normal subjects (controls). Epidemiological data, blood samples, and buccal smears were collected from all the subjects.

3.1.2 Ethical Concern

The study was planned and conducted in accordance with the declaration of Helsinki (2008). The samples were collected from Department of Surgical Oncology, Pt.B.D.Sharma, P.G.I.M.S., Rohtak. Therefore, ethical clearance for conducting this study was obtained from the Institutional Ethics Committee, Kurukshetra University, Kurukshetra, vide Letter No. IEC/10/229 dated 15-05-2010 (Appendix I) as well as from Institutional Ethics Committee, Pt.B.D.Sharma, P.G.I.M.S., Rohtak, vide Letter No. Surg-V/IEC/12/468 dated 03-11-2012 (Appendix II). An informed consent was taken from each individual, who took part in the study, prior to obtaining the samples (Appendix III, IV).

3.1.3 Epidemiological Survey

A personal questionnaire was prepared to analyze the profile of the study group and the data was recorded on a standard proforma (Appendix V). While the individuals were interviewed to obtain their detailed personal history, hospital records were referred to for obtaining their medical history. The personal information of all the subjects were kept confidential and coding was done to protect their identities.

3.1.4 Buccal Mucosa Samples

Exfoliated buccal epithelial cells were obtained from the middle part of the inner cheek using a moistened stainless steel spatula and smears of the cells were
prepared on pre-cleaned microscope slides. All precautions were taken to avoid infections of any type.

Exfoliated buccal epithelial cells were selected for the present study due to the following advantages:

1. The samples could be collected by non-invasive techniques.
2. The cells of basal lamina are in constant dividing state.
3. More than 90% of cancers are epithelial in origin; therefore, it is appropriate to use exfoliated buccal epithelial cells for the study of genetic damage caused by progress of cancer.
4. Basal lamina cells which have been repeatedly exposed to oxidative stress can pass the mutation, if any, to newly formed buccal epithelial cells.
5. The life cycle of buccal epithelial cells is quite short (2-3 weeks), hence, effect of recent oxidative stress can be studied.

3.1.5 Blood Samples

The help of a medical practitioner was taken for the collection of blood samples. Venous blood samples (2 ml each) were drawn concurrently from each diseased donor and matched healthy donor into a vacuum tube containing EDTA (Vacutainer, Becton Dickinsen, France) using sterile disposable needles. Every care was taken to avoid infections of any type. The samples were immediately transported to the Human Cytogenetics laboratory, Department of Zoology, Kurukshetra University, Kurukshetra for further processing and analysis. The samples were transported in a dark ice box to prevent further DNA damage.

For the present investigation, PBLs were the tissue of choice for the following reasons:

1. PBLs can be obtained easily by the least invasive methods.
2. They require very little manipulation, therefore, reducing the possibility of damage.
3. They are in a state of mitotic arrest with decreased efficiency for DNA repair. Thus, actual damaged DNA can be assessed.
4. They circulate through the body collecting the free radicals and therefore, expressing the real oxidative burden on the body.
5. Damage accumulated in the PBLs expresses itself when the cells are stimulated to divide in vitro or it can be studied directly with the help of suitable technique.

3.2 LABORATORY METHODS

In the present study, MN assay and comet assay were used as the biomarkers of choice for the evaluation of genetic damage in the tissues under observation.

3.2.1 Micronucleus (MN) Assay

During the present investigation the frequency of micronucleated exfoliated buccal epithelial cells of the diseased as well as normal subjects were investigated for the assessment of DNA damage, if any. The standard techniques of Tolbert et al. (1992) and Thomas et al. (2009) were used for the purpose, and the mean frequencies of micronucleated cell (MNC), total micronuclei (TMN) and other phenomena, like, broken egg (BE), bi-nucleated cells (BN), karyolysis (KL) and karyorrhexis (KH) were calculated to monitor the genetic damage.

