ASSESSMENT OF DNA DAMAGE IN CULTURED PERIPHERAL BLOOD LYMPHOCYTES OF THE TOBACCO USERS IN MIZORAM

A thesis submitted in partial fulfillment
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in
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by

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CERTIFICATE

This is to certify that **Shri B. Lalruatfela** carried out his research work under my supervision since 2015. The thesis entitled **"Assessment of DNA damage in cultured peripheral blood lymphocytes of the tobacco users in Mizoram"** is an original piece of work and has not been submitted for any other degree of any other university.

Place: Aizawl
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DECLARATION

I, B. Lalruatfela, hereby declare that the subject matter of this thesis entitled **"Assessment of DNA damage in cultured peripheral blood lymphocytes of the tobacco users in Mizoram."** is the record of work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University, Aizawl for the award of the degree of Doctor of Philosophy in Zoology.

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Ph.D. Course Work Award-Sheet

The following is the assessment record of ***B. Lalruatfela****, Roll No.* ***Zoo/Ph. D-CW/14/12****, Registration No.* ***111 of 2015*** *in the Ph. D. Course Work Examination held in December,* ***2014****.*

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The performance of the scholar shall be evaluated in the following grades : ***'O'*** *– Outstanding,* ***'A'*** *and* ***'B'*** *with grade point valuation in the 10 point scale, i.e., 7.00-10.00 corresponding to* ***'O'*** *grade, 6.00-6.99 corresponding to* ***'A'*** *grade, 5.00-5.99 corresponding to* ***'B'*** *grade. Those securing less than 5.00 points shall be graded as* ***'C'****. A scholar will be eligible for admission into Ph. D. programme if he/she secures at least* ***'B'*** *grade in each course.*

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ABBREVIATIONS

2MNBNC	:	Binucleated cell with two micronuclei.
8-OHdG	:	8-hydroxydeoxyguanosine.
AC	:	Aberrant cell.
AF	:	Acentric fragment.
B[a]P	:	Benzo[a]pyrene.
CAT	:	Catalase.
CB	:	Chromatid break.
CBMN	:	Cytokinesis blocked micronucleus assay.
CDNB	:	1-chloro-2,4-dinitronbezene
CON	:	Control group or Tobacco non-users.
CpG	:	Cytosine and guanine linked by phosphate diester bond.
CSB	:	Chromosome break.
DC	:	Dicentric chromosome.
DNA	:	Deoxyribonucleic acid.
DTNB	:	5,5'-dithiobis-2-nitrobenzoic acid.
G	:	Guanine.
GSH	:	Reduced glutathione.
GST	:	Glutathione-s-transferase.
h	:	Hour.
HPBL	:	Human peripheral blood lymphocyte.
IARC	:	International Agency for Research on Cancer.
KCl	:	Potassium chloride.
LOO	:	Lipid peroxidation.
M	:	Molar.
ml	:	Milliliter.
MMNBNC	:	Binucleated cell with multiple micronuclei.
MN	:	Micronucleus.
MNBNC	:	Binucleated cell with micronucleus.
mtDNA	:	Mitochondrial deoxyribonucleic acid.
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

NAB	:	N’-nitrosoanabasine.
NADH	:	Nicotinamide adenine dinucleotide.
NAT	:	N’-nitrosoanatabine.
NBT	:	Nitroblue tetrazolium.
NDMA	:	N-Nitrosodimethylamine.
nm	:	Nanometer.
NNK	:	4-(methylnitrosamino)-1-(3’-pyridyl)-1-butanone).
NNN	:	N-nitrosonornicotine.
OD	:	Optical density.
OR	:	Odds ratio.
PAH	:	Poly-aromatic hydrocarbon.
PBS	:	Phosphate buffer saline.
PHA	:	Phytohemagglutinin.
PMS	:	Phenazine methosulphate.
RNS	:	Reactive nitrogen species.
ROS	:	Reactive oxygen species.
rpm	:	Revolution per minute.
RPMI-1640	:	Roswell Park Memorial Institute-1640 culture medium.
SEM	:	Standard error of mean.
SOD	:	Superoxide dismutase.
T	:	Thymine.
TA	:	Total aberration.
TBA	:	Thiobarbituric acid.
TCA	:	Trichloroacetic acid.
TOB	:	Tobacco group or Tobacco users.
WHO	:	World Health Organization.
β	:	Beta-coefficient.

CHAPTER 1

GENERAL INTRODUCTION

TOBACCO

Tobacco is an important agricultural product processed from the plant of the genus *Nicotiana* belonging to the family Solanaceae. There are seventy two known species of *Nicotiana,* however, *Nicotiana tabacum* and *Nicotiana rustica* are of major economic importance as they are consumed in various forms. Tobacco plants are known to prosper in tropical and subtropical climatic conditions and are either cultivated or found in the wild. The body of a tobacco plant consists of a hollow, spongy stem and is mostly one to two meters tall depending on the species and variety (Figure 1.1). The leaves are covered by minute viscid glandular hairs (Figure 1.2 & 1.3) and the plants mostly thrive in moist soil and humid environment. Any part of the plant is processed into other products for consumption. These products are referred to as tobacco while the plant is referred to as a tobacco plant. The method of processing and consumption varies considerably throughout the world. It is consumed singly or in combination with other ingredients (Kishore, 2014).

SYSTEMATIC CLASSIFICATION

Kingdom- Plantae

Phylum- Magnoliophyta

Class- Magnoliopsida

Order- Solanales

Family- Solanaceae

Genus- *Nicotiana*

Species- *N. tabacum; N. rustica*

Bionomial name: *Nicotiana tabacum* L., *Nicotiana rustica* L.

The first known users of tobacco are the Native Americans, which was observed by Christopher Columbus and his expedition crew around the 15th century. The European explorers, during the great exploration era, introduced it to other parts of the world, initially as a medicine and then as a recreational product. The introduction to other parts of the world was heavily criticized and banned by kings and religious leaders. However, the impact of tobacco on a country's economy and the comprehensive admiration among the general populous obligated these leaders to accept it (West and Shiffman, 2007). The initial use of tobacco by the Native Americans was said to be of medicinal and spiritual purposes. The many chemicals present in tobacco have hallucinogenic properties and this particular property was known to be desired by the Native Americans to make contact with the spiritual world. The medicinal purpose of tobacco consumption is its property to alleviate pain. It was used to cure earache, toothache, asthma, tuberculosis, common cold and some skin diseases of domestic swines (Bhardwaj and Gakhar, 2004).

Tobacco consumption is popular all over the world. In fact, every nation throughout the world is a nation with tobacco users in its population (WHO, 2015). It is estimated that there are approximately 1 billion men and 250 million women who used tobacco globally (WHO, 2002; Ezzati and Lopez, 2003; Hecht, 2003). As a general consequence, an individual's habit of consuming any form of tobacco is an influence of peers or popular culture. Most tobacco users started the practice during adolescent or early adulthood. During the early stage of usage, users claimed to have pleasurable sensations which served as a positive reinforcement. This positive reinforcement gradually compelled the users to be habituated to it. This habituation is an effect of nicotine, the most powerful known addictive chemical in the tobacco.

Once the users are habituated to tobacco, or more specifically nicotine, they found it exceptionally challenging to discontinue its consumption. The discontinuance of tobacco consumption by habituated users leads to withdrawal symptoms, which include anxiety, restlessness, irritability, shortened attention span and intense craving for tobacco or nicotine (Benowitz, 1998).

Although nicotine is non-carcinogenic (Biesalski *et al*., 1998; Hecht, 1999; Hecht, 2012), its effect on the physiological and psychological construct of the body is immense. It elevated heart rate, blood pressure, cardiac output and oxygen consumption; relaxed the musculo-skeletal dynamics and cognizance; increased alertness and brought euphoria to the users. It also decreases muscular strength and lowers anaerobic performance. Nicotine has also been reported to have damaging effect on DNA of cultured epithelial and non-epithelial human cells (Kleinsasser *et al*., 2003; Chague *et al*., 2015).

TYPES OF TOBACCO

The kind of tobacco and its associated products used worldwide vary considerably from place to place and also depend upon the choice of individual consumers. The choice of tobacco used often reflects the socio-economic status of the users. Certain tobacco products are rather expensive and their consumptions, in a way, reflect the users' luxurious way of life. The availability of such tobacco products is also an important factor in the users' selection for consumption. Generally, tobacco can be divided into two types, smoked (combustible) and smokeless (non-combustible) tobacco. Smoking is the most common practice of tobacco consumption all over the world and consists of great number of varieties. Every cultures and communities have different specific names or brands for the

smoked tobacco products. Some of the most popular varieties include cigarette, cigar, pipe, hookah, bidi, cheroot, cigarillos, little cigar, kreteks, etc. Smoked tobacco products are initially hand rolled, but after the industrial revolution, factories and industries took over its production (WHO, 2006) and is currently one of the biggest industries.

Smokeless tobaccos are another form of consumable tobacco products. Many smokeless tobaccos are either sun dried of fire dried and are mostly orally consumed, although some are nasally used. There also exist liquid form of tobacco in the north eastern part of India. Some of the popular smokeless tobaccos include chewing tobacco, tuibur or hidakphu (tobacco brew), snuff, khaini, raja, mawa, mishri, snus, betel nut quid, spit tobacco, gutkha, tiranga, shikhar, hogesoppu, kaddipudi,gundi, kiwam, zarda, pattiwala, mainpuri, mawa, kharra, bajjar, gul, gudakhu, naswar, etc. (Foulds *et al*., 2003; Gupta and Ray, 2003; Lo *et al.*, 2015). The means of consumption of these tobaccos vary greatly; some are ingested while other are simply kept in the mouth and spitted out after sometime.

CHEMICAL COMPOSITION OF TOBACCO

Tobacco is known to contain more than eight thousand chemicals, out of which more than eighty have probable carcinogenic properties (IARC, 2004; Cooper, 2006; Ding *et al.*, 2008; Perfetti and Rodgman, 2011; Arimilli *et al.*, 2012). The soil on which a tobacco plant grows plays a significant role in determining some of the chemicals found in the plant. The plant often absorbs toxic chemicals from the soil along with other nutrients and water and store it in certain parts of their structure. Therefore, the type and number of chemicals vary among different types of tobacco products (Hoffmann *et al.*, 2001). Tobacco smoke has been known to exhibit toxic,

genotoxic, mutagenic, teratogenic and carcinogenic properties (Chiba and Masironi, 1992; Stohs *et al*., 1997; Demarini, 2004; Eyre *et al*., 2004; Husgavfel-Pursiainen, 2004). Some of the common toxic chemicals found in tobacco include benzo[a]pyrene, N'-nitrosonornicotine (NNN), N'-nitrosoanatabine (NAT), N'-nitrosoanabasine (NAB), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-Nitrosodimethylamine (NDMA), nitrite, cadmium, lead, arsenic, nickel, chromium, isoprene, styrene, acrylonitrile, acetaldehyde, formaldehyde, benzene, *p*-Cresol, p-Benzoquinone, N-Nitrosodiethylamine, ethylenethiourea, chrysene, , mercury, zinc, copper, nicotine, etc. (Stepanov and Hecht, 2005; Talhout *et al*., 2011; Borgerding *et al.*, 2012).

Many of these chemicals have cytotoxic properties and some of them are classified under class I carcinogens. The types of chemicals present in smoked and smokeless tobaccos also differ. Many chemicals are synthesized during the burning/pyrolysis of the tobacco in the process of smoking. In fact, every smoked and smokeless tobacco will have different chemical constituents depending on the method of processing and geographical area of the crop production (Pryor *et al*., 1983; Calafat *et al*., 2004). A study on the chemical composition of the tar phase of tuibur, a locally manufactured tobacco brew by the Mizos showed the presence of mainly non-polar organic substances that may be described as semivolatile and non-volatile (Lalmuanpuii and Muthukumaran, 2016).

TOBACCO AND HEALTH

Although the Native Americans were known to use tobacco as medicine, their detrimental effects on health came to light in the mid-19th century. From then onwards, the injurious health effect of tobacco have been recorded thousands of

times from all over the world. As some of the chemicals of tobacco are carcinogenic, the consumption of tobacco has been associated with many forms of cancer (Wynder & Wright, 1957; Hoffmann & Wynder, 1970; Talhout *et al.,* 2011; Bassiony *et al.*, 2015). In fact, almost all known cancers can be correlated to tobacco consumption. However, the most direct involvement of cancer can be seen in cancer of the mouth, lungs, esophagus, stomach, liver, pharynx, colon, etc. Besides cancer, it has also been known to cause several other diseases mostly involving the pulmonary system like bronchitis, asthma, tuberculosis, laryngitis, pharyngitis, etc. (Musk and De Klerk, 2003; Elmasry *et al.*, 2015).

Many studies have shown that individuals smoking a pack of cigarette per day showed 50% increase in colon cancer than non-smokers and even those who discontinued smoking remained at increased risk for the development of cancer, even if they stopped the habit very early. Contrastingly, some studies have established a protective effect for terminated smoking. The amount an individual smoked may have been a more important factor than the duration of smoking in the development of cancer. The consumption of alcohol in addition to tobacco has also been found to significantly increase the risk of colon cancer (Wynder *et al.,* 1977; Mashberg *et al.,* 1993; Slattery *et al.,* 1997). Tobacco smoking, alcohol consumption and betel quid chewing have been observed to significantly increase the risk of lung cancer (Phukan *et al.*, 2014; Saikia *et al.,* 2014). Lung, laryngeal and pharyngeal cancers have highest relative risk in current smokers than former smokers (Gandini *et al.,* 2008). Smokeless tobacco has also been attributed to increase the risk of many tobacco related cancers (Gupta and Ray, 2003; Rose *et al.*, 2016).

Tobacco not only causes cancer deaths but is also responsible for great number of deaths from cardiovascular, chronic obstructive pulmonary and degenerative diseases (IARC, 2007; Norman *et al.*, 2011). In 2000, 4.83 million premature deaths worldwide were attributed to tobacco use, of which 2.41 million were in developing and 2.43 million in developed industrialized countries and these numbers are expected to increase up to 10 million a year by 2030 (Peto *et al*., 2000; Ezzati and Lopez, 2003). The use of tobacco has also been known to cause adversities in pregnancy outcomes, possibly leading to abortion (Kallen, 1999; Little *et al*., 2004). Some studies estimated that tobacco use will result in an annual death exceeding approximately 12 million and each year 6.7 million new tobacco related cancer cases diagnosed (WHO, 2002; Lee and Hashibe, 2014).

Tobacco has been known to affect both the genetic and epigenetic components of the cell, thus resulting in abnormal cell proliferation and apoptosis; up regulation of certain oncogenes and inhibition and down regulation of tumour suppressor genes. The genetic effects may be in the form of point mutation, deletion, insertion, recombination, transversion, transition, and chromosomal aberrations including aneuploidy and polyploidy. These gross changes lead to the overexpression or inactivation of certain key cancer related genes (Valko *et al*., 2004). The epigenetic effects include hypermethylation of CpG islands in the promoter region of tumour suppressor genes, hypomethylation in the promoter region of proto-oncogenes, acetylation, phosphorylation, ubiquitylation, sumoylation, ribosylation and citrullination of some amino acids of the histone proteins (Jenuwein and Allis, 2001; Esteller, 2011). These changes affect the structure and ultimately the function of DNA, thereby leading to abnormal cellular functions and eventually leading to cancer.

The mechanism of action of DNA damage caused by tobacco involved variety of pathways. One pathway involves oxidative stress caused by highly reactive compounds called free radicals. These free radicals were formed within the cell or can come from external source such as tobacco tar. Free radicals present in tobacco tar had been found to penetrate viable cells, bound to DNA and produce nicks in the DNA, thus altering the DNA structure (Sun, 1990; Pryor, 1997). Tobacco is a rich source of oxidants and thus depletes the antioxidants of the body causing increase oxidative stress. This oxidative stress had been observed to damage sperms. The spermatozoa of smokers had been found to have a significantly higher level of DNA fragmentation, DNA strand break and 8-OHdG DNA adducts than non-smokers (Fraga *et al*., 1996; Potts *et al*., 1999; Ezzati and Lopez, 2003; Sepaniak *et al*., 2006). Other studies had also shown that smoking reduced sperm production and motility. However some reported smoking to have a negative impact on intracellular antioxidants but did not necessarily increase oxidative DNA damage (Mostafa, 2010; Viloria *et al*., 2010). In a study involving bladder cancer patients, 4-aminobiphenyl-DNA adducts were higher in current smokers than ex-smokers (Martone *et al*., 1998; Faraglia *et al*., 2003).

One of the carcinogen present in tobacco, NNK had been found to alter the structure of XRCC1, a DNA repair protein, therefore decreasing the protein's ability to repair damaged DNA (Abdel-Rahman and El-Zein, 2000). One of the most important tumour suppressor gene, p53 had been known to be negatively affected by the use of tobacco in many studies. In head and neck squamous cell carcinoma, p53 and cyclin D1, cell cycle checkpoint genes were found to be mutated and over expressed (Xu *et al*., 1998; Vahakangas *et al*., 2001; Calvez *et al*., 2005). In non-small cell lung cancer, exposure to tobacco carcinogens caused mutation in p53, EGFR, hMLH1, FHIT,

hMSH3 and D9S157 loci. Majority of these mutations were deletion of a single base pair or more, thus, resulting in loss of heterozygosity (Hirao *et al*., 2001; Zienolddiny *et al*., 2001; Krishnan *et al*., 2014). Among lung cancer patients with history of tobacco use and none at all, the pattern of mutation differed. It was found that there was an excess of guanine to thymine transversions in smoking related lung cancers. This G to T transversion was 30% prevalent in smokers while it was only 12% among the non-smokers (Pfeifer *et al*., 2002). Smokers were also found to have higher number of small chromatid exchanges (van Poppel *et al*., 1993).

Besides smoking tobacco, the use of smokeless tobacco also demonstrated many genetic alterations between tobacco users and non-users. p53, p21, K-ras, Bax, IL-6, TNF-α, iNOS and Cox-2 genes were found to be highly expressed among smokeless tobacco users, whereas Bcl-2 (an important apoptotic gene) expression seemed to decrease (Biswas *et al*., 2015; Tam *et al*., 2016). However, some studies did not found any correlation between tobacco use and mutations in important genes including H-ras, EGRF, K-ras, p53 and Cox-2 (Xu *et al*., 1998; Vahakangas *et al*., 2001; Calvez *et al*., 2005; Nelson *et al*., 2016).

MIZO

The Mizos are a group of tribals inhabiting the state of Mizoram in the north eastern part of India. Mizoram is located between 92.16°E to 93.26°E longitude and 21.56°N to 24.3°N latitude and shares an international boundary with Myanmar in the east and south and Bangladesh in the west and has an area of 21,081 sq.km. (Lalthanzara and Lalthanpuii, 2009). The lifestyle and dietary habits of the Mizos are rather unique when compared with other parts of the mainland India. Some of the indigenous foods of the Mizos contained smoked and fermented meats and

vegetables, and the use of alkali in the form of soda for the preparation of local food called “Bai”, which is particularly common (Phukan *et al.,* 2006; Lalthanpuii *et al.,* 2015).

Great number of the Mizos also use tobacco and tobacco related products regularly (Phukan *et al.,* 2005). These tobacco products included smoked and smokeless tobaccos. Smoked tobacco comprised of a local cigarette called “Zozial” (Figure 1.4), branded cigarettes of many forms (Figure 1.5), pipe, cheroot, bidi, etc. Zozial and branded cigarettes are currently considered to be the most common type of smoked tobacco used by the Mizos. The used of smokeless tobacco locally manufactured called “Sahdah” (Figure 1.6) and “Tuibur” (Figure 1.7) are also very common in Mizoram. Tuibur is a form of tobacco brew, produced as a cottage industry product with no standardized or properly documented production method and is freely available in the local market. Usually, tuibur is kept in the mouth without swallowing for some time and spitted thereafter. This duration of keeping tuibur in the mouth is also not fixed and varies from individual to individual, however, it mostly depends on the decline in alkalinity of the tuibur (Lalruatfela *et al*., 2017). Other smokeless form of tobacco products include, zarda paan, gutkha, khaini, raja tobacco, tiranga, etc, which are in common use among the Mizos. These habits of using tobacco may be responsible for the high incidences of cancer in Mizoram than the rest of India (NCRP, 2010; Malakar *et al.,* 2014). However, epidemiological research in this area is largely lacking. Some of the epidemiological studies had shown that smoke and smokeless tobacco increased the risk of gastric cancer among the Mizos (Phukan *et al.,* 2005; Lalpawimawha *et al*., 2015). Smoking tobacco alone also increased the risk of developing lung cancer (Lalpawimawha and

Lalruatfela, 2016). In this population, individuals having GSTM1 null genotype and GSTT1 non-null genotype with the habit of using smoked tobacco and tuibur were shown to have a higher risk of developing gastric cancer (Malakar *et al.,* 2012). Looking into the greater use of tobacco and higher incidence of cancer among Mizos, the present study aims to determine to level of DNA damage among the tobacco users of Mizoram.