3.2.1.1 Principle

MNi originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division and hence can be used as a measure of both chromosome breakage and chromosome loss (Schmid, 1975). Kinetochore or centromere detection methods can be used to distinguish between MN caused by chromosome breakage and MN caused by chromosome mal-segregation (Fenech et al., 1999; Shimizu et al., 1998, 2000). Therefore, the induction of MN is considered to be an effective biomarker of diseases and processes associated with induction of DNA damage. Besides MN other nuclear anomalies can also be used as indicators of genetic damage. Broken egg is an indicator of gene amplification and dicentric chromosomes while binucleated cells show a defect in cytokinesis and karyorrhexis is an indicator of mitotic failure (Holland et al., 2008).

3.2.1.2 Materials

Exfoliated buccal epithelial cells were taken from the oral cavity of the subjects for conducting MN assay in the present investigation.
3.2.1.3 Preparation of reagents

A) Aceto-orcein stain

A 2% solution of Aceto-orcein stain (2% in 60% acetic acid) was prepared using the following:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>60 ml</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>40 ml</td>
</tr>
<tr>
<td>Orcein</td>
<td>1 g</td>
</tr>
</tbody>
</table>

A 60% solution of Acetic acid was prepared by dissolving 60 ml of Acetic acid in 40 ml of double distilled water. In 50 ml of this solution 1g Orcein was dissolved by slow heating to prepare the working Aceto-orcein stain. The stain was cooled, filtered, and stored for further use.

Aceto-orcein stain was used as the primary stain during the present investigation.

B) Fast green stain

A 0.1% solution of Fast Green stain was prepared using the following:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast Green</td>
<td>100 mg</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

100 mg of Fast green stain was dissolved in 100 ml of double distilled water. The stain was filtered and stored for future use.

Fast green stain was used to counter-stain the cells so that the nucleus can be easily observed against the contrasting background.

3.2.1.4 Methodology

The methodology used was as follows:

A) Cytological preparations

1. Prior to sampling the subjects were asked to rinse their mouth to remove food and particles which may cause artifacts.
2. Exfoliated buccal epithelial cells were obtained from the middle part of the inner cheek using a moistened stainless steel spatula. All precautions were taken to avoid injuries or infections of any type.
3. Smears of the cells were prepared on pre-cleaned coded microscope slides. Two slides were prepared for each individual and air dried.
4. The air dried slides were immediately transported to the laboratory in ice (to
avoid further damage) where they were again air dried and then hydrolyzed in 1N HCl at 60°C for 8 minutes and rinsed in water.

5. Then they were stained with Aceto-orcein (2% in 60% acetic acid) for 20 minutes at 40°C and washed in ethanol and distilled water for two to three times.

6. The slides were counter-stained with 0.1% Fast green solution for 12 minutes at room temperature and again rinsed in ethanol and distilled water.

7. After that the slides were air dried.

**B) Instrumentation and scoring of MNi**

1. Coded slides were analyzed using an Olympus CX-41 trinocular research microscope at 1000× magnification.

2. At least 1000 cells for each individual were examined and the number of cells with nuclear anomalies were scored following the criteria given by Tolbert *et al.* (1992).

3. In addition to MN, other nuclear anomalies were also scored (Figure 10), like:
   a. **Broken eggs (BE)** or nuclei that appear to be broken but still connected to main nuclei with a thin band,
   b. **Binucleated cells (BN)** or the presence of two nuclei within a cell,
   c. **Karyorrhexis (KH)** or nuclear disintegration involving loss of integrity of the nucleus and
   d. **Karyolysis (KL)** or nuclear dissolution, in which aceto-orcein negative, ghost-like image of the nucleus remains.

4. In addition to these, there were other phenomena occurring in the cells such as pycnosis and chromocenters, which were observed during scoring of the anomalies. However, these were not counted in the anomalies. These are considered as a part of normal epithelial cell differentiation and maturation.

5. Image acquisitions of representative anomalies as well as normal cells were done with the help of Jenoptik ProRes® CapturePro 2.0 CTE camera and software package for future record and study.
C) Criteria for scoring MNi

According to Tolbert et al. (1992), in order for the cell to be considered micronucleated, the putative MN is required to meet the following criteria:

1. Rounded, smooth perimeter suggestive of membrane;
2. Less than 1/3rd of the diameter of the associated nucleus, but large enough to discern shape and color;
3. Staining intensity similar to that of nucleus;
4. Same focal plane as nucleus.