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Figure 1.1: Tobacco plant (*Nicotiana sp.*).

Figure 1.2: Leaves of tobacco plant.

Figure 1.3: Dried leaves of tobacco plant with wrapper.

Figure 1.4: Zozial (Local cigarette).

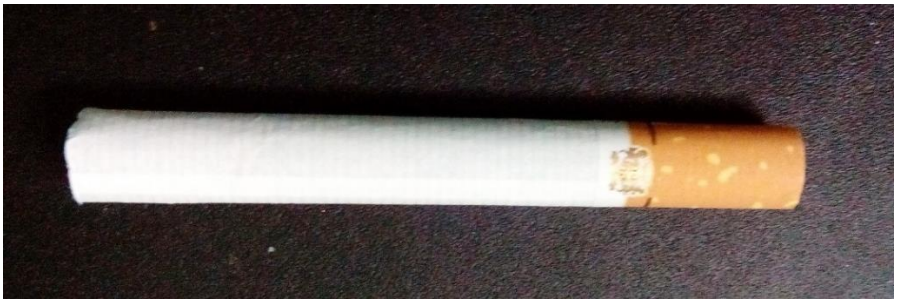

Figure 1.5: Branded cigarette.

Figure 1.6: Branded smokeless tobacco (Shikhar).

Figure 1.7: Tuibur (Tobacco brew).

CHAPTER 2

SELECTION OF SUBJECTS

Preparation of consent form and structured questionnaire

A bilingual (English and Mizo languages) consent form stating the volunteers' willingness to participate in the study was prepared. A structured questionnaire consisting of lifestyle and dietary habits, family history of cancer, and other relevant information related to the volunteers was also developed. This structured questionnaire had also been translated into Mizo language so that all the volunteers do not have any difficulty in answering the questions. Both these documents were submitted and approved by the Human Ethics Committee of the Mizoram University, Aizawl, India.

Selection of subjects

The volunteers were divided into two groups, viz. CON and TOB groups, based on their habit of tobacco consumption. Information of the volunteers is shown in Tables 2.1-2.9.

CON group: The control (CON) group consisted of any male or female volunteers who had no known history of any form of tobacco consumption.

TOB group: This group of volunteers comprised of habitual tobacco (TOB) users of either sex in any form. The type of tobacco used may either be smoked or smokeless tobacco or both.

Consent form

The individual from the control and study groups were contacted and explained about the study and given printed consent form thereafter to have their written consent and willingness to participate in the study. The consent form is reproduced in the next page.

Questionnaire

A set of questions relevant to this study was prepared and all the participants were asked these questions individually and all the informations given by the participants has been noted in this form. The form containing various structured questions that were posed to the volunteers of both groups is also reproduced after the consent form in the following pages.

DEPARTMENT OF ZOOLOGY,
MIZORAM UNIVERSITY,
AIZAWL, MIZORAM.

Consent form

I,……..…., have no objection to participate in the research study entitled "Assessment of DNA damage in cultured peripheral blood lymphocytes of the tobacco users in Mizoram" conducted by the Department of Zoology, Mizoram University. I do not object to give personal food and lifestyle habits for investigation and have been fully informed about the purpose and nature of this study. I have been informed that my identity will be kept fully confidential throughout the study and for any future publication that may be related to the study.

This consent form has been read out to me and I clearly understand the details of it.

Kei, …… hian, Department of Zoology, Mizoram University ten "Assessment of DNA damage in cultured peripheral blood lymphocytes of the tobacco users in Mizoram" tih zirbingna an neih turah hian tel ve ka rem ti e. Ka nun dan leh ei leh in chung chang hriattir ka remti a, he zirbingna thiltum hi chiang taka hrilhfiah ka ni. Ka nihna hi he zirbingna neih chhung leh hmalam hun thuchhuahah pawh tih lan a ni ngai lovang tih min hrilh bawk.

He phalna lehkha hi min chhiar chhuahsak a, ka hrethiam vek a ni.

Signature of the Investigator — Signature of the Volunteer

Date: — Date:

Assessment of DNA damage in cultured peripheral blood lymphocytes of the tobacco users in Mizoram

Volunteer Questionnaire

Cases/Controls

Serial Number:_____

Date:_____/_____/_____

1. Hming/Name:_____ 2. Kum/Age: _____

3. Pian ni leh kum/Date of birth:_____/_____/_____ 3. Sex: Mipa/Hmeichhia (Male/Female).

4. Address:

_____

_____

Phone No._____

5. **San zawng/Height**:_____ (cm/feet-inch). **Rih zawng/Weight**: _____ (Kg).

Zuk leh hmuam chungchang (Life style and habits):

1. Kuhva (Betel chewing habit):

(a) Ei ngailo (Never) [ ] (b) Nghei tawh (Past chewer) [ ] (c) La ei mek (Current chewer) [ ] (d) Chinai nen (With lime): Yes/No.

Kuhva chi (Main ingredients)	Ni khat a ei zat (Frequency/day)	Ei tan kum (Age started)	Nghei kum (Age stopped)	Ei hun chhung (Duration)
Kuhva hring (Fresh betel)				
Kuhva ro (Dried betel)				
Zarda				
A dangte (Any other)				

2. Sahdah (Tobacco):

(a) Hmuam ngailo (Never) [ ] (b) Nghei tawh (Past chewer) [ ](c) La hmuam mek (Current chewer [ ]

(i) I mut laiin Sahdah/Khaini/Raja I hmuam ngai em? (Do you keep Sahdah/Khaini/Raja in your mouth while sleeping?) _____

(ii) I Sahdah/Khaini/Raja hmuam I lem ngai em? (Do you swallow Sahdah/Khaini/Raja?)_____

Main ingredients	Ni khat a hmuam zat (Frequency/day)	Hmuam tan kum (Age started)	Nghei kum (Age stopped)	Hmuam hun chhung (Duration)
Sahdah (Local tobacco)				
Khaini (Golden tobacco)				
Raja				
Guthka/Panmasala/Tiranga				
Tuibur (Tobacco brew)				
A dang (Any other)				

3. Meizial zuk dan (Smoking habit):

(a) Zu ngailo [ ] (b) Nghei tawh [ ] (c) La zu mek [ ]

Meizial (Type)	Ni khata zuk zat (Frequency/day)	Zuk tan kum (Age started)	Nghei kum (Age stopped)	Zuk hun chhung (Duration)
Zozial (Local cigarette)				
Cigarette				
Cigar				
Churot				
Vaibel (Pipe)				
Tuibur				
A dang (Others)				

4. Zu in dan (Alcohol drinking habit):

(a) In ngailo (Never user) [ ] (b) Nghei tawh (Past user) [ ] (c) La in mek (Current user) [ ]

Zu chi (Type of alcohol)	In zeuh zeuh (Occasional) <5 drinks/week	In thin (Moderate) 5-10 drinks/week	In nasa (Heavy) >10 drinks/week	In tan kum (Age started)	Nghei kum (Age stopped)	In hun chhung (Duration)

Ei leh in chungchang (Fooding habit):

A. **Sa I ei ngai em? (Vegetarian/Non-vegetarian): _____**

Type		Kar khat a ei zin zawng (Frequency/week)					Siam dan (Preparation)	
		Nil	1	2-4	4+	Hriatloh (Unknown)	Chhum (Boiled)	Kan (Fried)
Sa rep (Smoked)	Vawk (Pork)							
	Bawng (Beef)							
	Ar (Chicken)							
	Kel (Mutton)							
	Sangha (Fish)							
	A dangte (Others)							
Sa chi al (Salted)	Sangha (Fish)							
	Kaikuang (Prawn)							
	A dangte (Others)							
Pickled	Bawng (Beef)							
	A dangte (Others)							
Sa chi dang ei thin (Any other)								

C. Khawtual ei leh in (Locally available food items):

Type	Kar khat a ei zin zawng (Frequency/week)				
	Nil	1	2-4	4+	Hriatloh (Unknown)
Bekang					
Sa-um					
Dangpuithu					
Nghapih					
Ai-um					
A dangte					

D. Chaw bawlhlo (Additives and spices):

Type	Kar khat a ei zin zawng (Frequency/week)				
	Nil	1	2	Nitin (Daily)	Hriatloh (Unknown)
Soda					
Ajinomoto(MSG)					
Ching-al (Ash filtrate)					
Ai-eng (Turmeric)					

6. Chaw chhumna hmanrua (Techniques of cooking):

(i) Lungalhthei(Coal) (ii) Meihawl thuk(Charcoal stove) (iii) Thing(Wood) (iv) Khawnvartui thuk(Kerosene stove) (v) Liquefied Petroleum Gas (LPG) (vi) Electric thuk(Electric stove) (vii) Microwave oven (viii) A dangte(Others).

7. Chhungkaw natna vei (Family history of subject):

I thisen zawmpui la dam leh boral tawh cancer natna vei an awm em? Khawngaihin han sawi chiang teh (Any of your alive/deceased relatives (***Father, mother, grandparents, uncles, aunts, aunties, cousins, etc***) suffer/suffered from cancer? Please specify):

1.

2.

"I puihna avangin ka lawm e (Thank you for your kind assistance)"

RESULTS

In this study, a total of 245 volunteers participated and out of this, 42 volunteers were tobacco non-users and served as CON cohort whereas the remaining 203 subjects were tobacco users and served as TOB cohort. The CON group consisted of 23 males and 29 females. The TOB group included 129 males and 74 females. The age of all the participants of both the groups ranged between 18 years and 67 years. The mean age of the CON group was 32.77 years whereas that of the TOB group was 42.61 years (Table 2.2). Majority of the participants resided in urban area and are married (Table 2.1). The majority of the participants (26.27%) were young and belonged to the18-27 age group whereas 16.86% of the individuals belonged to 48-57 age (Table 2.3).

When the individuals of both group were categorized by their habit of betel (pan) chewing, 88.18% of TOB group were either current or past consumers of pan whereas 69.23% of the individuals belonging to the CON group are either current or past consumers of pan. Among the pan consumers, majority of the TOB group (62.01%) consumed more than 10 pieces of pan per day while majority of the individuals of CON group (27.78%) consumed more than 10 pieces of pan per day. Most of the individuals of both the groups consumed pan without the addition of tobacco (Table 2.4). Among the TOB group, 54.68% were sahdah consumers whereas 29.56% used tuibur, in the past (33.33%) or the present (66.77%). Female individuals of TOB group indulged more in using smokeless tobacco than the corresponding male subjects (Table 2.5). 130 individuals (64.04%) belonging to TOB group used tobacco in the form of smoking while 73 individuals (35.96%) did

not smoke tobacco. The form of smoking tobacco consisted mostly of zozial (local cigarette) and cigarettes (branded). 73.08% of the smokers smoked both zozial and cigarette while 26.92% smoked only either zozial or cigarette. More than 60% of the smokers of both genders started the practice of smoking after 16 years of age. 65.38% of the smokers smoked for more than ten years while 34.62% smoke for ten years or less (Table 2.6).

In both the groups, a total of 7.69% individuals have used alcohol currently or in the past. Among this, only 25% consumed alcohol currently. The type of alcohol consumed were either locally made or branded alcohol. Majority of the alcohol consumers used both the alcohol types. 27.08% of the TOB group and 50% of the CON group started consuming alcohol only after the age of twenty (Table 2.7). All the volunteers are non-vegetarian, i.e. they consumed meat and its related product in one form or another. 98.52% of the TOB group and 100% of the CON group consumed smoked meat of any kind. More than half participants of both the groups are smoked vegetable consumers while pickled meat was consumed by quite a few participants. The consumption of pickled vegetables and fruits, salted foods, fermented foods, monosodium glutamate and soda were more common among the TOB group than the CON groups (Table 2.8). 46.31% of the subjects belonging to TOB group and 44.23% of the CON group have one or more members of their blood relatives suffered from cancer (Table 2.9).

Table 2.1: Distribution of control and tobacco groups by socio-demographic factors.

Variable	Category	Tobacco group				Control group			
		M	(%)	F	(%)	M	(%)	F	(%)
Residence	Rural	48	19.59	56	22.86	5	2.04	2	0.82
	Urban	81	33.06	18	7.35	15	6.12	20	8.16
Marital status	Married	50	20.41	48	19.59	7	2.86	6	2.45
	Unmarried	79	32.24	26	10.61	13	5.31	16	6.53

M= Male; F= Female; %= Percentage.

Table 2.2: Distribution of control and tobacco groups by age and gender.

Variable	Category	TOB		CON	
		n	(%)	n	(%)
Age	18-27	42	20.69	25	59.52
	28-37	42	20.69	11	26.19
	38-47	38	18.72	4	9.53
	48-57	38	18.72	2	4.76
	58-67	43	21.18	-	-
	Mean±SD	42.61±15.01		27.21±8.81	
Sex	Male	129	63.55	20	47.62
	Female	74	36.45	22	52.38

TOB= Tobacco group; CON= Control group; n= Number of individuals; %= Percentage.

Table 2.3: Distribution of control and tobacco groups by age.

Age	TOB		CON		Total (%)
	Male	Female	Male	Female	
18-27	19	23	10	15	67 (27.35)
28-37	27	15	5	6	53 (21.63)
38-47	23	15	3	1	42 (17.14)
48-57	25	13	2	0	40 (16.33)
58-67	35	8	0	0	43 (17.55)
Total (%)	129 (52.65)	74 (30.21)	20 (8.16)	22 (8.98)	245 (100.00)

TOB= Tobacco group; CON= Control group; %= Percentage; OR= Odds ratio; 95% CI= Confidence interval at 95% (Lower limit-Upper limit).

Table 2.4: Distribution of control and tobacco groups by habit of betel consumption.

Variable	Category	TOB		CON		OR (95% CI)
		n	%	n	%	
Betel	Yes	179	88.18	36	69.23	**3.32** (1.60-6.86)
	No	24	11.82	16	30.77	
Current user	Yes	150	83.80	26	72.22	**1.99** (0.09-4.57)
	No	29	16.20	10	27.78	
Frequency (per day)	<10	68	37.99	26	72.22	**0.29** (0.13-0.63)
	≥10	111	62.01	10	27.78	
Duration of use (year)	≤10	57	31.84	17	47.22	**0.61** (0.29-1.27)
	≥11	122	68.16	19	52.78	
With lime	Yes	162	90.50	32	88.89	**1.19** (0.38-3.77)
	No	17	9.50	4	11.11	
With tobacco	Yes	23	12.85	6	16.67	**0.74** (0.28-1.97)
	No	156	87.15	30	83.33	

TOB= Tobacco group; CON= Control group; n= Number of individuals; %= Percentage; OR= Odds ratio; 95% CI= Confidence interval at 95% (Lower limit-Upper limit).

Table 2.5: Distribution of tobacco group by smokeless tobacco consumption.

Variable	Category	Tobacco group				OR (95% CI)
		Male	Female	Total	%	
Sahdah	Yes	49	62	111	54.68	**8.44** (4.13-17.21)
	No	80	12	92	45.32	
Sahdah current user	Yes	25	49	74	66.67	**3.62** (1.58-8.29)
	No	24	13	37	33.33	
Sahdah duration of use (year)	≤10	21	9	30	27.03	**0.23** (0.09-0.56)
	≥11	28	53	81	72.97	
Tuibur	Yes	31	29	60	29.56	**2.04** (1.10-3.78)
	No	98	45	143	70.44	
Tuibur current user	Yes	18	22	40	66.67	**2.27** (0.75-6.89)
	No	13	7	20	33.33	
Tuibur duration of use	≤10	9	5	14	23.33	**0.51** (0.15-1.75)
	≥11	22	24	46	76.67	
Any other	Yes	55	33	88	43.35	**1.08** (0.61-1.93)
	No	74	41	115	56.65	

TOB= Tobacco group; CON= Control group; %= Percentage; OR= Odds ratio; 95% CI= Confidence interval at 95% (Lower limit-Upper limit).

Table 2.6: Distribution of tobacco group by smoking habit.

Variable	Category	Tobacco group				OR (95% CI)
		Male	Female	Total	%	
Smoker	Yes	118	12	130	64.04	**0.02** (0.01-0.05)
	No	11	62	73	35.96	
Current smoker	Yes	73	5	78	64.00	**0.44** (0.13-1.47)
	No	45	7	52	40.00	
Zozial	Yes	13	2	15	11.54	**1.62** (0.32-8.19)
	No	105	10	115	88.46	
Cigarette	Yes	18	2	20	15.38	**1.11** (0.23-5.50)
	No	100	10	110	84.62	
Both Zozial and Cigaratte	Yes	87	8	95	73.08	**0.71** (0.20-2.53)
	No	31	4	35	26.92	
Dose per day in butts	≤10	75	10	85	65.38	**5.73** (0.71-46.33)
	≥11	43	2	45	34.62	
Age started smoking	≤15	47	1	48	36.92	**0.14** (0.02-1.10)
	≥16	71	11	82	63.08	
Duration of smoking (year)	≤10	44	1	45	34.62	**0.15** (0.02-1.23)
	≥11	74	11	85	65.38	

TOB= Tobacco group; CON= Control group; %= Percentage; OR= Odds ratio; 95% CI= Confidence interval at 95% (Lower limit-Upper limit).

Table 2.7: Distribution of control and tobacco groups by alcohol consumption.

Variable	Category	TOB		CON		OR (95% CI)
		n	%	n	%	
Alcohol	Yes	48	23.65	1	2.38	**3.62** (1.24-10.55)
	No	155	76.35	41	97.62	
Current user	Yes	26	54.17	1	100.00	**3.55** (0.34-36.56)
	No	22	45.83	0	0.00	
Branded alcohol only	Yes	11	22.92	1	100.00	**4.36** (2.60-7.33)
	No	37	77.08	0	0.00	
Local alcohol only	Yes	5	10.42	0	0.00	**1.12** (1.01-1.23)
	No	43	89.58	0	0.00	
Both alcohol	Yes	32	66.67	0	0.00	**3.00** (2.01-4.48)
	No	16	33.33	0	0.00	
Drinks per week	Daily	13	27.08	0	0.00	**1.37** (1.15-1.63)
	<5	35	72.92	1	100.00	
Age started drinking (year)	≤20	35	72.92	1	100.00	**1.00** (0.13-7.70)
	≥21	13	27.08	0	0.00	
Duration of drinking (year)	≤10	28	58.33	1	100.00	**1.71** (1.35-2.18)
	≥20	20	41.67	0	0.00	

TOB= Tobacco group; CON= Control group; n= Number of individuals; %= Percentage; OR= Odds ratio; 95% CI= Confidence interval at 95% (Lower limit-Upper limit).

Table 2.8: Distribution of control and tobacco groups by dietary habit.

Variable	Category	TOB		CON		OR (95% CI)
		n	%	n	%	
Smoked meat	Yes	200	98.52	42	100.00	-
	No	3	1.48	0	0.00	
Smoked vegetable	Yes	105	51.72	21	50.00	**0.99** (0.54-1.83)
	No	98	48.28	21	50.00	
Pickled meat	Yes	91	44.83	7	16.67	**3.42** (1.63-7.21)
	No	111	55.17	35	83.33	
Pickled vegetable and fruits	Yes	124	61.08	13	30.95	**3.87** (1.99-7.51)
	No	79	38.92	29	69.05	
Any salted food	Yes	50	24.63	3	7.14	**5.33** (1.59-17.88)
	No	152	75.37	39	92.86	
Any tin food	Yes	92	45.32	22	52.38	**0.87** (0.47-1.61)
	No	110	54.68	20	47.62	
Fermented foods	Yes	148	72.91	17	40.48	**2.13** (1.15-4.08)
	No	55	27.09	25	59.52	
Monosodium glutamate	Yes	160	78.82	23	54.76	**2.73** (1.42-5.20)
	No	43	21.18	19	45.24	
Soda	Yes	171	84.24	24	57.14	**4.24** (2.18-8.24)
	No	32	15.76	18	42.86	

TOB= Tobacco group; CON= Control group; n= Number of individuals; %= Percentage; OR= Odds ratio; 95% CI= Confidence interval at 95% (Lower limit-Upper limit).