Recently, there has been a tremendous increase in interest in the evaluation of cytogenetic damage in epithelial cells and the frequency of MN was extensively used as a biomarker of genotoxic exposure and early biological effect in human biomonitering studies (Norppa and Falck, 2003; Nersesyan and Llin, 2007).

3.2.2 Single Cell Gel Electrophoresis/ Comet Assay

In the present study, the genetic damage in the PBLs of the people affected with cancer as well as normal healthy individuals was evaluated using the SCGE which is popularly known as Comet Assay. The protocol given by Ahuja and Saran (1999) was followed with minor adjustments for laboratory conditions.

3.2.2.1 Principle

Comet assay is based upon the fact that DNA molecules, when placed in an electric field, migrate in accordance with their molecular size. Single cell suspension of PBLs is made in the low melting point agarose (LMPA) and then, this suspension is layered on to a microscopic slide, previously coated with another layer of normal melting point agarose (NMPA). Most of the proteins and membranes (organelle’s and cellular) are lysed due to the high salt concentration during treatment with lysis solution. Due to the removal of most of the histones the nucleosomes are disrupted, but the DNA remains super coiled. These loops of DNA can be regarded as independent topological units. Thus, one SSB (caused by any agent) leads to the relaxation of the super coiling in the loop in which it occurs, allowing that loop to extend under the influence of an electrophoretic field. DNA, which is negatively charged, moves towards anode. The extent of migration of DNA is inversely related
to the size of fragments of DNA. This can be used to calculate the extent of damage present in the DNA. The DNA fragments produced by the SSBs give a characteristic appearance of a comet where the intact DNA is represented by the head and damaged DNA by the tail. Therefore, this assay is also called comet assay (Collins, 1992). The undamaged cells too have a characteristic structure called “halo”. Comet tail length is a measure of DNA damage and longer tail is an indicator of more damage. Nowadays, many more comet parameters can be analysed with the help of imaging softwares (Collins, 1992, 2004; Armalyte and Zukas 2002; Villela et al., 2007).

3.2.2.2 Materials

Venous blood samples (2 ml each) were drawn from each subject into a vacuum tube containing EDTA (Vacutainer, Becton Dickinsen, France) using sterile disposable needles.

3.2.2.3 Preparation of reagents

A) **Phosphate buffered saline, PBS (Ca++, Mg++ free):**

Dulbecco's PBS 1 l packet

Added 990 ml ddH$_2$O to the Dulbecco's PBS 1 litre packet, adjusted the pH to 7.4 and made the final volume to 1000 ml. Stored at room temperature.

B) **Lysing solution:**

Stock solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>37.2 g</td>
</tr>
<tr>
<td>NaOH pallets</td>
<td>12.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>146.1 g</td>
</tr>
<tr>
<td>Tris base</td>
<td>1.2 g</td>
</tr>
</tbody>
</table>

pH=10

All the ingredients were added to about 700 ml of double distilled water and dissolved by stirring. The pH was adjusted to 10 using conc. HCl or NaOH and the volume was made up to 1000 ml with double distilled water. This stock solution of lysing solution was filtered and stored at room temperature.
Final lysing solution:

Lysing stock solution   89 ml
Triton X-100    1ml
DMSO     10 ml

1% Triton X-100 and 10% dimethyl sulphoxide (DMSO) were added to the stock lysing solution to prepare 100 ml of the final lysing solution which was then refrigerated for 30-60 minutes prior to use. The purpose of the DMSO in the lysing solution is to scavenge radicals generated by the iron released from haemoglobin.

C) Electrophoresis buffer (300 mM NaOH / 1 mM EDTA):

Stock solutions:

Solution A:
EDTA (200mM) 14.89 g in 20 ml of ddH₂O

Solution B
NaOH (10 N) 200 g in 500 ml of ddH₂O

Both the solutions A and B were stored at room temperature. Working electrophoresis buffer (made fresh before each electrophoresis run) was prepared by mixing 5 ml of Solution A with 30 ml of Solution B and making up the final volume to 1000 ml with distilled water. The pH of the buffer was maintained at >13.

D) Neutralization buffer:
Tris (0.4 M) 48.5 g in 1000 ml ddH₂O
pH was adjusted to 7.5 using conc. HCl, and buffer was stored at room temperature.