Table 2.9: Distribution of control and tobacco groups by family history of cancer.

Variable	Category	TOB		CON		OR (95% CI)
		n	%	n	%	
Family history of any cancer	Yes	94	46.31	19	45.24	**1.09** (0.59-2.01)
	No	109	53.69	23	54.76	

TOB= Tobacco group; CON= Control group; n= Number of individuals; %= Percentage; OR= Odds ratio; 95% CI= Confidence interval at 95% (Lower limit-Upper limit).

CHAPTER 3

ACCELERATION OF MICRONUCLEI FREQUENCY IN THE CONSUMERS OF TOBACCO IN MIZO COMMUNITY

Abstract

Many factors can threaten the genetic integrity of the genome of individuals and out of which the use of tobacco in any form is one of the crucial factors that constantly threaten the genome. Cytokinesis blocked micronucleus assay is an excellent tool to assess any DNA damage precisely. Lymphocyte by virtue of their longevity and non-proliferation are able to retain signature of DNA damage over the years and later express it in the form of micronuclei once they are stimulated to divide. Blood from tobacco users and non-users was collected from healthy Mizo volunteers and cultured in the laboratory. The buffy coat of cells was inoculated into RMPI medium containing phytohemagglutinin and allowed to grow for 44 h. Cytochalasin-B was added to block cytokinesis and the micronuclei were studied at 72 h post culture. Data analysis revealed that the frequency of micronuclei increased significantly in individuals regularly consuming tobacco in any form when compared to those individuals who did not take any form of tobacco. It was found that the individuals who were using smokeless tobacco in the form of tuibur (tobacco brew) had higher frequency of micronuclei than the other forms of tobacco. Our study clearly indicates that the use of tobacco in any form is a risk factor for DNA damage.

INTRODUCTION

The integrity of the genome is under constant threat due to various internal and external factors. Although human beings have very efficient repair system, exposure to certain detrimental factors put this system at receiving end and the repair capacity of the genome gets compromised leading to various disorders. Therefore, the integrity of the

genome must be preserved by the cells to maintain its normal structure and function and keep them healthy. The failure of cells to do so leads to devastating consequences including mutation, which may be defined as any change in the native structure of the DNA (Chakarov *et al*., 2013). Mutation very often leads to chromosomal aberrations (Mateuca *et al*., 2006). There are many mechanisms by which the cells maintain any change in the genome and this includes a repertoire of enzymes or proteins which play key roles in the execution of various repair mechanisms that will maintain the integrity of the genome. These enzymes or proteins include DNA repair enzymes, cell cycle proteins, apoptotic proteins, etc. The cell has inbuilt mechanisms to detect DNA damage and the moment any damage is detected, these become operational. Cell cycle proteins detect any damage in the DNA before the cell undergoes division, where DNA repair enzymes will repair the damage. However, if the DNA damage is too severe to be repaired, the cell with the damaged DNA will be directed to commit suicide by programmed cell death or apoptosis (Helleday *et al.*, 2014). If this cell with damaged DNA somehow manages to evade apoptosis and continues to divide, it leads to development of cancer and eventually death of the individual (Christmann *et al.*, 2003; Petrini and Stracker, 2003; Valko *et al.*, 2004; Jeggo *et al*., 2016).

The damage in the DNA can be triggered either by external or internal sources (Iyama and Wilson, 2013). The internal sources of DNA damage are spontaneous in nature and include errors made by the cell's replication machinery in the course of DNA replication or other cellular processes (Sancar *et al.*, 2004; Acuna-Hidalgo *et al.*, 2016). The external sources may be physical, chemical or biological in origin and are called

mutagens (Iyama and Wilson, 2013). Mutagens cause mutations in either one or both strands of the DNA. If the damage happens on both strands of the DNA with the same genetic locus, it leads to chromosomal aberration where a piece of the chromosome is separated from the main chromosome. If this cell undergoes cell division, the broken piece of chromosome can be seen, after karyokinesis but before cytokinesis, as a tiny dot in a separate nucleus inside the cell. This small nucleus containing the broken piece of chromosome is referred to as a micronucleus (MN). It often occurs that there can be more than two or more numbers of micronucleus inside a single cell if the DNA damage happens on multiple loci of the same or different chromosomes, such are referred to as micronuclei (Fenech, 2000; Fenech *et al.*, 2011). Basically, the composition of a micronucleus is chromosome. The size of the chromosome inside a micronucleus vary significantly, some may even contain a whole chromosome, whereas others may have fragments of chromosome. The length of the chromosome inside a micronucleus directly determines its size. Fragments of chromosome present in micronucleus arise as a result of DNA double strand breaks, whereas the presence of a whole chromosome may arise because of damages in the centromere or mitotic spindle proteins, thus, disallowing the chromosome to be separated along with other chromosomes (Norppa and Falck, 2003; Fenech *et al.*, 2011).

Tobacco has been known to have many adverse effects on the health of its users, which include toxic, cytotoxic, genotoxic, mutagenic, teratogenic and carcinogenic events. These effects are a result of more than eight thousand chemicals present in tobacco, out of which 500 are toxic and more than 80 are carcinogenic in nature (Hecht, 2003). Many

studies had shown that tobacco induced micronuclei in buccal mucosa epithelial cells and cell line *in vitro* treated with tobacco smoke (Massey *et al.,* 1998). The consumption of tobacco has been associated with many forms of cancer (Talhout *et al.,* 2011; Bassiony *et al.*, 2015). Cancer of the mouth, lungs, esophagus, stomach, liver, pharynx, colon, etc., have been known to be directly linked to tobacco use. Besides cancer, tobacco use is known to cause several other diseases like bronchitis, asthma, tuberculosis, laryngitis, pharyngitis, cardiovascular, chronic obstructive pulmonary, degenerative diseases, etc. (Musk and De Klerk, 2003; IARC, 2007; Norman *et al.*, 2011; Elmasry *et al.*, 2015). The consumption of alcohol along with tobacco has been reported to significantly increase the risk of colon cancer (Slattery *et al.,* 1997). Tobacco smoking, alcohol consumption and betel quid chewing have also been observed to significantly increase the risk of developing lung cancer (Phukan *et al.,* 2014; Saikia *et al.,* 2014). Smokeless tobacco has also been attributed to the increase risk of many tobacco related cancers (Gupta and Ray, 2003; Rose *et al.*, 2016).

Therefore, the present study was undertaken to determine the frequency of micronuclei in peripheral blood lymphocytes of the Mizos who have been consuming tobacco.

METHODOLOGY

Blood collection

The subjects were divided into two groups as described in the previous chapter, i.e. Chapter 2.

Control group: This group consisted of volunteers in the age group of 18-67 years of either sex who had no known history of any form of tobacco consumption.

Tobacco group: This group consisted of volunteers belonging to the age group of 18-67 years of either sex, who consumed any form of tobacco on regular basis. The type of tobacco used may either be smoked or smokeless tobacco or both.

After identifying suitable volunteers for this study, the consent form and questionnaire described in Chapter 2 were distributed to the volunteers for filling. Approximately 10 ml of blood sample was collected from each individual in a separate sterile heparinized vacutainer using sterile blood collection technique. The collected peripheral blood samples were transported to Cancer and Radiation Biology Laboratory, Department of Zoology, Mizoram University for further processing.

Human peripheral blood lymphocyte culture

Human peripheral blood lymphocyte (HPBL) culture was performed according to standard protocol (Jagetia *et al.*, 2001). The blood was allowed to settle for half an hour and the buffy coat was aseptically collected for lymphocyte culture. Approximately one million nucleated cells were inoculated into several sterile culture tubes containing 2 ml RPMI-1640 medium supplemented with 25 μl/ml of phytohemagglutinin (PHA) as

the mitogen to stimulate cell division of the lymphocytes. The cultures were set in triplicate for each volunteer and transferred to a CO_2 incubator maintained at 37°C.

Cytokinesis blocked micronucleus assay

Cytokinesis blocked micronucleus assay was performed according to standard protocol (Fenech and Morley, 1985; Kirsch-Volders *et al*., 2003). Briefly, after 44 h of initial incubation, 5 μg/ml of cytochalasin-B was added into each culture to inhibit cytokinesis and the cells were allowed to grow for another 28 h. The cells were harvested 72 h after the initiation of the culture. The cells were centrifuged at 1000 rpm for 10 mins and the supernatant was discarded. The remaining pellet containing lymphocytes was subjected to mild hypotonic treatment (KCl 0.75%) and incubated at 37°C for 5 minutes. After incubation, the tubes were centrifuged at 1000 rpm for 5 mins and the supernatant was discarded. The pellet was fixed with Carnoy's fixative (3:1, methanol:acetic acid) for 15 mins and centrifuged at 1500 rpm for 5 mins. After centrifugation, the cells were resuspended in a small volume of fixative and dropped on to pre-cleaned coded chilled slides. The slides containing cells were stained with acridine orange (0.125%) and washed with PBS. The slides were observed under a microscope (DM2500, Leica Mikrosystems GmBH, Wetzlar, Germany) fitted with epifluorescence attachment for the presence of micronuclei in the binucleated lymphocytes (BN). BN cells with one micronucleus (MNBNC), containing two (2MNBNC) and BN cells with more than two MN (MMNBNC) were scored and 1000 BN cells were counted from each culture tube and a total of 3000 cells were scored for each individual.

Statistical analyses

The statistical analyses between the control and study cohorts were carried out by Mann-Whitney U test for micronuclei frequencies and Pearson's correlation coefficient was calculated to determine the correlation of micronuclei formation with age. Linear regression was used to see the effect of different variables on the frequency of micronuclei.

RESULTS

The micronuclei frequency has been expressed as mean ± standard error of the mean (SEM) and results of both the control and tobacco groups are shown in Tables 3.1 and 3.2 respectively.

The mean frequency of MNBNC in the control group was 26.93±1.53. The control group also showed the presence of two and multiple micronuclei (Table 3.3). The consumption of tobacco in any form resulted in drastic elevation in the frequencies of micronuclei in both males and females (Table 3.2, Figure 1). Gender wise analysis revealed that the frequency of MNBNC was significantly higher in females than males, whereas the frequency of 2MNBNC and MMNBNC did not show such statistical correlation (Table 3.4 Figure 3.2). Out of 245 individuals screened the maximum frequency was 109.33 and the lowest frequency of MNBNC was 17.67. The analysis of micronuclei frequency in relation to age indicated that the frequency of micronuclei elevated significantly with age *i.e.* it was higher in the older individuals than the younger individuals (Table 3.5)

The correlation between different variables and micronuclei frequency was determined by linear regression, especially with respect to age, betel consumption, alcohol use, dietary habits including the use of sodium bicarbonate, monosodium glutamate and habits of tobacco consumption. The analysis indicated that age, the consumption of betel, betel with lime, betel with tobacco, frequency of betel consumed every day, salted meat, sodium bicarbonate, monosodium glutamate, pickled vegetables and fruits and alcohol use in the past or present, and duration of alcohol intake played an important role in increasing the frequency of MNBNC. Despite this increase, only age and alcohol use showed significant correlation at $p < 0.05$ (Table 3.6). The frequency of two micronuclei has been found to be affected by age, use of betel currently, betel with tobacco consumption, duration of betel use, alcohol use, drinking of branded or local alcohol or both the alcohol types. Similarly, daily consumption of alcohol, consumption of smoked vegetables, pickled vegetables and fruits, fermented foods and sodium bicarbonate increased the frequency of 2MNBNCs. Among these variables, age, alcohol use and drinking of branded or local alcohol or both the both branded and local alcohol have been found to increase the frequency of 2MNBNC significantly (Table 3.7). When the analysis of data for MMNBNC and different variables was carried out it was found that age, consumption of betel, betel with lime, betel with tobacco, duration of betel use, alcohol use, drinking of branded or local alcohol or both branded and local alcohol, daily drinking of alcohol, age of drinking alcohol, consumption of smoked vegetables, salted meats, pickled meat and sodium bicarbonate, and family history of any form of

cancer increased the frequency of MMNBNC, however, significant correlation was detected only for age and consumption of salted meat (Table 3.8).

When the frequency of micronuclei was analyzed with respect to consumption of different forms of tobacco, it was found that the frequency of micronuclei increase in the TOB group with the current use of sahdah and tuibur, tuibur consumption, smoking and duration of smoking. Smoking, tuibur use and duration of smoking were found to increase the frequency of micronuclei in the TOB group significantly (Table 3.9). Similarly, there has been a correlation between the consumption of sahdah, tuibur, duration of tuibur use, smoking of zozial only, zozial and cigarette, frequency and duration of smoking and the frequency of 2MNBNC. The use of tuibur and duration of smoking were found to have significant correlation (Table 3.10). Despite the fact that the frequency of MMNBNC increased in the individuals consuming sahdah, duration of sahdah use, duration and use of tuibur, smoking, smoking of zozial only and frequency of smoking, the correlation was not statistically significant (Table 3.11).

DISCUSSION

The human genome is constantly threatened due increased environmental pollution and adoption of life style that is detrimental to cellular DNA. Even though there are many unavoidable factors that may have deleterious effects on DNA beyond an individual's control, individual's choice of life style may aggravate these changes in the genome. Tobacco use has been associated with several diseases that include ischemic heart disease, stroke, myocardial infarction, chronic lung diseases, lung fibrosis, emphysema, oral diseases including staining of dentures, Parkinson's disease, male

infertility and many types of cancer (Miller and Das, 2007; Morse and Rosas, 2014). It was estimated that there are 1.3 billion smokers in the world and 50% of them die due to tobacco smoking related diseases (Britton, 2017). Despite this fact, the trend for tobacco use has not been declining. Many individual are also taking other forms of tobacco besides smoking. In the North eastern part of India, tobacco use is highly prevalent and Mizoram in particular has high incidence of cancer presumably because of the use of tobacco and other unique life style patterns. Therefore the present study was designed to study DNA damage in the form of micronuclei in individuals of the Mizo ethnic group who are using tobacco in any form.

In addition, the consumers of tobacco or tobacco related products are also exposed to other additional factors like non-ionizing radiations, diesel exhausts, electromagnetic field, chemicals, etc., which may further aggravate the condition. Lymphocytes are model system to study the effect of long term exposure of any agent. As they do not divide once they are formed, they can retain the signature of DNA damage which is expressed as MN once they are forced to divide *in vitro* (Jagetia and Venkatesha, 2005). Tobacco consumption has been found to induce micronuclei in peripheral blood lymphocytes earlier (Christobher *et al.*, 2017). The exfoliated cells of buccal mucosa from tobacco users have shown increased frequency of micronuclei (Palaskara and Jindal, 2010; Bansal *et al.*, 2012, Dosi *et al.*, 2015). Among smokers alone, studies have indicated significant rise of MN frequency in buccal mucosa, exfoliative cells and peripheral blood lymphocytes when compared to corresponding control subjects (Nerseyan *et al.,* 2006; Zamani *et al.,* 2011; Khanna *et al.*, 2012; Pradeep *et al.*, 2014).

Individuals who have been using tobacco have shown not only an increase in MNBNCs but also that of 2MNBNCs and MMNBNCs indicating that tobacco induce complex multiply sites of DNA damage (Jagetia and Vanketsha, 2015; Nikitaki *et al.*, 2015).

The duration and frequency of tobacco smoked were also found to have a direct relationship with the MN frequency, which was significantly higher in our study cohorts. A similar effect has been reported earlier (Motgi *et al.,* 2014; Gangadharan *et al.,* 2016). Smokeless tobacco consumption has been found to increase MN frequency in comparison to control cohorts (Sellapa *et al.,* 2009; Jyoti *et al.,* 2012). Interestingly, the effect of smoking and smokeless tobacco differs as many new chemicals are formed during the process of pyrolysis in smoking tobacco (Pryor *et al*., 1983; Calafat *et al*., 2004). The individuals who used smokeless tobacco had a higher frequency of MNBNCs than smokers, however, the differences were statistically non-significant. Likewise, no significant difference between smokeless tobacco users and smokers has been reported (Ozkul *et al.,* 1997; Motgi *et al.,* 2014). In contrast, others had shown that the effect of smoking tobacco caused a significant elevation in the MN frequency than smokeless tobacco in buccal mucosa cells (Bansal *et al.,* 2012; Iyer *et al.*, 2015). NNK, a carcinogens present in tobacco has been reported to induce MN in mononucleated cells (El-Zein *et al.,* 2008). The smoking of tobacco alone has been reported to significantly increase MN frequency in buccal mucosa, exfoliative cells and peripheral blood lymphocytes when compared to control subjects (Nerseyan *et al.,* 2006; Zamani *et al.,* 2011; Khanna *et al.,* 2012; Nefic and Handzic, 2013; Pradeep *et al.,* 2014).

Females showed higher frequency of MN than males (Maffei *et al.,* 2002; Reis *et al.,* 2006; Nefic and Handzic, 2013). A similar effect has been observed in the present study where females had higher number of micronucleated lymphocytes than the males. However, no difference in MN frequency in buccal mucosa and urothelial cells have been reported in women who smoked tobacco. This may due to small sample size, smoking patterns and paucity of information about the number of cigarettes smoked (Blaszczyk and Mielzynska-Svach, 2014).

The use of tobacco coupled with consumption of alcohol showed positive correlation as the frequency of MN was higher than tobacco use alone. Alcohol consumption has been shown to increase the frequency of MN in human buccal mucosa (Jeeva *et al.,* 2015). The use of tobacco, alcohol and betel quid have been found to synergistically increase the frequency of MN in buccal mucosa cells of tobacco consumers (Bharali and Arangham, 2014). Similarly, age has also influenced the outcome of MN in both groups as both groups showed positive correlation. However, tobacco consumers had an elevated frequency of MN than the controls. Similarly, increasing age has been found to rise the frequency of micronuclei (Wojda *et al.,* 2007; Jones *et al*., 2011; Nefic and Handzic, 2013). This difference in frequency of MN has been proposed to be an important genetic indicator for the predisposition of certain types of cancers. It had been shown that there are significant differences in MN frequency between cancer patients and healthy controls. However, the variables affecting micronucleus induction have been reported to differ between two study groups. Among controls, only tobacco had been reported to influence MN frequency but not age or sex. Contrastingly, in cancer

patients only age had been found to significantly influence MN frequency (Duffaud *et al.,* 1997; Milosevic-Djordjevic *et al.,* 2010).

The population of Mizoram, although not highly populated in comparison to other states of India, has one of the highest incidence of cancer in the country. There are many hypotheses for this reason and one such hypothesis is that the Mizos have been using many forms of smoking and smokeless tobacco that may have increased genetic instability leading to higher incidence of cancer. Our study indicates an increased genomic instability in the ethnic Mizo population, which could be the main cause of higher cancer incidence. Epidemiological studies had shown the relation of tobacco use and cancer of certain sites among the Mizos (Phukan *et al.,* 2005; Lalpawimawha *et al.,* 2015; Lalpawimawha and Lalruatfela, 2016). Tobacco affected the genetic and epigenetic components of cells, thus affecting the structure and function of many key genes. The genetic effects may be in the form of point mutation, deletion, insertion, recombination, transversion, transition, and chromosomal aberrations including aneuploidy and polyploidy (Valko *et al*., 2004).

The exact mechanism of increased micronuclei frequency in the tobacco users is not known and multiple putative mechanisms may have been responsible for this observation. Tobacco smoke has been reported to trigger the production of quinone/hydroquinone free radicals that in turn produces superoxide radicals inducing oxidative stress (Church and Pryor, 1985; Valavanidis *et al.*, 2009). A recent study has shown free radical production by various brands of US cigarettes (Goel *et al.*, 2017). The free radicals produced by tobacco may induce DNA adducts, which may be converted into DNA strand breaks and subsequently into micronuclei after cell division. In fact, the use of tobacco has been

reported to produce DNA and protein adducts. Tobacco also induces sugar damage, apurinic/apyrimidinic sites, small base damages, bulky DNA adducts, DNA cross links and DNA strand breaks (Hang, 2010; Phillips and Venitt, 2012).

Epidemiological studies have shown that consumption of tobacco leads to several mutations in the genome and 30 different base substitutions have been identified in individuals who have been smoking tobacco and suffering from cancers related to tobacco (Alexandrov *et al*., 2016). Free radicals present in tobacco tar had been known to produce nicks in the DNA and increase adducts formation (Pryor, 1997; Sepaniak *et al*., 2006). In a study involving bladder cancer patients, 4-aminobiphenyl-DNA adducts were higher in current smokers than ex-smokers (Martone *et al*., 1998; Faraglia *et al*., 2003). One of the carcinogens present in tobacco, NNK had been found to alter the structure of XRCC1, a DNA repair protein, therefore decreasing the protein's ability to repair damaged DNA and also induces formation of DNA adducts (Abdel-Rahman and El-Zein, 2000; Hang, 2010). In addition, the use of tobacco had been known to influence the structure and function of important genes like p53, p21, K-ras, H-ras, Bax, IL-6, TNF-α, iNOS, BCl-2, cyclin D1, EGFR, hMLH1, FHIT, hMSH3 and Cox-2 genes (Calvez *et al*., 2005; Krishnan *et al*., 2014; Biswas *et al*., 2015; Tam *et al*., 2016) that may have contributed in various ways to inhibit the DNA repair mechanisms and increase the micronuclei frequency in the tobacco consumers.

CONCLUSIONS

The frequency of micronuclei increased in the tobacco users and it was higher in smokeless tobacco users than those of smokers. The consumption of alcohol and betel and

age have been confounding factors that increased the MN frequency. The increase in cancer incidence among the Mizos may be because of elevation in genomic instability as indicated by the formation of greater number of MN. The formation of MN in tobacco consumers may be due to the formation of free radicals and DNA adducts by tobacco and negative alteration in the p53, p21, K-ras, H-ras, Bax, IL-6, TNF-α, iNOS, BCl-2, cyclin D1, EGFR, hMLH1, FHIT, hMSH3 and Cox-2 genes.

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Table 3.1: Frequency of micronucleus, two micronuclei and more than two micronuclei of control group.

ID	Mean±SEM			ID	Mean±SEM		
	MNBNC	2MNBNC	MMNBNC		MNBNC	2MNBNC	MMNBNC
C1	42.00±2.08	18.00±1.53	5.33±0.67	**C2**	42.67±1.45	15.67±0.88	7.00±0.58
C3	37.33±0.67	15.33±0.88	6.33±0.33	**C4**	32.00±2.52	16.67±0.67	4.33±0.67
C5	32.33±1.86	15.00±0.58	4.67±0.67	**C6**	37.67±1.76	14.33±0.33	7.33±0.67
C7	33.33±2.33	15.67±0.67	3.67±0.67	**C8**	25.33±0.67	18.33±1.20	4.33±0.33
C9	28.67±0.88	14.33±1.86	5.33±0.88	**C10**	31.67±0.33	13.33±1.76	6.67±0.67
C11	34.67±0.88	14.00±1.00	5.00±0.58	**C12**	29.67±1.45	12.00±0.58	5.33±0.88
C13	23.00±1.15	14.33±1.76	5.67±1.45	**C14**	20.33±0.88	15.67±0.88	6.33±0.67
C15	17.33±0.67	12.67±1.45	5.00±0.58	**C16**	19.67±0.67	10.67±0.88	5.67±1.20
C17	19.33±1.20	12.33±0.88	6.00±1.15	**C18**	18.00±1.15	13.33±0.33	5.33±1.45
C19	17.33±0.88	15.33±1.76	3.67±0.33	**C20**	17.67±0.33	19.33±0.67	5.67±0.67
C21	46.67±1.45	20.33±0.88	4.67±0.33	**C22**	48.67±1.20	16.33±0.67	4.00±0.58
C23	45.33±1.86	16.67±0.67	4.33±1.20	**C24**	35.00±1.00	18.67±1.20	4.67±0.88
C25	35.00±1.53	17.00±1.53	4.33±0.33	**C26**	33.67±0.88	11.67±0.88	3.33±0.88
C27	34.67±2.40	10.67±0.88	5.00±0.58	**C28**	33.33±2.19	12.67±0.88	4.67±0.88
C29	28.33±1.20	12.67±0.33	5.67±0.88	**C30**	23.00±1.15	18.00±1.15	5.00±0.58
C31	19.00±1.00	19.67±0.88	5.33±0.88	**C32**	18.33±1.45	16.00±1.00	5.00±0.58
C33	18.67±1.20	15.33±0.33	4.33±0.88	**C34**	15.00±1.73	14.67±0.33	4.00±0.58
C35	17.67±0.67	14.33±1.20	6.33±0.33	**C36**	17.33±1.45	12.33±0.33	5.33±0.33
C37	16.67±0.88	12.00±1.15	4.33±0.67	**C38**	16.67±1.33	10.00±1.00	6.00±1.15
C39	17.67±1.20	10.67±0.33	3.67±0.88	**C40**	18.00±1.15	11.67±0.88	5.33±0.33
C41	16.33±0.88	10.67±0.88	6.00±1.53	**C42**	16.00±1.73	12.67±0.88	5.33±1.45

ID= Identification number; C= Control group; MNBNC= Binucleated cells with one micronucleus; 2MNBNC= Binucleated cells with two micronuclei; MMNBNC= Binucleated cells with more than two micronuclei; SEM= Standard error mean; N= 3.

Table 3.2: Frequency of micronucleus, two micronuclei and more than two micronuclei of tobacco group.

ID	Mean±SEM			ID	Mean±SEM		
	MNBNC	2MNBNC	MMNBNC		MNBNC	2MNBNC	MMNBNC
T1	106.67±2.03	34.00±2.08	10.67±0.88	**T2**	106.00±1.15	33.00±1.53	9.33±0.88
T3	105.67±2.03	29.33±0.67	10.00±1.15	**T4**	101.33±3.48	22.33±0.88	9.67±0.88
T5	97.00±2.08	33.00±1.53	5.67±0.88	**T6**	102.00±1.53	26.33±0.88	5.00±0.58
T7	107.67±0.67	29.33±1.20	6.33±0.67	**T8**	102.33±0.88	26.67±1.20	6.33±0.33
T9	92.67±1.67	24.67±0.67	8.00±0.58	**T10**	97.00±3.46	24.67±0.33	8.67±0.88
T11	92.33±2.96	26.00±0.58	10.33±0.33	**T12**	99.67±1.86	22.00±1.15	11.00±1.00
T13	98.33±1.45	23.67±1.20	3.67±0.88	**T14**	103.00±1.73	25.33±0.67	7.00±0.58
T15	91.00±1.73	23.67±0.67	6.67±0.33	**T16**	105.67±0.88	22.67±0.33	6.00±0.58
T17	102.67±1.86	26.67±0.88	9.67±0.33	**T18**	93.67±2.03	25.00±0.58	7.67±0.67
T19	99.67±1.20	24.33±0.88	6.67±0.33	**T20**	90.67±2.33	10.67±0.88	3.33±1.20
T21	99.67±0.88	12.67±0.88	3.67±0.67	**T22**	104.67±1.45	12.67±0.33	4.33±0.88
T23	100.33±1.45	18.00±1.15	10.67±0.88	**T24**	94.00±1.00	19.67±0.88	7.00±1.73
T25	92.33±0.88	16.00±1.00	6.67±1.45	**T26**	99.33±1.86	14.67±0.88	7.33±1.76
T27	89.67±1.45	10.33±0.88	8.67±0.33	**T28**	100.00±1.53	12.33±0.88	11.67±2.40
T29	96.00±2.89	12.33±0.67	9.00±0.58	**T30**	101.67±1.67	14.67±2.19	10.00±1.00
T31	91.33±0.88	20.33±1.45	6.67±1.45	**T32**	99.67±3.48	19.33±1.45	8.33±0.88
T33	83.00±1.00	16.00±0.58	5.67±1.20	**T34**	86.67±1.86	17.33±0.67	6.67±0.88
T35	90.67±2.91	19.33±1.20	7.67±0.88	**T36**	94.67±1.45	22.67±1.86	6.00±1.53
T37	96.00±2.08	19.00±1.73	5.00±1.53	**T38**	91.00±1.73	15.67±1.20	6.67±0.67
T39	100.33±1.20	16.67±0.33	5.67±1.20	**T40**	97.33±4.06	11.00±1.00	6.00±1.53
T41	82.67±1.20	15.67±0.88	6.33±1.20	**T42**	84.00±1.00	15.67±2.03	6.00±1.15
T43	95.33±1.45	20.33±1.45	6.67±0.67	**T44**	82.67±0.88	19.33±1.45	6.67±0.33
T45	87.00±1.73	16.00±0.58	4.67±0.88	**T46**	93.33±2.96	17.33±0.67	5.00±0.58
T47	93.00±2.89	19.33±1.20	6.00±1.15	**T48**	92.67±2.19	19.33±3.18	5.33±1.45
T49	92.00±2.65	16.00±0.58	3.67±0.33	**T50**	85.00±2.31	12.33±2.33	5.67±0.67
T51	79.67±1.20	14.67±1.20	4.67±0.67	**T52**	91.67±2.03	15.67±0.33	4.67±0.88
T53	82.33±1.20	15.67±0.88	4.33±0.33	**T54**	87.67±2.33	12.67±1.20	5.33±1.20
T55	87.33±2.73	18.00±2.52	5.33±0.33	**T56**	89.67±1.86	17.00±1.53	4.67±0.88
T57	83.00±2.52	16.00±0.58	5.00±0.58	**T58**	83.33±2.33	18.33±0.33	5.67±1.20
T59	82.33±1.33	20.00±0.58	4.33±1.20	**T60**	69.33±1.20	20.33±1.20	6.67±0.88
T61	80.00±2.08	16.33±0.67	5.33±1.45	**T62**	85.33±2.33	14.00±1.53	5.67±1.45
T63	69.33±0.88	13.00±1.00	6.00±1.00	**T64**	82.33±2.03	13.33±0.33	5.67±0.88
T65	76.00±2.08	15.67±0.33	5.67±0.33	**T66**	62.67±2.03	15.00±0.58	6.00±1.00
T67	70.67±0.88	13.67±1.20	7.67±0.33	**T68**	76.67±2.33	15.00±0.58	5.33±0.67
T69	64.33±2.96	14.00±0.58	7.00±0.58	**T70**	65.00±2.08	12.00±1.00	6.33±0.33
T71	56.00±1.73	15.00±0.58	4.33±0.67	**T72**	73.00±1.53	15.33±0.88	4.67±0.67
T73	67.00±1.53	14.00±1.53	5.67±0.33	**T74**	71.67±0.88	13.00±1.00	5.33±0.88

T75	50.33±0.33	13.33±0.33	4.33±1.20	**T76**	48.67±0.88	16.67±0.67	5.33±0.88
T77	51.67±2.33	15.00±0.58	4.33±0.88	**T78**	44.67±1.20	18.67±1.20	4.00±1.15
T79	47.00±1.53	21.33±1.86	7.00±0.58	**T80**	45.67±2.03	15.67±0.33	6.33±0.67
T81	41.00±2.08	13.00±0.58	5.00±0.58	**T82**	46.00±1.73	15.33±0.33	5.67±1.20
T83	44.67±2.03	12.33±1.20	6.00±1.15	**T84**	43.00±1.73	13.33±0.33	5.33±1.45
T85	48.67±1.20	15.00±0.58	3.67±0.33	**T86**	43.00±2.31	14.67±0.88	5.67±0.67
T87	45.00±2.08	14.00±0.58	5.00±1.53	**T88**	41.67±2.19	15.00±0.58	7.67±1.45
T89	44.00±2.08	13.00±1.15	6.33±1.45	**T90**	45.33±2.33	12.33±0.33	7.00±1.15
T91	43.67±1.86	13.33±0.33	8.00±0.58	**T92**	45.67±1.45	11.67±0.33	6.33±0.88
T93	44.00±2.52	13.67±0.67	5.67±0.88	**T94**	41.00±2.08	13.67±0.33	6.00±1.53
T95	35.67±1.45	11.33±0.67	6.33±1.20	**T96**	43.00±1.73	13.00±0.58	3.67±0.33
T97	42.33±2.03	10.33±0.33	5.33±0.88	**T98**	47.67±0.33	15.00±0.58	5.00±1.00
T99	46.33±1.20	14.67±1.86	5.00±0.58	**T100**	48.67±1.45	13.33±0.88	4.67±0.67
T101	34.33±0.67	20.00±0.58	5.00±1.15	**T102**	37.67±1.33	15.67±1.20	6.00±1.00
T103	42.33±1.20	17.33±0.88	7.00±1.15	**T104**	40.00±1.15	18.33±0.33	5.67±0.88
T105	35.33±1.45	21.00±0.58	5.00±0.58	**T106**	35.67±2.40	18.67±1.20	6.00±1.53
T107	40.00±0.58	16.00±0.58	5.67±0.67	**T108**	43.00±1.15	12.67±1.20	6.00±1.15
T109	45.33±1.76	13.33±0.88	4.33±0.33	**T110**	34.00±2.52	14.33±0.88	7.33±0.67
T111	41.00±2.08	15.33±0.67	3.67±0.67	**T112**	32.67±2.03	15.67±0.33	4.33±0.33
T113	31.67±2.03	13.00±0.58	4.00±1.00	**T114**	29.67±0.88	15.33±0.33	6.00±0.58
T115	38.67±1.86	12.33±1.20	4.33±0.88	**T116**	34.67±2.03	13.33±0.33	7.33±0.33
T117	33.00±2.08	15.00±0.58	4.67±0.33	**T118**	36.00±1.15	14.67±0.88	6.33±0.88
T119	39.33±0.67	14.00±0.58	5.00±0.58	**T120**	37.67±1.20	15.00±0.58	3.67±0.88
T121	28.33±1.86	13.00±1.15	4.33±0.33	**T122**	39.00±0.58	12.33±0.33	4.67±0.88
T123	29.67±0.88	13.33±0.33	4.00±1.00	**T124**	38.33±1.20	11.67±0.33	4.33±0.88
T125	27.00±1.53	13.67±0.67	4.67±0.33	**T126**	20.67±0.33	13.67±0.33	3.33±0.88
T127	27.67±1.20	11.33±0.67	5.00±0.58	**T128**	17.67±0.33	13.00±0.58	4.33±0.88
T129	18.00±1.00	10.33±0.33	6.33±0.33	**T130**	105.67±1.86	17.00±1.73	4.67±0.33
T131	106.33±2.33	35.67±1.86	5.67±0.88	**T132**	109.33±1.76	35.33±0.88	4.00±0.58
T133	102.33±4.67	34.67±1.86	4.67±0.67	**T134**	100.33±0.88	34.67±1.45	5.00±1.15
T135	107.67±0.67	36.00±0.58	5.67±0.33	**T136**	102.33±0.88	33.33±2.19	4.67±0.88
T137	94.67±2.33	32.67±1.20	4.67±0.33	**T138**	100.00±1.73	35.00±1.73	6.33±0.88
T139	101.33±2.40	33.67±0.88	3.67±0.88	**T140**	103.33±1.86	25.33±1.76	6.00±0.58
T141	99.00±2.08	26.00±1.00	5.33±1.45	**T142**	108.67±1.45	31.00±1.53	6.33±1.67
T143	108.00±1.00	24.00±1.00	5.33±0.33	**T144**	98.33±2.73	25.67±1.20	6.33±1.20
T145	105.00±3.06	25.33±2.19	6.00±0.00	**T146**	104.00±1.73	25.67±0.88	5.33±0.88
T147	96.00±3.21	16.33±2.40	7.33±0.33	**T148**	96.67±1.76	17.33±2.73	6.00±1.15
T149	98.00±3.61	19.00±2.52	6.67±0.67	**T150**	105.33±1.33	21.67±0.67	6.67±0.33
T151	105.33±3.48	25.33±1.76	4.67±0.88	**T152**	91.67±0.67	22.00±2.00	5.00±0.58
T153	96.00±2.08	21.00±1.15	5.00±0.58	**T154**	101.33±2.91	20.33±2.96	5.67±0.88
T155	104.67±1.67	21.67±0.88	5.33±0.33	**T156**	95.00±1.53	10.67±0.88	4.67±1.45
T157	96.33±0.88	18.33±2.85	4.33±0.88	**T158**	94.00±2.08	22.67±0.33	4.67±0.67

T159	92.33±3.84	21.67±2.19	5.00±1.53	**T160**	99.33±3.48	19.00±1.73	7.33±0.33
T161	91.67±1.20	16.33±0.88	4.67±0.33	**T162**	95.00±2.52	18.00±1.00	6.33±0.88
T163	92.00±9.54	17.33±1.45	4.67±0.67	**T164**	104.67±1.20	14.00±0.58	6.33±1.67
T165	92.67±2.85	15.00±0.58	4.00±0.58	**T166**	90.33±0.88	12.00±0.58	4.67±0.33
T167	86.00±2.65	12.67±0.33	4.33±1.20	**T168**	84.67±4.06	13.00±0.58	7.33±1.45
T169	86.33±1.20	12.00±0.58	7.00±1.53	**T170**	82.67±1.67	14.00±0.58	7.33±0.88
T171	71.67±1.45	12.33±1.20	7.33±1.20	**T172**	76.67±1.45	12.33±0.33	7.00±0.58
T173	76.00±1.15	12.33±0.88	6.00±0.58	**T174**	73.67±1.20	11.00±1.00	5.00±1.53
T175	68.67±1.76	14.00±0.58	6.33±1.20	**T176**	71.33±0.88	15.33±0.67	4.67±1.20
T177	69.67±1.45	14.33±0.67	4.33±0.33	**T178**	69.00±0.58	14.00±0.58	6.00±1.00
T179	65.67±1.20	15.00±0.58	4.67±0.67	**T180**	59.67±0.88	12.00±0.58	5.00±0.58
T181	59.67±1.33	12.67±0.33	4.00±0.58	**T182**	65.67±2.33	13.00±0.58	6.67±0.88
T183	56.00±2.08	12.00±0.58	7.00±1.15	**T184**	53.67±2.19	14.00±0.58	5.00±0.58
T185	48.33±0.67	12.33±1.20	5.33±0.88	**T186**	51.00±1.73	12.33±0.33	5.67±1.45
T187	48.00±0.58	12.33±0.88	6.33±0.67	**T188**	48.67±1.20	11.33±1.33	5.00±0.58
T189	48.67±0.88	18.33±0.88	5.67±1.20	**T190**	43.00±1.53	17.67±0.33	6.00±1.15
T191	35.67±1.20	22.33±0.88	5.33±1.45	**T192**	41.33±1.86	16.67±0.33	3.67±0.33
T193	39.00±1.53	14.67±0.67	5.33±0.33	**T194**	41.67±0.88	16.67±0.88	5.00±1.53
T195	32.00±1.53	15.67±0.33	5.00±0.58	**T196**	35.00±1.53	14.67±0.67	6.00±1.53
T197	37.67±1.45	14.67±0.33	5.67±0.67	**T198**	28.00±2.31	15.00±0.58	6.00±1.15
T199	33.67±1.86	13.33±0.88	5.00±0.58	**T200**	23.33±1.20	12.67±0.67	3.67±0.88
T201	23.33±1.76	10.00±1.15	4.67±0.33	**T202**	19.33±0.67	10.67±0.33	4.00±0.58
T203	18.00±1.15	10.33±0.67	5.00±0.58				

ID= Identification number; T= Tobacco group; MNBNC= Binucleated cells with one micronucleus; 2MNBNC= Binucleated cells with two micronuclei; MMNBNC= Binucleated cells with more than two micronuclei; SEM= Standard error mean; N= 3.

Table 3.3: Summary of the overall frequency of micronucleus, two micronuclei and more than two micronuclei of both the study group.

Variable	Group	N	Mean	SD	SEM	95% CI	
						Lower	Upper
MNBNC	CON	42	26.93	9.92	1.53	23.84	30.02
	TOB	203	71.19	27.50	1.93	67.38	74.99
2MNBNC	CON	42	14.54	2.71	0.42	13.70	15.39
	TOB	203	17.50	6.05	0.42	16.67	18.34
MMNBNC	CON	42	5.13	0.93	0.14	4.84	5.42
	TOB	203	5.89	1.96	0.14	5.62	6.16

CON= Control group; TOB= Tobacco group; N= Total; SD= Standard deviation; SEM= Standard error mean; CI= Confidence interval; MNBNC= Binucleated cells with one micronucleus; 2MNBNC= Binucleated cells with two micronuclei; MMNBNC= Binucleated cells with more than two micronuclei.