E) Staining solution:
Ethidium Bromide (10× Stock - 20 µg/ml): added 10 mg EtBr powder to 50 ml ddH₂O. Mixed it and stored at room temperature.
For 1× stock - mix 1 ml of the 10× stock with 9 ml ddH₂O.
Appropriate precautions were taken while working with EtBr stain as it is a DNA intercalating agent.
3.2.2.4 Methodology

The methodology used was as follows:

A) Experimental procedure

1. Preparation of slides

For every experiment slides were prepared in duplicate for each subject. Slides were coded prior to the experiment. Prepared 1.0% NMPA (500 mg per 50 ml) in double distilled water and 0.75% LMPA (375 mg per 50 ml) in PBS. Heated in the microwave oven till boiling point and the agarose dissolved completely. Placed LMPA vial in a 37ºC water bath to cool and stabilize the temperature.

i) First layer

Conventiona microscopic slides were made grease free by dipping in rectified spirit for about an hour followed by flame drying. Then layering was done with 150-200 µl of NMPA. Slides were allowed to dry at room temperature or by a brief exposure to 40-50ºC for about 10 minutes in hot air oven. After that slides were stored in dust free environment for future use. NMPA layer provides a strong anchorage for the future layers and helps in preventing slippage of gel layers during the experiment.

ii) Second layer

About 75 µl of LMPA (37ºC) was mixed with 5-10 µl of blood sample (~10,000 cells) and this suspension was used to make a second layer. A 24mm×60mm coverslip was placed over the gel to flatten out the molten agarose layer. The slides were placed on a slide tray resting on the ice packs until the agarose layer hardened (~3 to 5 minutes). Care was taken while putting or removing the cover slips to avoid the entry of air bubbles and wear and tear to gel layer.

iii) Third layer

The coverslip was gently slid off and a third layer of 75-100 µl LMPA was applied, and the coverslip was replaced. The slide was returned to the slide tray until the agarose layer hardened again (~3 to 5 minutes). With this layer the middle sample containing layer got sandwiched between the other two layers. Third layer was added to fill in any residual holes in the second layer, to increase the distance between the cell and gel surface, and to protect the sample containing layer from wear and tear during experimentation.
2. **Lysing**

After the solidification of the agarose gel, the slides were placed in a chilled, freshly prepared lysing solution consisting of high concentration of salt and detergents. The lysing solution was chilled for 1 hour prior to use, mainly to maintain the stability of the agarose gel. The DMSO in the lysing solution prevents radical-induced DNA damage associated with the iron released from the lysis from erythrocytes present in the blood (Tice *et al.*, 1991). Most of the proteins and membranes (organelle’s and cellular) are lysed by the lysis solution to expose the nucleoids. Most of the histones are removed, and nucleosomes are disrupted, but the DNA remains super coiled. While the duration of lysis employed by different investigators varies considerable, from less than 1 hour to weeks, we achieved optimum results with the time duration of 1-2 hours during the present study.

3. **Alkali unwinding**

After lysis, the slides were taken out and put into horizontal electrophoretic tank. Slides were placed side by side, leaving no space between them, in one direction with agarose pointing towards anode. Freshly prepared electrophoretic solution was carefully poured into the tank to submerge the slides completely. The level was kept approximately 2-3 mm above the slides with no bubbles on the slides. The slides were then left to incubate in the buffer for about 40 minutes prior to the electrophoresis. This allows for the DNA to unwind and for the expression of ALS as SSBs before the electrophoresis.

4. **Electrophoresis**

After alkali unwinding, electrophoresis was carried out at room temperature. The single stranded DNA in the gel was electrophoresed for 30 minutes under alkaline conditions to produce comets with different degrees of DNA migration. The voltage was fixed at 25V or 1.0 V/cm and the current was maintained at 300 mA (milliamperes) by raising or lowering the buffer level in the tank. Usually, the optimal voltage/ampereage and electrophoreses duration depend on the extent of DNA migration desired for the control cell and the range of responses being evaluated among the treated cells.
5. **Neutralization**

After electrophoresis, the power supply was turned off. The slides were lifted gently from the electrophoretic tank and placed on a staining tray. The slides were coated with neutralization buffer 3 times, each time for about 10 minutes. This was done to remove any traces of detergent and alkali, which would otherwise interfere with staining. Slides were washed with distilled water and kept on an aluminum tray and allowed to dry completely at room temperature for an hour.