Table 3.4: Mann-Whitney U rank test showing difference in population mean between both gender, both genders of control group and tobacco group, male sex of control and tobacco group, female sex of control and tobacco group, male and female control group, male and female tobacco group.

Test between	Variable	t-test between	Variable
All Male & Female	MNBNC	**CON and TOB**	MNBNC*
	2MNBNC		2MNBNC*
	MMNBNC		MMNBNC*
CON and TOB (Male)	MNBNC*	**CON and TOB (Female)**	MNBNC*
	2MNBNC		2MNBNC*
	MMNBNC		MMNBNC*
CON (Male & Female)	MNBNC	**TOB (Male & Female)**	MNBNC*
	2MNBNC		2MNBNC
	MMNBNC*		MMNBNC

*CON= Control group; TOB= Tobacco group; MNBNC= Binucleated cells with one micronucleus; 2MNBNC= Binucleated cells with two micronuclei; MMNBNC= Binucleated cells with more than two micronuclei; *Significant at 0.05 level of significant; No symbol= Not significant.*

Table 3.5: Pearson correlation coefficient of different groups with age.

Group	Correlation with age	Value	Group	Correlation with age	Value
CON	MNBNC	0.831*	**TOB**	MNBNC	0.959*
	2MNBNC	0.476*		2MNBNC	0.675*
	MMNBNC	0.072		MMNBNC	0.328*
CON (Male)	MNBNC	0.911*	**CON (Female)**	MNBNC	0.936*
	2MNBNC	0.429		2MNBNC	0.644*
	MMNBNC	0.146		MMNBNC	-0.380
TOB (Male)	MNBNC	0.958*	**TOB (Female)**	MNBNC	0.969*
	2MNBNC	0.648*		2MNBNC	0.723*
	MMNBNC	0.478*		MMNBNC	0.099

*CON= Control group; TOB= Tobacco group; MNBNC= Binucleated cells with one micronucleus; 2MNBNC= Binucleated cells with two micronuclei;MMNBNC= Binucleated cells with more than two micronuclei; *Significant at 0.05 level of significant; No symbol= Not significant.*

Table 3.6: Linear regression showing the effects of socio-demographic variables, dietary habits and familial history of cancer on the frequency of MNBNC. R^2: 0.968.

Variable	β	SEM	t	p
Age	2.267	0.359	6.319	0.000
Sex	-13.104	10.820	-1.211	0.246
Residence	-1.943	4.356	-0.446	0.662
Betel	0.033	5.874	0.469	0.640
Betel: current user	-1.262	5.334	-0.237	0.816
Betel with lime	2.181	7.084	0.308	0.763
Betel with tobacco	3.999	9.987	0.400	0.695
Betel frequency/day	0.165	0.313	0.527	0.607
Betel use (year)	-0.293	0.357	-0.821	0.425
Alcohol user	2.148	15.426	0.139	0.041
Alcohol: Current user	3.851	8.301	0.464	0.650
Branded alcohol only	-18.276	13.402	-1.364	0.194
Local alcohol only	-12.173	14.102	-0.863	0.403
Branded and local alcohol	-14.972	14.745	-1.015	0.327
Daily alcohol drinker	-12.130	8.639	-1.404	0.166
Not daily alcohol drinker	-6.472	6.421	-1.008	0.331
Alcohol: age at start	-0.372	0.664	-0.560	0.584
Alcohol drink duration (year)	0.464	0.298	1.560	0.134
Smoked vegetables	-3.957	4.246	-0.932	0.367
Pickled vegetables and fruits	2.934	5.715	0.513	0.616
Any smoked meat	-1.728	1.554	-1.112	0.285
Any salted meat	0.354	4.889	0.073	0.943
Any pickled meat	-0.191	5.171	-0.037	0.971
Any tinned foods	-1.943	4.704	-0.413	0.686
Fermented foods	-0.862	7.076	-0.122	0.905
Sodium bicarbonate	1.997	7.868	0.254	0.803
Monosodium glutamate	6.850	6.398	1.071	0.302
Familial cancer	-2.226	4.114	-0.541	0.597

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05.

Table 3.7: Linear regression showing the effects of socio-demographic variables, dietary habits and familial history of cancer on the frequency of 2MNBNC. R^2: 0.889.

Variable	β	SEM	t	p
Age	0.365	0.128	2.855	0.013
Sex	-1.002	3.860	-0.260	0.799
Residence	-1.728	1.554	-1.112	0.285
Betel	0.086	1.242	1.289	0.199
Betel: current user	0.942	1.903	0.495	0.628
Betel with lime	-0.122	2.527	-0.048	0.962
Betel with tobacco	5.704	3.563	1.601	0.132
Betel frequency/day	-0.203	0.112	-1.821	0.090
Betel use (year)	0.096	0.127	0.751	0.465
Alcohol user	16.436	5.503	2.987	0.010
Alcohol: Current user	4.889	2.961	1.651	0.121
Branded alcohol only	10.796	4.781	2.258	0.040
Local alcohol only	14.783	5.030	2.939	0.011
Branded and local alcohol	13.027	5.260	2.477	0.027
Daily alcohol drinker	1.513	1.589	-0.952	0.346
Not daily alcohol drinker	-3.129	2.290	1.366	0.193
Alcohol: age at start	-0.689	0.237	-2.906	0.011
Alcohol drink duration (year)	0.008	0.154	0.053	0.959
Any smoked meat	2.213	1.515	1.461	0.166
Pickled vegetables and fruits	0.690	2.039	0.339	0.740
Any salted meat	-1.102	1.744	-0.632	0.538
Any pickled meat	-0.455	1.844	-0.247	0.809
Any tinned foods	-0.857	1.678	-0.511	0.618
Fermented foods	2.709	2.524	1.073	0.301
Sodium bicarbonate	0.950	2.807	0.338	0.740
Monosodium glutamate	-3.640	2.282	-1.595	0.133
Familial cancer	-0.324	1.468	0.221	0.828

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05.

Table 3.8: Linear regression showing the effects of socio-demographic variables, dietary habits and familial history of cancer on the frequency of MMNBNC. R^2: 0.713.

Variable	β	SEM	t	p
Age	0.229	0.097	2.360	0.033
Sex	-5.051	2.924	-1.727	0.106
Residence	-0.672	1.177	-0.571	0.577
Betel	0.558	1.442	-0.387	0.705
Betel: current user	-3.260	1.915	-1.703	0.111
Betel with lime	1.181	2.699	0.438	0.668
Betel with tobacco	0.065	0.085	0.775	0.451
Betel frequency/day	-0.558	1.442	-0.387	0.705
Betel use (year)	0.013	0.096	0.131	0.898
Alcohol user	5.966	4.169	1.431	0.174
Alcohol: Current user	1.883	2.244	0.839	0.415
Branded alcohol only	3.262	3.622	0.901	0.383
Local alcohol only	1.006	3.811	0.264	0.796
Branded and local alcohol	-0.817	3.985	-0.205	0.840
Daily alcohol drinker	1.402	0.708	-1.981	0.053
Not daily alcohol drinker	-0.202	1.735	0.116	0.909
Alcohol: age at start	0.146	0.180	0.812	0.430
Alcohol drink duration (year)	-0.207	0.117	-1.772	0.098
Any smoked meat	0.738	1.148	0.643	0.530
Pickled vegetables and fruits	-3.720	1.544	-2.409	0.030
Any salted meat	3.052	1.321	2.310	0.037
Any pickled meat	2.605	1.397	1.864	0.083
Any tinned foods	-0.921	1.271	-0.724	0.481
Fermented foods	-0.647	1.912	-0.338	0.740
Sodium bicarbonate	1.814	2.126	0.853	0.408
Monosodium glutamate	-2.294	1.729	-1.327	0.206
Familial cancer	0.096	1.112	0.087	0.932

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05.

Table 3.9: Linear regression showing the effects of different tobacco products on the frequency of MNBNC among the tobacco group. R^2: 0.786.

Variable	β	SEM	t	p
Sahdah	-3.063	5.924	-0.517	0.612
Sahdah: current user	6.274	8.670	0.724	0.480
Sahdah use (Year)	-0.181	0.293	-0.617	0.546
Tuibur	0.337	4.221	4.675	0.000
Tuibur: current user	7.242	5.819	1.245	0.231
Tuibur use (Year)	-0.015	0.440	-0.035	0.973
Smoker	6.711	4.016	-1.671	0.026
Current smoker	-8.436	7.422	-1.137	0.272
Zozial only	-8.388	10.093	-0.831	0.418
Cigarette only	-5.962	6.687	-0.892	0.374
Zozial and cigarette	-7.021	7.229	-0.971	0.346
Smoke/day	-0.112	0.463	-0.242	0.812
Duration of smoking (Year)	1.231	0.318	3.869	0.001

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05.

Table 3.10: Linear regression showing the effects of different tobacco products on the frequency of 2MNBNC among the tobacco group. R^2: 0.690.

Variable	β	SEM	t	p
Sahdah	3.800	2.676	1.420	0.175
Sahdah: current user	1.787	3.917	0.456	0.654
Sahdah use (Year)	-0.266	0.132	-2.012	0.061
Tuibur	0.289	0.892	4.205	0.000
Tuibur: current user	-2.463	2.629	-0.937	0.363
Tuibur use (Year)	0.326	0.199	1.639	0.121
Smoker	-1.314	0.886	-1.484	0.139
Current smoker	-3.781	3.353	-1.128	0.276
Zozial only	3.017	4.559	0.662	0.518
Cigarette only	-0.875	1.332	-0.657	0.512
Zozial and cigarette	1.015	3.266	0.311	0.760
Smoke/day	0.214	0.209	1.023	0.322
Duration of smoking (Year)	0.321	0.144	2.233	0.040

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05.

Table 3.11: Linear regression showing the effects of different tobacco products on the frequency of MMNBNC among the tobacco group. R^2: 0.407.

Variable	β	SEM	t	p
Sahdah	1.087	0.884	-1.229	0.237
Sahdah: current user	-0.792	1.294	-0.612	0.549
Sahdah use (Year)	0.059	0.044	1.344	0.198
Tuibur	0.085	0.247	1.257	0.210
Tuibur: current user	1.478	0.869	1.701	0.108
Tuibur use (Year)	0.035	0.066	0.532	0.602
Smoker	0.042	0.288	0.147	0.883
Current smoker	0.255	1.108	0.230	0.821
Zozial only	1.032	1.507	0.685	0.503
Cigarette only	-0.337	0.457	-0.736	0.463
Zozial and cigarette	-0.474	1.079	-0.439	0.666
Smoke/day	0.007	0.069	0.103	0.919
Duration of smoking (Year)	-0.018	0.047	-0.381	0.708

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05.

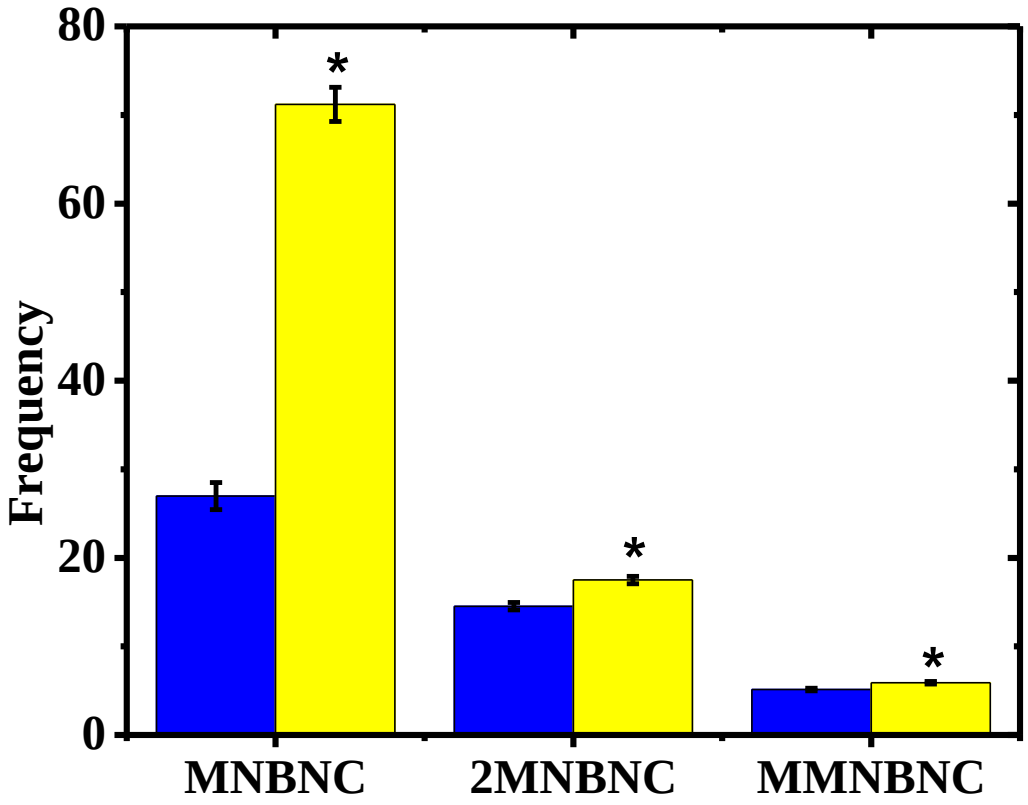


Figure 3.1: Comparison of frequency of micronucleus, two micronuclei and more than two micronuclei between control and tobacco groups.

Blue bar= Control group; Yellow bar= Tobacco group; MNBNC= Binucleated cells with one micronucleus; 2MNBNC= Binucleated cells with two micronuclei; MMNBNC= Binucleated cells with more than two micronuclei.

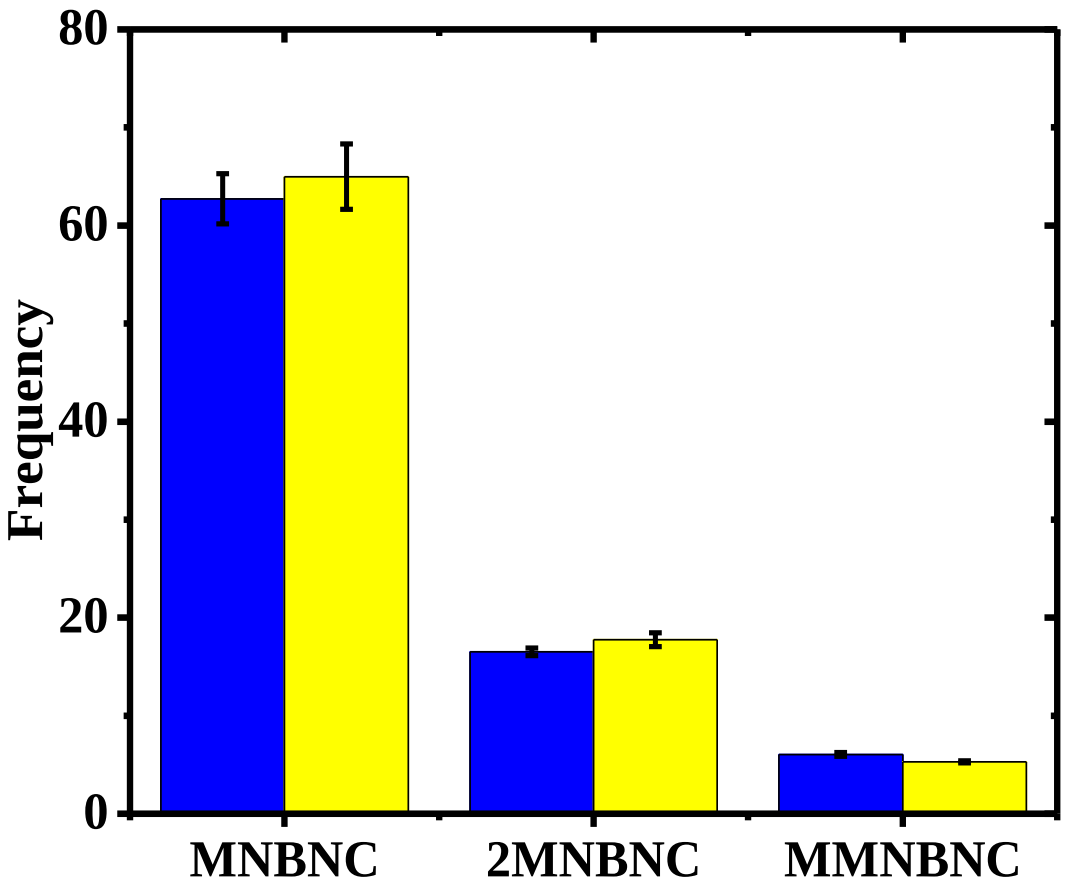


Figure 3.2: Gender wise comparison of frequency of micronucleus, two micronuclei and multiple micronuclei.

Blue bar= Male; Yellow bar= Female; MNBNC= Binucleated cells with one micronucleus; 2MNBNC= Binucleated cells with two micronuclei;
MMNBNC= Binucleated cells with more than two micronuclei.

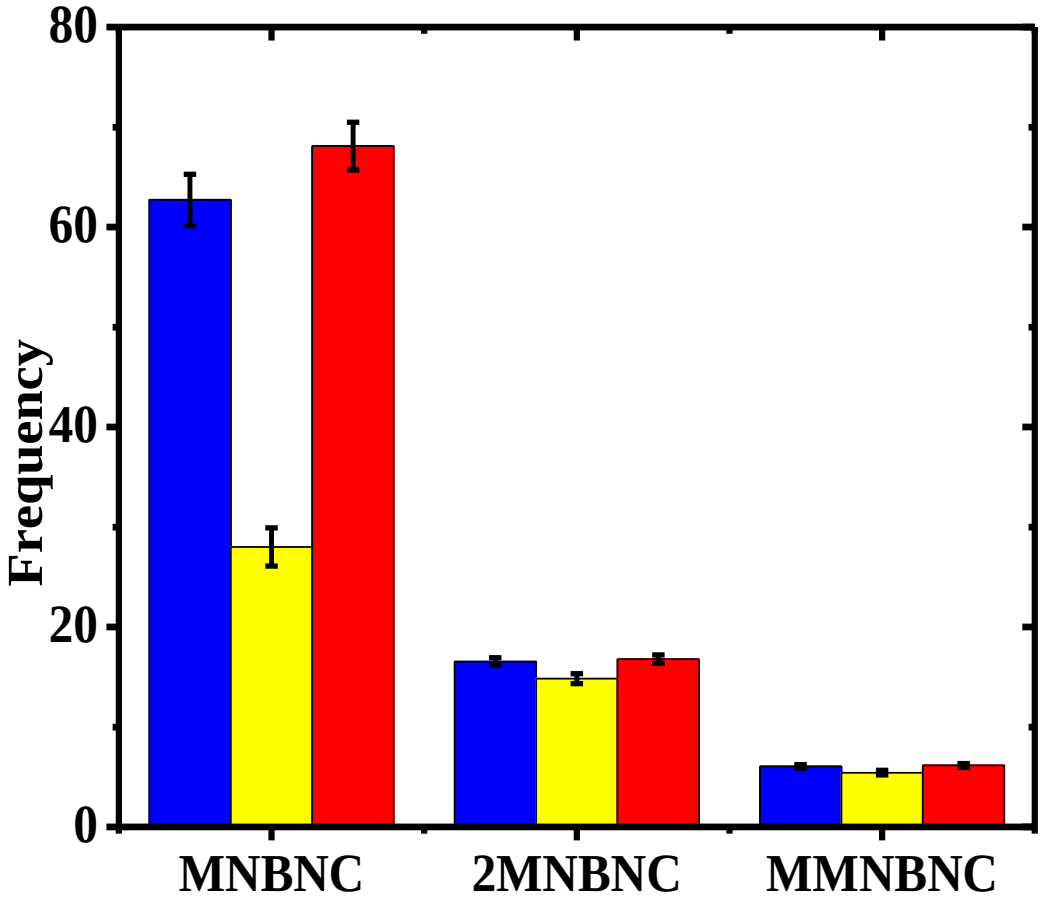


Figure 3.3: Comparison of frequency of micronucleus, two micronuclei and more than two micronuclei for male sex.

Blue bar= All male sex of both groups; Yellow bar= Male sex of the control group; Red bar= Male sex of the tobacco group; MNBNC= Binucleated cells with one micronucleus; 2MNBNC= Binucleated cells with two micronuclei;
MMNBNC= Binucleated cells with more than two micronuclei.

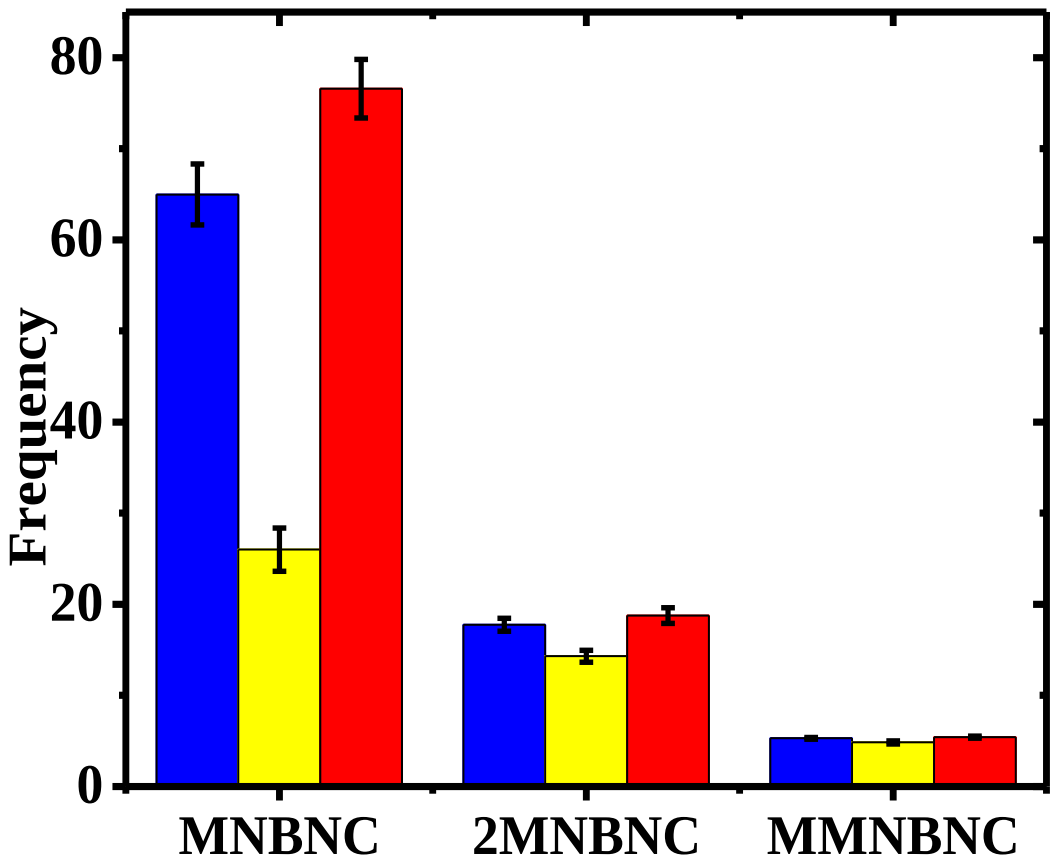


Figure 3.4: Comparison of frequency of micronucleus, two micronuclei and more than two micronuclei for female sex.

Blue bar= All female sex of both groups; Yellow bar= Female sex of the control group; Red bar= Female sex of the tobacco group; MNBNC= Binucleated cells with one micronucleus; 2MNBNC= Binucleated cells with two micronuclei; MMNBNC= Binucleated cells with more than two micronuclei.

PHOTOPLATES

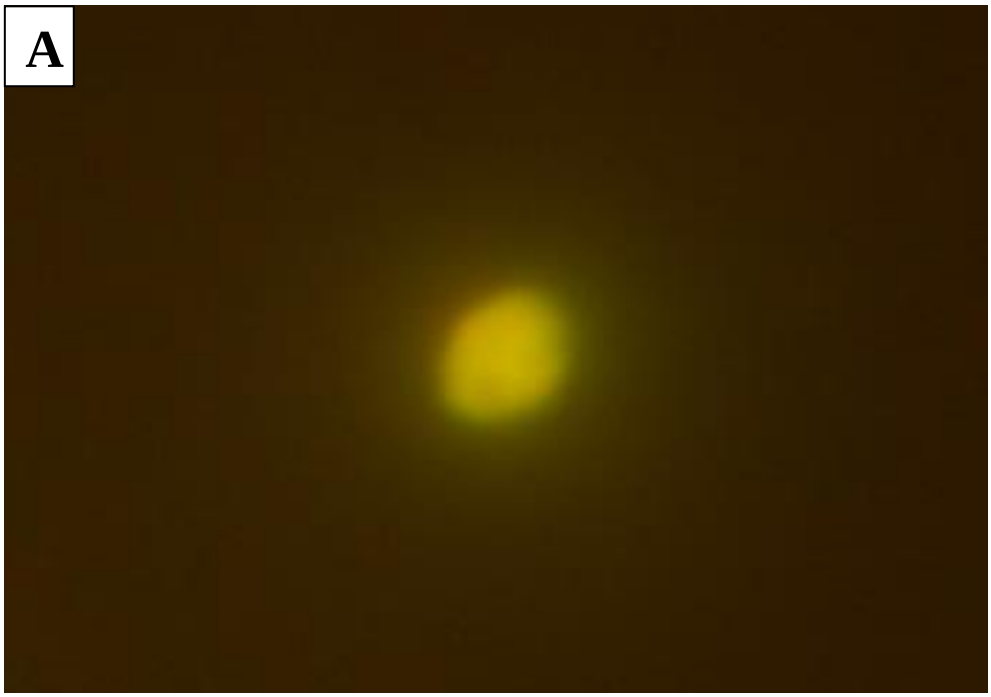
Plate A: Mononucleated lymphocyte.

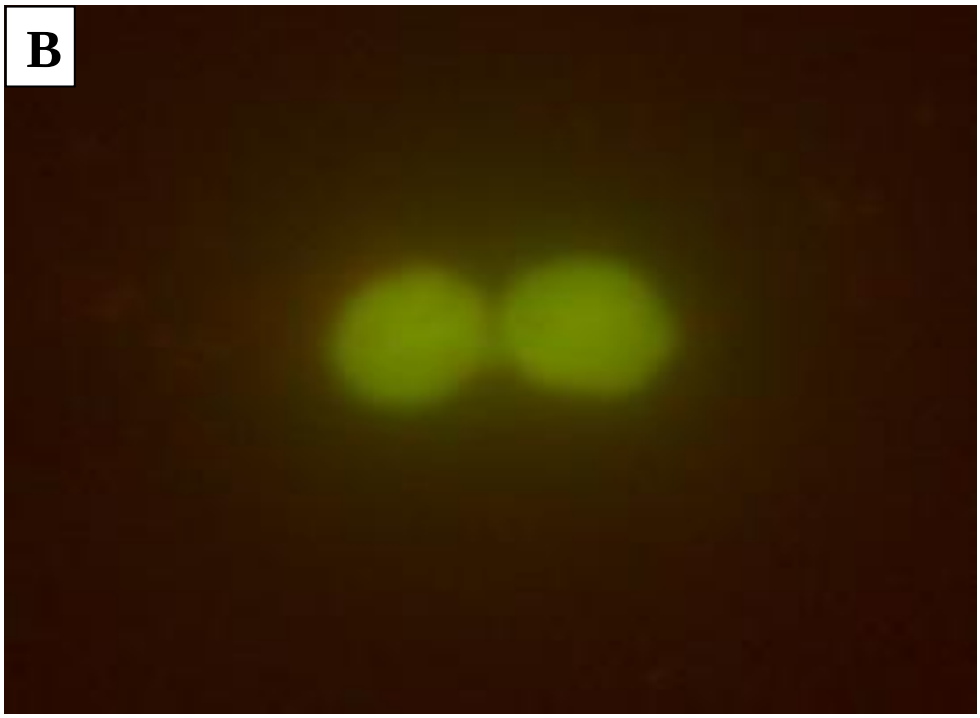
Plate B: Binucleated lymphocyte.

Plate C: Binucleated lymphocyte with one micronucleus.

Plate D: Binucleated lymphocyte with two micronuclei.

Plate E: Binucleated lymphocyte with multiple micronuclei.

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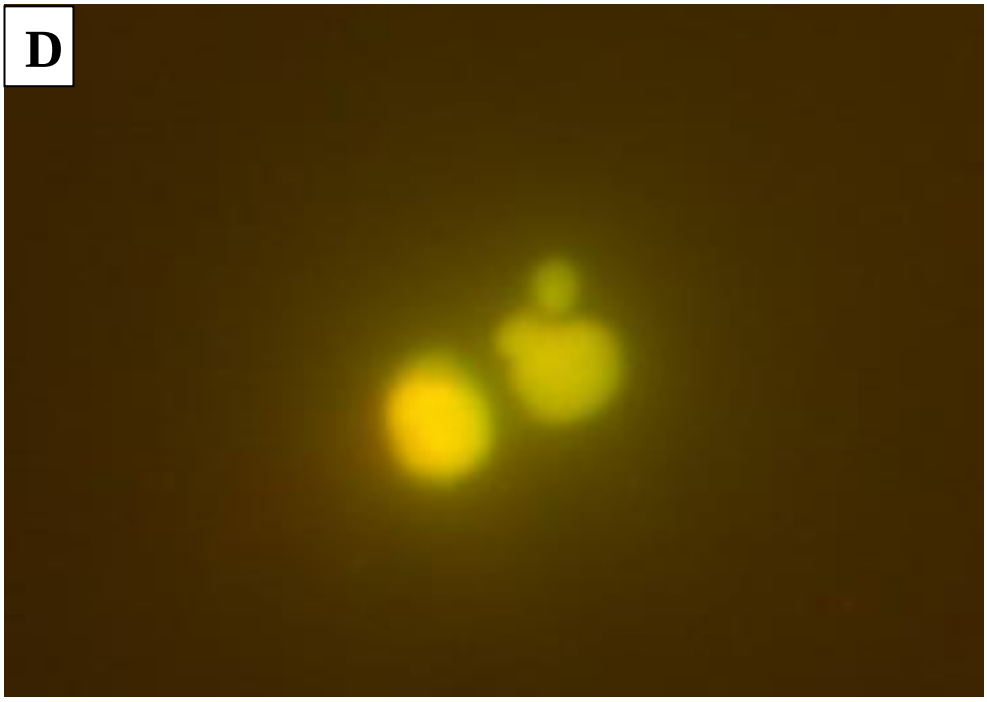
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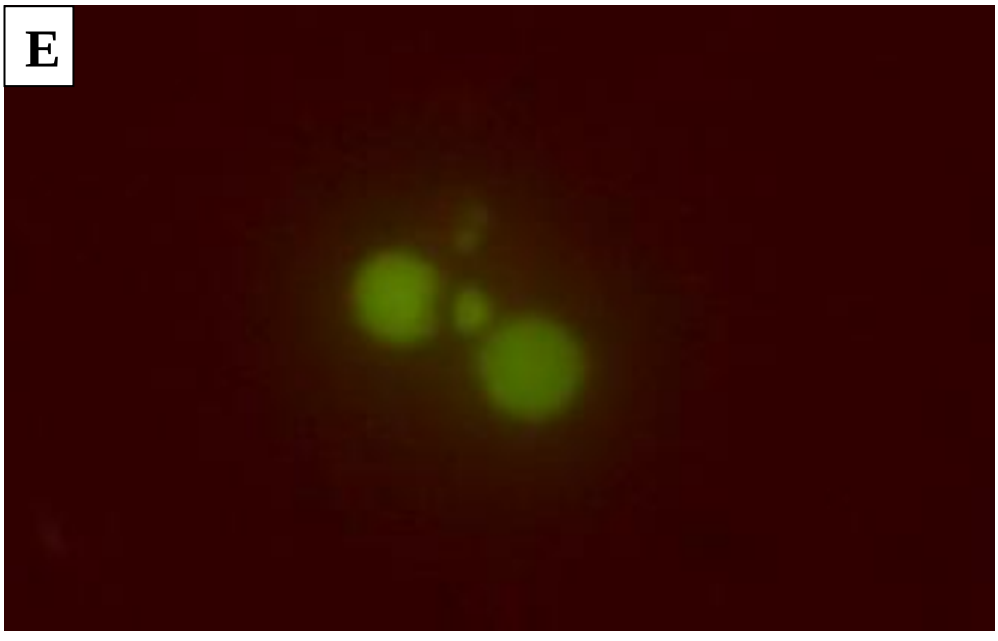
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CHAPTER 4

AUGMENTATION OF OXIDATIVE STRESS BY TOBACCO CONSUMPTION IN MIZOS

Abstract

The human body is subjected to many types of biological, chemical and physical factors that threaten the integrity of the genome. These factors often result in the production of highly reactive species called free radicals. The free radicals cause oxidative stress in the human body, which is often detrimental to the cells. The human body synthesizes antioxidants to defend against free radicals induced oxidative stress. The antioxidant molecules remove the unwanted free radicals from the body. Therefore, the increase or decrease in the amount and activities of enzymatic and non-enzymatic antioxidants is a reflection of increased or reduced oxidative stress. In the present study, the level glutathione, glutathione-s-transferase, catalase, superoxide dismutase and lipid peroxidation were estimated in the serum of Mizo tobacco users. Tobacco consumption has resulted in depletion in the glutathione concentration and the activities of catalase and superoxide dismutase accompanied by rise in the activity of glutathione-s-transferase and lipid peroxidation significantly than the control group. The use of tobacco in any form was also found to greatly enhance the level of oxidative stress as indicated by the increased in lipid peroxidation and reduction in the glutathione concentration and the activities of catalase and superoxide dismutase.

INTRODUCTION

The human body is a network of interacting molecules that works perfectly in homeostatic equilibrium to keep the body healthy. Changes in this equilibrium often result in devastating consequences. Human body is constantly exposed to many internal and external agents that threatens the normal structure and function of the human genome. The external threats can be biological including viruses and bacteria; chemical like mutagens or carcinogens or physical like ionizing radiations, ultra violet radiation, and electromagnetic radiations emanating from cosmos (Jacobson-Kram and Contrera, 2007; Lau *et al.,* 2014; Palaszewska-Tkacz *et al.,* 2015). These agents cause unwanted chemical reactions inside the body leading to the formation of reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). These reactive molecules are otherwise known as free radicals. Free radicals can come from external or internal source. One examples of external sources that instigate free radical formation is tobacco use (Leonard *et al.,* 1995; Singh *et al.,* 2015). Free radicals owing to their high chemical reactivity cause oxidation of various important biomolecules and cellular components leading to detrimental consequences (Gupta *et al.,* 2014; Lushchak, 2014). The free radicals also often steal electrons from lipids which are essential part of the lipid bilayer of plasma membrane and subsequently cause damage to the plasma membrane (Ayala *et al.,* 2014; Niki, 2014). The interaction of different free radicals with cellular genome leads to the formation of base damages, DNA adducts and strand breaks, which cause mutations or even death of the cells. (Suzuki *et al.,* 2002; Chung *et al.,* 2014).

A group of molecules called antioxidants and antioxidant enzymes maintain the oxidation equilibrium and protect the body from the harmful effects of free radicals by scavenging them immediately after their production. Therefore a negative change in the level or activities of antioxidants and antioxidant enzymes can be a symptom of cellular or physiological oxidative stress (Jain *et al.,* 2015; Siti *et al.,* 2015; Jagetia and Shetty, 2016). The change in the level of enzymatic and non-enzymatic antioxidants can be a result of a direct effect of free radicals on their production and activities or an indirect effect as a response to the damage caused by free radicals to intracellular organelles. The lifestyle and dietary habits which an individual choose to adopt often present greater or lesser threat to one's health. The consumption of tobacco, alcohol or other toxic substances are known to increase the production of oxidants in the body, hence, they have adverse effect on the health of the users of such products (Lowe *et al.,* 2013; Buldak *et al.,* 2014; Mladenov *et al.,* 2015).

Tobacco is known to be a rich source of oxidants. Therefore the consumption of tobacco is known to deplete the quantity of antioxidant molecules (Gallagher *et al.,* 2009). Chemicals present in tobacco also possess the ability to induce the formation of free radicals inside the body as a byproduct of reactions from the metabolism of the tobacco chemicals. The combination of these biochemical events lead to change in the oxidative state of tobacco users causing decline in antioxidant molecules and activating antioxidant enzymes such as catalase, glutathione-s-transferase, glutathione peroxidase, superoxide dismutase, etc. (Mesaros *et al.,* 2012; Sirisha and Manohar, 2013; Pace *et al.,* 2014; Sajid and Bano, 2015). Therefore, the current study aims to determine the

level of glutathione, glutathione-s-transferase, catalase, superoxide dismutase and lipid peroxidation in the serum of Mizo tobacco users of Mizoram.

MATERIALS AND METHODS

Chemicals

Nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), 1-chloro-2,4-dinitronbezene (CDNB), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH) and thiobarbituric acid (TBA) were obtained from Sigma Chemical Co. (Bangalore, India). Dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), trichloroacetic acid (TCA), and hydrogen peroxide (H_2O_2) were procured from SD Fine Chemicals, Mumbai, India, whereas disodium hydrogen phosphate (Na_2HPO_4), hydrochloric acid (HCl), and n-butanol were purchased from Merck India Limited, Mumbai, India. Nicotinamide adenine dinucleotide (NADH) and acetic acid were purchased from HiMedia, Mumbai, India.

Experimental

The volunteers were divided into two groups as described in Chapter 2.

Control group: This group (CON) comprised of volunteers in the age group of 18-67 years of either sex who had no known consumption of any form of tobacco and its related products.

Tobacco group: This group (TOB) consisted of volunteers in the age group of 18- 67 years of either sex, who consumed any form of tobacco on regular basis. The type of tobacco used may either be smoked or smokeless tobacco or both.

Collection of blood and separation of serum

Blood was aseptically collected by venipuncture and the whole blood was left to stand for half an hour and then centrifuged at 4400 rpm for two hours to separate the serum from the whole blood. The serum from each individual was collected separately in sterile cryovials and all biochemical assays were performed on sera stored overnight in deep freezer maintained at -80°C. The stored sera were thawed one hour before the biochemical analyses.

Estimation of glutathione concentration

The amount of glutathione present in the sera of the volunteers were estimated using standard protocol as described earlier (Moron *et al.*, 1979). Glutathione concentration was measured by its reaction with DTNB (Ellman's reaction) to give a compound that absorbs light at 412 nm. Briefly, 970 µl of 0.2 M Na_2HPO_4 was mixed with 20 µl 10 mM DTNB and 30 µl of serum. The blank consisted of distilled water instead of the serum. The mixture was allowed to stand for 2 minutes and the absorbance was read against the blank at 412 nm in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India).

Study of glutathione-s–transferase activity

Glutathione-s-transferase activity was studied following the method of Beutler (1984). In brief, 850 µl of 0.1 M phosphate buffer pH 6.5 and 50 µl of 20 mM CDNB were mixed and incubated at 37°C for 10 min followed by the addition of 50 µl of 20 mM GSH and 50 µl of serum. For blank, distilled water was used instead of the serum.

The absorbance was read against the blank at 340 nm at 1 min intervals for 5 minutes in UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India).

Study of catalase activity

The activity of catalase was determined according to protocol of Aebi (1984). Briefly, in a 1 ml cuvette, 10 µl of sample was diluted with 90 µl of 50 mM phosphate buffer (pH 7.0), thereafter 900 µl of buffer and 30 mM of H_2O_2 were added. The decrease in absorbance was monitored at 240 nm for 30 seconds in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India).

Study of superoxide dismutase activity

The activity of SOD was estimated using standard protocol (Fried, 1975) and the principle depends on the fact that SOD catalyzes the dismutation of two superoxide anions (O_2^-) into hydrogen peroxide and molecular oxygen. Briefly, 1.2 ml of serum was diluted with distilled water in the ratio of 1:9. Thereafter, 100 µl of 186 µM phenazene methosulfate, 300 µl of 3.0 mM nitroblue tetrazolium, 200 µl of 780 µM NADH were added and the mixture was incubated for 90 seconds at 30°C. The reaction was terminated by adding 1 ml of acetic acid and 4 ml n-butanol. The tubes were left to stand at room temperature for 10 min and centrifuged at 1000 rpm for 10 min. The upper butanol layer was collected and the absorbance was recorded at 560 nm using a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India). The percent inhibition was calculated by measuring the absorbance of blank without SOD enzyme and the test with SOD enzyme samples using the formula (Blank-Sample)/Blank X 100.