6. **Staining of slides**

After neutralization, the slides were stained with 80µl of 1× EtBr for 5 minutes. The slides were then washed thrice by dipping in chilled distilled water to remove excess stain and give a debris free background. As EtBr is known to be a DNA intercalating agent, appropriate precautions were taken to avoid any contact with the stain.

The whole procedure was performed under dim light to avoid photolysis and artifactual DNA damage.

B) **Acquisition of images and visual scoring of images:**

For comet visualization, Nikon Eclipse 90i Trinocular Research microscope was used at 100× magnification. Previously coded slides were scored without any personal bias. 100 cells were randomly chosen (50 from each duplicate slide) from each subject and analyzed under microscope and images were acquired using the DS-QiMC camera and Lucia Comet Assay v6.22 software integrated with the microscope and saved on the system. The microscope was pre-calibrated using the software. The saved image files were later on retrieved and analyzed. The scoring was done by single observer to minimize the inter observer variability.

C) **Criteria for scoring comets**

Comet can be divided into head and tail regions. For the calculation of damage index (DI), following comet parameters were included in the study: % DNA in tail, integral intensity, tail length, tail moment, Olive tail moment and tail area.
1. **% DNA in tail** is the total tail intensity divided by total comet intensity and multiplied by 100.

2. **Integral intensity** is mean intensity of pixels in comet.

3. **Tail length** is calculated by subtracting the head diameter from comet length where as some scientists consider the tail length from the centre of the comet head.

4. **Tail moment** is calculated by multiplying %DNA in tail by tail length.

5. **Olive tail moment** is summation of tail intensity profile values multiplied by their relative distance to the head center, divided by total comet intensity.

6. **Tail area** is the number of pixels in tail.

Migration length is directly related to fragment size and is expected to be proportional to the extent of DNA damage (Kumaravel and Jha, 2006; Villela et al., 2007).

While parameters like Comet length, Tail length, Tail moment and Olive moment are considered very reliable and used preferentially by many workers for studying DNA damage (Tice et al., 1991; Collins, 1992, 2004; Ahuja and Saran, 1999; Armalyte and Zukas 2002; Kumaravel and Jha, 2006; Villela et al., 2007, Yadav and Sharma, 2008b) yet the simpler method of different comet types, where cells are sorted out into four classes based on the tail size and shape i.e. 0-4 types (Collins, 2004), has also been used to study the genotoxic effects by various investigators (Collins, 2004; Villela et al., 2007) (Figure 11).

### 3.2.3 ABO and Rh (D) Allele Frequencies

During the present investigation the frequency of ABO and Rh (D) alleles in case of both cancer patients and normal subjects were calculated to find out if a particular blood group type was susceptible to cancer. The standard technique of Yasuda (1984) was used for the purpose.

#### 3.2.3.1 Materials

Venous blood samples (2 ml each) were drawn from each subject into a vacuum tube containing EDTA (Vacutainer, Becton Dickinsen, France) using sterile disposable needles.
3.2.3.2 Methodology

The methodology used was as follows:

a) ABO and Rh (D) blood group systems were typed using whole blood by slide method.

b) Standard techniques of serology and manufacturer’s directions enclosed with the different blood grouping reagents were followed. The antisera of Span clone make were used in the present study.

c) The allele frequency in the ABO blood group system was calculated according to Yasuda (1984).

d) The frequency of d allele in the Rh (D) system was estimated by square root method (Mourant et al. 1976).

3.3 STATISTICAL ANALYSIS

Statistical softwares, SPSS v16.0 and Microsoft Excel 2007 were used for the analysis of the epidemiological data and the experimental results obtained during the current investigation as well as preparation of graphs and tables for the proper representation of these findings. Following tests were applied to determine the significant differences in genetic damage between different study groups and the correlation between different factors and the genetic damage observed:

a) Student’s t-Test

b) Pearson’s correlation coefficient

c) One-way analysis of variance (ANOVA) with Duncan’s post hoc test