Estimation of lipid peroxidation

The estimation of lipid peroxidation (LOO) was performed by the method of Buege and Aust (1978). Briefly, 1 ml of serum was mixed with 1 ml of TCA and centrifuged at 1500 rpm for 10 minutes. One ml of the supernatant was collected and mixed with 1 ml TBA-HCl reagent. The reaction mixture was heated at 95°C in a dry water bath for 25 minutes and cooled immediately to room temperature. It was centrifuged at 1000 rpm for 10 min and the supernatant was collected and its absorbance was read at 540 nm against the blank in a UV-VIS Biospectrophotometer (Eppendorf India Limited, Kolkata, India). The blank contained all the reagents minus the cell homogenate substituted with distilled water. The MDA concentration of the sample was calculated using standard graph.

Statistical analyses

The statistical significance for different biochemical assays between the CON and TOB groups were carried out by Student's t-test. Pearson's correlation coefficient was used to determine the correlation of the different biochemical parameters with age. Linear regression was performed to see the effect of different variables on the different biochemical assays performed.

RESULTS

The results are expressed as mean±standard error of the mean (SEM) in Table 4.1- 4.23 and Figure 4.1-4.3.

Glutathione

The concentration of GSH was found to be significantly lower in the TOB group than the CON group (Tables 4.1, 4.2 and 4.11). The mean GSH concentration was found to be 3.456 and 2.394 for the CON and TOB groups respectively. A negative correlation was observed between GSH concentration and age (Table 4.13). The consumption of betel, alcohol, pickled fruits and vegetables, any tinned foods and monosodium glutamate were found to have positive correlation with the level of GSH. However, a statistical significant correlation was observed only for the consumption of betel with tobacco (Table 4.14). The use of sahdah, tuibur and smoking were also found to have positive correlation with GSH level in the serum. However, a statistically significant correlation was observed only for the duration of tuibur use (Table 4.19).

Glutathione-s-transferase

The activity of GST was found to be significantly higher in the TOB group (0.235±0.161) than the CON group (0.073±0.035) (Tables 4.3, 4.4, 4.11 and figure 4.1). A significantly higher GST activities were also seen in males and female TOB group when comparisons were made between the concurrent CON group (Table 4.12 and figures 4.2 and 4.3). A positive correlation was observed for age and GST activity (Table 4.13). Age, the consumption of betel, alcohol, any smoked meat and sodium bicarbonate were found to positively influence the activity of GST, however, age and the use of betel were found to only have significant correlation (Table 4.15). The use of smokeless and smoking tobacco also have correlation with GST activity. However, the

use of tuibur, duration of sahdah and smoking tobacco use were observed to have significant correlation with GST activities (Table 4.20).

Catalase

Catalase activity was found to be significantly higher in the CON group than the TOB group (Figure 4.1). Females were found to have higher catalase activity than males (Tables 4.5, 4.6, 4.11, 4.12 and figures 4.2, 4.3 and 4.4). A negatively weak correlation of catalase activity and age was observed for the CON group while the TOB group showed a moderate negative correlation. Female tobacco users showed strong negative correlation for catalase activity with age (Table 4.13). The use of betel and alcohol and family history of cancer were found to be correlated with catalase activity, however, none of these variables showed significant correlation (Table 4.16). The consumption of sahdah, tuibur and cigarette also showed positive but non-significant correlation with catalase activity (Table 4.21).

Superoxide dismutase

The TOB group exhibited significantly lower SOD activity than the CON group (Figure 4.1). The female volunteers were also found to have higher SOD activity than the male volunteers (Tables 4.7, 4.8, 4.11 and 4.12). SOD activity was found to have weak negative correlation with age among the CON group while the TOB group showed moderately negative correlation. The female TOB group showed strong negative correlation for SOD activity and age (Table 4.13). The consumption of betel and alcohol and family history of any type of cancer were found to be correlated with SOD activity. Among these variables, the current use of alcohol was found to have significant effect

(Table 4.17). The use of sahdah, tuibur, zozial, cigarette and amount of smoke per day showed positive correlation with SOD activity. However, only the current use of smoking tobacco showed significant relationship (Table 4.22).

Lipid peroxidation

The CON group showed a moderate positive correlation between LOO and age while the TOB group displayed strong positive correlation. The CON male as well as male and female of the TOB group showed strong positive correlation for LOO and age while female CON showed weak positive correlation (Table 4.13). The variables affecting LOO were age, consumption of betel, alcohol, monosodium glutamate and family history of cancer. However, none of these variables had significant effect (Table 4.18). The use of sahdah, tuibur, zozial and cigarette also positively affected LOO. The duration of sahdah use, tuibur and smoking showed positive relationship with LOO (Table 4.23).

DISCUSSION

The role of antioxidants and antioxidant enzyme is important in maintaining the health of the body. The adoption of unhealthy lifestyle including the consumption of tobacco and alcohol is known to adversely affect the health of its users by altering the level of the enzymatic and non-enzymatic antioxidants. Cigarette smoke has been found to damage protein-disulfide isomerase, induce lymphocyte apoptosis and suppress lymphocyte formation and the generation of dendritic cells through the ERK-dependent pathways (El-Zayadi, 2006; Kroening *et al.,* 2008; Kenche *et al.,* 2016). The consumers of pan masala tobacco users were found to be in a state of oxidative stress which is

reflected in the depletion of non-enzymatic antioxidants among the users (Shrestha *et al.,* 2012). Smokers also exhibited higher level of serum oxidative stress than controls (El-Zayadi, 2006; Tanriverdi *et al.,* 2006; Jain *et al.,* 2009). Individuals exposed to environmental tobacco smoke have been reported to exhibit an increase oxidative stress than others who has no tobacco smoke exposure (Howard *et al.,* 1998).

The level of GSH was found to be lower in the bronchoalveolar lavage and epithelial lining fluid among smokers than nonsmokers (Alatas *et al.,* 1999). Similar result was reported by other studies (Bloomer, 2007; Pacini *et al.,* 2012; Gould *et al.,* 2015), which is also consistent with our results where the tobacco consumers have shown depleted GSH concentration. Interestingly, the discontinuation of tobacco use has been reported to increase the level of GSH (Lane *et al.,* 1996; Mons *et al.,* 2016).

Besides antioxidants, the negative alteration in the activities of antioxidant enzymes is a reflection of many types of diseases including cardiovascular diseases, hypertension, diabetes, congenital heart disease, infertility, and cancer (Iguchi *et al.,* 2009; Koh *et al.,* 2011; Didziapetriene *et al.,* 2014; Kumar *et al.,* 2014; Li *et al.,* 2015; Gomez-Marcos *et al.,* 2016). GST is an important detoxifying enzyme that conjugates GSH with other xenobiotic compounds. This conjugate formed can be removed from the body through the excretion of urine or bile (Hayes *et al.,* 2005; Josephy, 2010). High level of GST had been implicated to reflect cellular injury (Beckett *et al.,* 1985; Hughes *et al.,* 1997; Loguercio and Federico, 2003). In the present experiment, we observed more than three folds increase in GST activities in the tobacco group when compared to the control group, which could be a reflection of cellular damage as a result of tobacco

consumption. SOD is an enzyme that catalyzes the dismutation of superoxide radicals into hydrogen peroxide and molecular oxygen (Hayyan *et al.,* 2016). The hydrogen peroxide formed from the activity of SOD is decomposed to water and oxygen by the enzyme catalase (Chelikani *et al.,* 2004). Smokers were found to have lower activity of SOD and lipid peroxides than non-smokers (Abdolsamadi *et al.,* 2011; Biswas *et al.,* 2015) which is consistent with our current findings where the controls group exhibited higher activities of SOD and CAT. Among the two groups studied, males were found to have lower SOD and CAT activities in the serum. Lung cancer patients who smoked were found to have low activity of SOD (Margaret *et al.*, 2011). Schizophrenics are reported to have higher activity of SOD and CAT and higher level of LOO (Rukmini *et al.*, 2004).

Besides tobacco use, age has been known to significantly affect the antioxidant system of the body (Celec *et al.,* 2005). The amount of oxidative events is estimated to increase throughout adult life in the human body (Jones *et al.,* 2002). A decrease in SOD activity with increase in age has been reported earlier (Andersen *et al.,* 1997; Ozbay & Dulger, 2002). These findings are similar to our finding where we reported a negative correlation between age and the activities of SOD and CAT although this correlation is non-significant among the control group, however, it is significant in the tobacco consumers. A significantly strong positive correlation was observed for GST activity and age in the tobacco group. Polymorphism of important antioxidant genes such as GSTT1, GSTM1, SULT1A1, MnSODAA, and NAT1 were found to be associated with many forms of diseases such as rheumatoid arthritis, congenital heart

diseases, cancer of the mouth, pharynx, larynx, prostate, esophagus, breast, lung, colorectum, liver, etc. (Mattey *et al.,* 2002; Kabesch *et al.,* 2004; Tamimi *et al.,* 2004; Peters *et al.,* 2006; Breton *et al.,* 2009; Iguchi *et al.,* 2009; Koh *et al.,* 2011; Kumar *et al.,* 2011; Sun *et al.,* 2013; Boccia *et al.,* 2015; Li *et al.,* 2015).

Indication of oxidative stress by non-enzymatic means also include the change in level of lipid peroxidation. Free radicals steal electron from lipid forming the lipid bilayer of the plasma membrane. The loss of electron in these lipids results in the loss of selective permeability of the plasma membrane making the cell vulnerable to any form of damaging agents which are otherwise normally blocked by the cell membrane under normal circumstances (Barrera, 2012; Gaschler and Stockwell, 2017). Among diesel exposed toll collectors and professional smelters, higher amount of LOO measured as malondialdehyde (MDA) in the serum was observed (Arbak *et al.,* 2004; Bizon & Milnerowicz, 2014). Besides this, LOO was found to be greatly affected by the use of tobacco. Tobacco users were found, in many studies, to have higher level of LOO than controls (Kalra *et al.,* 1991; Celec *et al.,* 2005; El-Zayadi, 2006; Bloomer, 2007). In particular, smokeless tobacco has been reported to cause difference in LOO between users and non-users (Biswas *et al.,* 2015). Similarly, there has been a significant rise in LOO among the tobacco users than controls. In contrast, no relationship between smoking and increase LOO has been also reported (Puri *et al,*. 2008).

CONCLUSIONS

The level of GSH and activities of CAT and SOD were lower in the tobacco users while the activities of GST and the amount of lipid peroxidation were found to be higher in the tobacco users than the control group. This indicated higher oxidative stress in the tobacco users when compared to tobacco non-users. Besides tobacco, the effect of age, alcohol use and betel consumption were prominent in increasing oxidative stress. Increase in oxidative stress in the tobacco users may be a result of tobacco induced free radicals entering the body or the formation of new free radicals as a result of chemical reactions induced by the tobacco chemicals.

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Table 4.1: Amount of glutathione in the control group.

ID	GSH µmol/mg protein Mean±SEM	ID	GSH µmol/mg protein Mean±SEM	ID	GSH µmol/mg protein Mean±SEM
C1	3.433±0.004	C20	3.461±0.002	C33	3.483±0.058
C3	2.571±0.015	C22	3.556±0.004	C34	3.567±0.051
C4	3.639±0.002	C26	3.484±0.002	C35	3.524±0.064
C9	3.507±0.002	C27	3.660±0.001	C36	3.462±0.069
C16	3.394±0.007	C30	3.495±0.003	C40	3.436±0.023
C17	3.594±0.003	C32	3.500±0.060		

ID= Identification number; C= Control group; GSH= Glutathione; SEM= Standard error of mean; N=3.

Table 4.2: Amount of glutathione in the tobacco group.

ID	GSH µmol/mg protein	ID	GSH µmol/mg protein	ID	GSH µmol/mg protein
	Mean±SEM		Mean±SEM		Mean±SEM
T2	2.277±0.005	**T77**	2.098±0.110	**T134**	2.311±0.107
T3	2.085±0.008	**T78**	2.291±0.136	**T135**	2.094±0.032
T4	2.357±0.012	**T80**	2.374±0.012	**T138**	2.043±0.034
T8	2.021±0.003	**T81**	2.124±0.117	**T142**	2.333±0.107
T10	1.996±0.004	**T82**	2.082±0.034	**T145**	2.161±0.036
T16	2.316±0.003	**T86**	2.234±0.128	**T146**	2.295±0.078
T19	1.999±0.017	**T88**	2.234±0.113	**T147**	2.379±0.012
T20	2.417±0.011	**T91**	2.444±0.049	**T148**	2.326±0.038
T24	2.360±0.002	**T92**	2.498±0.055	**T149**	2.251±0.118
T25	2.009±0.003	**T97**	2.131±0.119	**T152**	2.359±0.009
T36	2.508±0.010	**T102**	2.259±0.131	**T154**	2.251±0.118
T37	2.106±0.004	**T103**	2.274±0.126	**T159**	2.022±0.012
T39	2.459±0.002	**T106**	2.227±0.118	**T160**	2.240±0.081
T40	2.123±0.002	**T108**	2.343±0.112	**T168**	2.141±0.132
T41	2.606±0.063	**T109**	2.328±0.202	**T170**	2.244±0.126
T46	2.393±0.003	**T110**	2.495±0.048	**T174**	2.162±0.104
T50	2.372±0.003	**T112**	2.493±0.057	**T180**	2.686±0.045
T51	2.012±0.002	**T116**	2.240±0.081	**T184**	3.093±0.105
T56	2.125±0.116	**T117**	2.588±0.217	**T186**	2.815±0.087
T58	2.240±0.123	**T119**	2.661±0.173	**T189**	2.357±0.012
T59	2.262±0.128	**T120**	2.612±0.159	**T190**	2.488±0.066
T61	2.396±0.143	**T122**	2.996±0.017	**T193**	2.996±0.004
T63	2.439±0.057	**T123**	2.422±0.291	**T194**	2.604±0.062
T69	1.981±0.011	**T127**	3.357±0.012	**T196**	3.006±0.006
T71	2.256±0.090	**T128**	3.021±0.003	**T200**	3.048±0.094
T72	2.141±0.119	**T129**	3.663±0.330	**T202**	3.431±0.280
T74	2.267±0.139	**T130**	1.982±0.036	**T203**	3.285±0.133
T76	2.345±0.029	**T132**	1.999±0.017		

ID= Identification number; T= Tobacco group; GSH= Glutathione; SEM= Standard error of mean; N=3.

Table 4.3: Glutathione-s-transferase activities in the control group.

ID	GST U/mg protein Mean±SEM	ID	GST U/mg protein Mean±SEM	ID	GST U/mg protein Mean±SEM
C1	0.101±0.005	**C20**	0.084±0.005	**C33**	0.036±0.007
C3	0.108±0.011	**C22**	0.025±0.012	**C34**	0.043±0.027
C4	0.115±0.008	**C26**	0.110±0.003	**C35**	0.029±0.008
C9	0.084±0.009	**C27**	0.112±0.003	**C36**	0.022±0.012
C16	0.069±0.012	**C30**	0.117±0.006	**C40**	0.037±0.015
C17	0.073±0.016	**C32**	0.072±0.010		

ID= Identification number; C= Control group; GST= Glutathione-s-transferase; SEM= Standard error of mean; U= Enzyme unit; N=3.

Table 4.4: Glutathione-s-transferase activities in the tobacco group.

ID	GST U/mg protein	ID	GST U/mg protein	ID	GST U/mg protein
	Mean±SEM		Mean±SEM		Mean±SEM
T2	0.473±0.014	**T77**	0.125±0.010	**T134**	0.510±0.005
T3	0.506±0.014	**T78**	0.102±0.004	**T135**	0.455±0.013
T4	0.492±0.012	**T80**	0.101±0.007	**T138**	0.452±0.012
T8	0.476±0.007	**T81**	0.129±0.006	**T142**	0.408±0.013
T10	0.466±0.003	**T82**	0.091±0.002	**T145**	0.366±0.013
T16	0.467±0.003	**T86**	0.103±0.006	**T146**	0.336±0.017
T19	0.450±0.004	**T88**	0.086±0.017	**T147**	0.374±0.009
T20	0.437±0.002	**T91**	0.086±0.001	**T148**	0.321±0.012
T24	0.434±0.003	**T92**	0.081±0.008	**T149**	0.317±0.008
T25	0.425±0.002	**T97**	0.065±0.007	**T152**	0.271±0.012
T36	0.418±0.002	**T102**	0.066±0.004	**T154**	0.252±0.012
T37	0.415±0.010	**T103**	0.074±0.011	**T159**	0.278±0.014
T39	0.399±0.001	**T106**	0.068±0.014	**T160**	0.273±0.016
T40	0.379±0.006	**T108**	0.066±0.004	**T168**	0.223±0.007
T41	0.384±0.004	**T109**	0.072±0.011	**T170**	0.222±0.012
T46	0.371±0.003	**T110**	0.076±0.006	**T174**	0.218±0.003
T50	0.337±0.003	**T112**	0.082±0.016	**T180**	0.211±0.009
T51	0.317±0.003	**T116**	0.061±0.010	**T184**	0.164±0.017
T56	0.291±0.020	**T117**	0.059±0.004	**T186**	0.177±0.008
T58	0.325±0.024	**T119**	0.063±0.004	**T189**	0.142±0.012
T59	0.320±0.014	**T120**	0.066±0.006	**T190**	0.110±0.005
T61	0.265±0.017	**T122**	0.059±0.002	**T193**	0.098±0.010
T63	0.317±0.003	**T123**	0.052±0.003	**T194**	0.078±0.003
T69	0.212±0.002	**T127**	0.047±0.003	**T196**	0.076±0.006
T71	0.117±0.003	**T128**	0.040±0.001	**T200**	0.075±0.009
T72	0.111±0.006	**T129**	0.038±0.003	**T202**	0.072±0.005
T74	0.105±0.003	**T130**	0.596±0.004	**T203**	0.043±0.003
T76	0.136±0.004	**T132**	0.541±0.006		

ID= Identification number; C= Control group; GST= Glutathione-s-transferase; SEM= Standard error of mean; U= Enzyme unit; N=3.

Table 4.5: Activity of catalase for control group.

ID	CAT U/mg protein Mean±SEM	ID	CAT U/mg protein Mean±SEM	ID	CAT U/mg protein Mean±SEM
C1	2.562±0.018	**C20**	2.594±0.007	**C33**	2.669±0.126
C3	2.521±0.027	**C22**	2.911±0.007	**C34**	3.076±0.170
C4	2.715±0.008	**C26**	2.556±0.033	**C35**	3.159±0.233
C9	2.484±0.092	**C27**	2.635±0.006	**C36**	3.564±0.076
C16	2.686±0.006	**C30**	2.814±0.057	**C40**	3.361±0.018
C17	2.456±0.017	**C32**	2.556±0.032		

ID= Identification number; C= Control group; CAT= Catalase; SEM= Standard error of mean; U= Enzyme unit; N=3.

Table 4.6: Activity of catalase for tobacco group.

ID	CAT U/mg protein Mean±SEM	ID	CAT U/mg protein Mean±SEM	ID	CAT U/mg protein Mean±SEM
T3	2.213±0.065	**T78**	2.223±0.009	**T135**	2.058±0.013
T4	2.192±0.012	**T80**	2.282±0.038	**T138**	2.274±0.007
T8	2.310±0.005	**T81**	2.436±0.010	**T142**	2.146±0.066
T10	2.024±0.014	**T82**	2.419±0.027	**T145**	2.268±0.018
T16	2.313±0.003	**T86**	2.290±0.012	**T146**	2.079±0.006
T19	2.054±0.036	**T88**	2.151±0.030	**T147**	2.151±0.030
T20	2.168±0.014	**T91**	2.615±0.009	**T148**	2.324±0.010
T24	2.316±0.002	**T92**	2.229±0.015	**T149**	2.396±0.022
T25	2.610±0.003	**T97**	2.403±0.084	**T152**	2.453±0.125
T36	2.575±0.007	**T102**	2.361±0.044	**T154**	2.403±0.084
T37	2.320±0.002	**T103**	2.429±0.014	**T159**	2.300±0.012
T39	2.193±0.007	**T106**	2.324±0.033	**T160**	2.218±0.008
T40	2.229±0.004	**T108**	2.344±0.025	**T168**	2.317±0.009
T41	2.205±0.003	**T109**	2.284±0.008	**T170**	2.364±0.082
T46	2.443±0.003	**T110**	2.880±0.059	**T174**	2.446±0.005
T50	2.115±0.010	**T112**	3.177±0.092	**T180**	2.557±0.025
T51	2.281±0.003	**T116**	2.310±0.002	**T184**	2.767±0.005
T56	2.274±0.007	**T117**	2.988±0.059	**T186**	2.546±0.031
T58	2.248±0.030	**T119**	3.287±0.027	**T189**	3.210±0.099
T59	2.250±0.040	**T120**	2.777±0.089	**T190**	2.674±0.030
T61	2.122±0.069	**T122**	2.842±0.033	**T193**	2.816±0.033
T63	2.262±0.013	**T123**	2.742±0.093	**T194**	3.302±0.025
T69	2.114±0.027	**T127**	3.170±0.030	**T196**	2.958±0.013
T71	2.348±0.032	**T128**	3.222±0.010	**T200**	3.214±0.033
T72	2.596±0.014	**T129**	3.205±0.003	**T202**	3.292±0.015
T74	2.553±0.016	**T130**	2.023±0.011	**T203**	3.448±0.003
T76	2.312±0.007	**T132**	2.082±0.024		

ID= Identification number; C= Control group; CAT= Catalase; SEM= Standard error of mean; U= Enzyme unit; N=3.

Table 4.7: Superoxide dismutase activities in the control group.

ID	SOD U/mg protein Mean±SEM	ID	SOD U/mg protein Mean±SEM	ID	SOD U/mg protein Mean±SEM
C3	3.558±0.015	**C22**	3.908±0.007	**C34**	3.943±0.037
C4	3.715±0.008	**C26**	3.549±0.027	**C35**	4.025±0.100
C9	3.484±0.092	**C27**	3.625±0.006	**C36**	4.697±0.108
C16	3.669±0.012	**C30**	3.827±0.046	**C40**	5.094±0.017
C17	3.500±0.013	**C32**	3.569±0.022		

ID= Identification number; C= Control group; SOD= Superoxide dismutase; SEM= Standard error of mean; U= Enzyme unit; N=3.

Table 4.8: Superoxide dismutase activities in the tobacco group.

ID	SOD U/mg protein Mean±SEM	ID	SOD U/mg protein Mean±SEM	ID	SOD U/mg protein Mean±SEM
T3	1.426±0.055	**T78**	2.223±0.009	**T135**	2.092±0.022
T4	2.192±0.012	**T80**	2.302±0.021	**T138**	2.267±0.005
T8	2.303±0.004	**T81**	2.410±0.005	**T142**	2.136±0.061
T10	2.058±0.027	**T82**	2.429±0.017	**T145**	2.278±0.014
T16	2.330±0.009	**T86**	2.296±0.010	**T146**	2.072±0.005
T19	2.061±0.040	**T88**	2.185±0.009	**T147**	2.185±0.009
T20	2.165±0.017	**T91**	2.601±0.018	**T148**	2.321±0.012
T24	2.317±0.003	**T92**	2.226±0.017	**T149**	2.398±0.023
T25	2.613±0.005	**T97**	2.336±0.017	**T152**	2.500±0.078
T36	2.574±0.008	**T102**	2.394±0.027	**T154**	2.336±0.017
T37	2.317±0.003	**T103**	2.419±0.010	**T159**	2.266±0.023
T39	2.201±0.005	**T106**	2.327±0.032	**T160**	2.211±0.009
T40	2.226±0.006	**T108**	2.331±0.017	**T168**	2.333±0.012
T41	2.199±0.004	**T109**	2.277±0.008	**T170**	2.431±0.015
T46	2.436±0.004	**T110**	2.863±0.043	**T174**	2.433±0.005
T50	2.122±0.009	**T112**	3.210±0.061	**T180**	2.547±0.017
T51	2.277±0.005	**T116**	2.316±0.006	**T184**	2.754±0.005
T56	2.268±0.008	**T117**	2.974±0.061	**T186**	2.580±0.002
T58	2.245±0.028	**T119**	3.283±0.026	**T189**	3.277±0.033
T59	2.247±0.038	**T120**	2.877±0.037	**T190**	2.664±0.026
T61	2.179±0.043	**T122**	2.875±0.031	**T193**	2.783±0.036
T63	2.255±0.007	**T123**	2.779±0.060	**T194**	3.285±0.036
T69	2.134±0.022	**T127**	3.203±0.003	**T196**	2.942±0.011
T71	2.365±0.029	**T128**	3.239±0.007	**T200**	3.194±0.013
T72	2.589±0.014	**T129**	3.202±0.005	**T202**	3.299±0.008
T74	2.559±0.009	**T130**	2.043±0.021	**T203**	3.445±0.004
T76	2.309±0.007	**T132**	2.092±0.014		

ID= Identification number; C= Control group; SOD= Superoxide dismutase; SEM= Standard error of mean; U= Enzyme unit; N=3.

Table 4.9: Level of lipid peroxidation in the control group.

ID	LOO µmol/mg protein	ID	LOO µmol/mg protein	ID	LOO µmol/mg protein
	Mean±SEM		**Mean±SEM**		**Mean±SEM**
C3	31.96±0.072	**C22**	21.97±0.031	**C34**	26.94±0.037
C4	30.01±0.110	**C26**	30.55±0.027	**C35**	25.36±0.713
C9	29.46±0.297	**C27**	31.99±0.036	**C36**	24.73±0.079
C16	26.80±0.122	**C30**	32.89±0.072	**C40**	22.09±0.017
C17	25.10±0.054	**C32**	28.70±0.155		

ID= Identification number; C= Control group; LOO= Lipid peroxidation; SEM= Standard error of mean; N=3.

Table 4.10: Level of lipid peroxidation in the tobacco group.

ID	LOO µmol/mg protein	ID	LOO µmol/mg protein	ID	LOO µmol/mg protein
	Mean±SEM		Mean±SEM		Mean±SEM
T3	51.776±0.135	**T78**	34.323±0.091	**T135**	52.092±0.022
T4	51.502±0.293	**T80**	34.068±0.102	**T138**	52.167±0.102
T8	50.970±0.064	**T81**	33.376±0.029	**T142**	49.136±0.061
T10	49.991±0.043	**T82**	32.429±0.017	**T145**	48.278±0.014
T16	49.063±0.073	**T86**	32.296±0.010	**T146**	48.106±0.031
T19	48.994±0.050	**T88**	32.045±0.136	**T147**	47.185±0.009
T20	48.098±0.083	**T91**	32.601±0.018	**T148**	47.021±0.144
T24	46.747±0.128	**T92**	30.226±0.017	**T149**	46.398±0.023
T25	47.780±0.170	**T97**	30.170±0.175	**T152**	45.500±0.078
T36	46.547±0.019	**T102**	28.161±0.129	**T154**	45.203±0.116
T37	45.917±0.059	**T103**	27.953±0.079	**T159**	43.933±0.356
T39	45.131±0.066	**T106**	26.827±0.098	**T160**	43.211±0.009
T40	45.060±0.165	**T108**	26.331±0.017	**T168**	42.917±0.049
T41	43.732±0.819	**T109**	26.177±0.092	**T170**	42.431±0.015
T46	43.003±0.030	**T110**	25.863±0.043	**T174**	40.289±0.148
T50	42.022±0.049	**T112**	24.667±0.342	**T180**	38.547±0.017
T51	42.257±0.015	**T116**	24.283±0.035	**T184**	32.754±0.005
T56	41.078±0.057	**T117**	23.948±0.033	**T186**	30.556±0.021
T58	41.245±0.028	**T119**	23.283±0.026	**T189**	28.277±0.033
T59	40.247±0.038	**T120**	23.877±0.037	**T190**	27.664±0.026
T61	37.879±0.111	**T122**	23.075±0.089	**T193**	26.883±0.090
T63	38.955±0.054	**T123**	22.779±0.060	**T194**	26.285±0.036
T69	36.134±0.022	**T127**	21.203±0.003	**T196**	25.942±0.011
T71	34.765±0.029	**T128**	23.122±0.094	**T200**	24.094±0.088
T72	34.723±0.147	**T129**	23.032±0.094	**T202**	23.299±0.008
T74	34.759±0.206	**T130**	54.983±0.043	**T203**	23.645±0.197
T76	35.942±0.113	**T132**	53.025±0.062		

ID= Identification number; C= Control group; LOO= Lipid peroxidation; SEM= Standard error of mean; N=3.

Table 4.11: Summary of the biochemical parameters under investigation of the control and tobacco groups.

Variable	Group	N	Mean	SD	SEM	95% CI	
						Lower	Upper
GSH	CON	17	3.456	0.239	0.058	3.333	3.380
	TOB	83	2.394	0.353	0.039	2.318	2.471
GST	CON	17	0.073	0.035	0.008	0.055	0.091
	TOB	83	0.235	0.161	0.018	0.199	0.270
CAT	CON	17	2.783	0.325	0.079	2.616	2.951
	TOB	83	2.468	0.362	0.040	2.389	2.547
SOD	CON	17	3.817	0.442	0.107	3.590	4.045
	TOB	83	2.463	0.380	0.042	2.380	2.546
LOO	CON	17	27.772	3.439	0.834	26.003	29.540
	TOB	83	37.461	10.020	1.100	35.274	39.649

CON= Control group; TOB= Tobacco group; N=Total; SD= Standard deviation; SEM= Standard error of mean; CI= Confidence interval; GSH= Glutathione; GST= Glutathione-S-transferase; CAT= Catalase; SOD= Superoxide dismutase; LOO= Lipid peroxidation.

Table 4.12: Mann-Whitney U rank test showing difference in population mean between different groups.

Test between	Variable	Test between	Variable
All Male & Female	GSH*	**CON and TOB**	GSH*
	GST		GST*
	CAT*		CAT*
	SOD*		SOD*
	LOO		LOO*
CON and TOB (Male)	GSH*	**CON and TOB (Female)**	GSH*
	GST*		GST*
	CAT*		CAT*
	SOD*		SOD*
	LOO*		LOO*
CON (Male & Female)	GTH	**TOB (Male & Female)**	GSH
	GST		GST
	CAT*		CAT
	SOD*		SOD
	LOO		LOO

*CON= Control group; TOB= Tobacco group; GSH= Glutathione; GST= Glutathione-S-transferase; CAT= Catalase; SOD= Superoxide dismutase; LOO= Lipid peroxidation; *Significant at 0.05 level of significance; No symbol= Not significant.*

Table 4.13: Pearson correlation coefficient of the different biochemical parameters studied with age.

Group	Correlation with age	Value	Group	Correlation with age	Value
CON	GSH	-0.332	**TOB**	GSH	-0.556*
	GST	0.496*		GST	0.944*
	CAT	-0.350		CAT	-0.660*
	SOD	-0.349		SOD	-0.672*
	LOO	0.520*		LOO	0.984*
CON (Male)	GSH	-0.317	**CON (Female)**	GSH	0.539
	GST	0.828*		GST	0.379
	CAT	0.042		CAT	-0.533
	SOD	-0.017		SOD	-0.576
	LOO	0.891*		LOO	0.225
TOB (Male)	GSH	-0.474*	**TOB (Female)**	GSH	-0.854*
	GST	0.970*		GST	0.929*
	CAT	-0.605*		CAT	-0.922*
	SOD	-0.640*		SOD	-0.918*
	LOO	0.992*		LOO	0.983*

*CON= Control group; TOB= Tobacco group; GSH= Glutathione; GST= Glutathione-S-transferase; CAT= Catalase; SOD= Superoxide dismutase; LOO= Lipid peroxidation; *Significant at 0.05 level of significance; No symbol= Not significant.*

Table 4.14: Linear regression showing the effects of non-tobacco variables on the level of glutathione. R^2: 0.696.

Variable	β	SEM	t	p
Age	-0.026	0.010	-2.521	0.015
Sex	-1.327	0.523	2.535	0.014
Residence	-0.026	0.085	-0.307	0.760
Betel	0.172	0.196	0.878	0.384
Betel: current user	-0.027	0.095	-0.290	0.773
Betel with lime	-0.156	0.125	-1.251	0.217
Betel with tobacco	0.210	0.098	2.141	0.037
Betel frequency/day	-0.003	0.005	-0.656	0.515
Betel use (year)	-0.004	0.006	-0.628	0.533
Alcohol user	0.075	0.435	0.173	0.863
Alcohol: current user	0.148	0.239	0.619	0.538
Branded alcohol only	-0.282	0.262	-1.074	0.288
Local alcohol only	-0.220	0.447	-0.491	0.625
Branded and local alcohol	-1.334	0.456	-2.925	0.767
Daily alcohol drinker	10.080	1.041	1.037	0.305
Not daily alcohol drinker	0.656	1.038	0.632	0.530
Alcohol: age at start	-0.039	0.034	-1.153	0.254
Alcohol drink duration (year)	-0.003	0.010	-0.268	0.789
Smoked vegetables	-0.013	0.076	-0.173	0.864
Pickled vegetables and fruits	0.201	0.094	2.128	0.238
Any smoked Meat	-0.088	0.314	-0.280	0.780
Any salted meat	-0.044	0.090	-0.484	0.630
Any pickled meat	-0.056	0.081	-0.692	0.492
Any tinned foods	0.006	0.096	0.062	0.951
Fermented foods	-0.070	0.085	-0.830	0.410
Soda	-0.098	0.117	-0.843	0.403
Monosodium glutamate	0.160	0.112	1.436	0.157
Familial cancer	-0.008	0.073	-0.116	0.909

β= Beta coefficient; SEM= Standard error of mean; t= Beta coefficient/Standard error of mean; p= Significant level at 0.05.

Table 4.15: Linear regression showing the effects of non-tobacco variables on the activities of glutathione-s-transferase. R^2: 0.962.

Variable	β	SEM	t	p
Age	0.007	0.002	4.602	0.000
Sex	-0.183	0.079	2.313	0.025
Residence	0.003	0.013	0.245	0.807
Betel	0.060	0.030	2.014	0.049
Betel: current user	-0.002	0.014	-0.133	0.895
Betel with lime	-0.029	0.019	-1.558	0.126
Betel with tobacco	-0.001	0.015	-0.099	0.922
Betel frequency/day	0.000	0.001	-0.382	0.704
Betel use (year)	-0.001	0.001	-1.669	0.102
Alcohol user	0.196	0.066	-2.983	0.004
Alcohol: Current user	-0.054	0.036	1.500	0.140
Branded alcohol only	0.000	0.040	0.007	0.995
Local alcohol only	0.093	0.068	1.369	0.177
Branded and local alcohol	-0.712	0.949	-0.750	0.179
Daily alcohol drinker	0.306	0.158	1.943	0.058
Not daily alcohol drinker	0.282	0.157	1.798	0.078
Alcohol: age at start	-0.007	0.005	-1.465	0.149
Alcohol drink duration (year)	0.001	0.002	0.622	0.537
Smoked vegetables	-0.005	0.011	0.448	0.656
Pickled vegetables and fruits	-0.007	0.014	-0.488	0.628
Any smoked meat	0.046	0.048	0.958	0.343
Any salted meat	-0.004	0.014	-0.262	0.794
Any pickled meat	0.017	0.012	1.396	0.169
Any tinned foods	-0.027	0.014	1.858	0.069
Fermented foods	-0.024	0.013	-1.831	0.073
Soda	0.005	0.018	0.298	0.767
Monosodium glutamate	-0.003	0.017	-0.167	0.868
Familial cancer	-0.012	0.011	-1.086	0.283

β= Beta coefficient; SEM= Standard error of mean; t= Beta coefficient/Standard error of mean; p= Significant level at 0.05.

Table 4.16: Linear regression showing the effects of non-tobacco variables on the activities of catalase. R^2: 0.791.

Variables	β	SEM	t	p
Age	-0.031	0.008	-3.768	0.000
Sex	-1.191	0.421	2.830	0.007
Residence	-0.169	0.068	-2.481	0.017
Betel	0.124	0.158	0.788	0.434
Betel: current user	0.078	0.076	1.025	0.310
Betel with lime	-0.080	0.100	-0.802	0.426
Betel with tobacco	0.029	0.079	0.372	0.711
Betel frequency/day	0.000	0.004	-0.180	0.858
Betel use (year)	0.003	0.004	0.754	0.455
Alcohol user	0.482	0.350	1.378	0.174
Alcohol: Current user	0.210	0.192	1.090	0.281
Branded alcohol only	-0.194	0.211	-0.920	0.362
Local alcohol only	-0.365	0.359	-1.016	0.315
Branded and local alcohol	-0.698	0.572	-1.220	0.422
Daily alcohol drinker	0.686	0.837	0.820	0.416
Not daily alcohol drinker	0.773	0.834	0.927	0.359
Alcohol: age at start	-0.058	0.027	-2.123	0.039
Alcohol drink duration (year)	-0.007	0.008	-0.826	0.413
Smoked vegetables	-0.101	0.061	-1.667	0.102
Pickled vegetables and fruits	-0.094	0.076	1.236	0.222
Any smoked meat	-0.180	0.253	0.713	0.479
Any salted meat	-0.097	0.073	-1.342	0.186
Any pickled meat	-0.072	0.065	1.112	0.271
Any tinned foods	-0.161	0.077	2.101	0.041
Fermented foods	-0.069	0.068	-1.003	0.321
Soda	-0.086	0.094	-0.914	0.365
Monosodium glutamate	-0.120	0.090	-1.338	0.187
Familial cancer	0.010	0.058	0.178	0.859

β= Beta coefficient; SEM= Standard error of mean; t= Beta coefficient/Standard error of mean; p= Significant level at 0.05.

Table 4.17: Linear regression showing the effects of non-tobacco variables on the activities of superoxide dismutase. R^2: 0.821.

Variables	β	SEM	t	p
Age	-0.030	0.009	-3.515	0.001
Sex	-1.070	0.437	2.447	0.018
Residence	-0.149	0.071	-2.105	0.040
Betel	0.131	0.164	0.800	0.428
Betel: current user	0.087	0.079	1.107	0.274
Betel with lime	-0.089	0.104	-0.853	0.398
Betel with tobacco	0.016	0.082	0.190	0.850
Betel frequency/day	0.001	0.004	0.324	0.747
Betel use (year)	0.003	0.005	0.656	0.515
Alcohol user	0.455	0.363	1.252	0.217
Alcohol: Current user	0.511	0.200	2.556	0.014
Branded alcohol only	-0.419	0.219	-1.913	0.062
Local alcohol only	-0.597	0.373	-1.597	0.117
Branded and local alcohol	-0.533	0.528	-1.009	0.174
Daily alcohol drinker	-0.518	0.870	-0.595	0.554
Not daily alcohol drinker	-0.438	0.867	-0.505	0.616
Alcohol: age at start	-0.023	0.028	-0.809	0.423
Alcohol drink duration (year)	-0.003	0.009	-0.320	0.750
Smoked vegetables	-0.092	0.063	-1.457	0.151
Pickled vegetables and fruits	-0.102	0.079	1.297	0.201
Any smoked meat	-0.171	0.262	0.652	0.517
Any salted meat	-0.099	0.075	-1.311	0.196
Any pickled meat	-0.104	0.068	1.539	0.130
Any tinned foods	-0.148	0.080	1.850	0.070
Fermented foods	-0.087	0.071	-1.227	0.226
Soda	-0.084	0.097	-0.864	0.392
Monosodium glutamate	-0.098	0.093	-1.047	0.300
Familial cancer	0.024	0.061	0.400	0.691

β= Beta coefficient; SEM= Standard error of mean; t= Beta coefficient/Standard error of mean; p= Significant level at 0.05.

Table 4.18: Linear regression showing the effects of non-tobacco variables on the level of lipid peroxidation. R^2: 0.988.

Variables	β	SEM	t	p
Age	0.644	0.055	11.816	0.000
Sex	-0.180	2.787	-0.064	0.949
Residence	0.269	0.450	0.597	0.553
Betel	0.973	1.044	0.931	0.356
Betel: current user	-0.669	0.504	-1.328	0.190
Betel with lime	0.655	0.663	0.987	0.329
Betel with tobacco	-0.303	0.523	-0.580	0.564
Betel frequency/day	0.025	0.025	1.023	0.311
Betel use (year)	-0.082	0.030	-2.765	0.008
Alcohol user	6.556	2.317	-2.830	0.007
Alcohol: Current user	0.572	1.275	0.449	0.655
Branded alcohol only	1.047	1.397	0.750	0.457
Local alcohol only	3.591	2.381	1.508	0.138
Branded and local alcohol	-4.634	2.749	-1.686	0.355
Daily alcohol drinker	10.655	5.546	1.921	0.061
Not daily alcohol drinker	-10.394	5.526	1.881	0.066
Alcohol: age at start	-0.205	0.180	-1.141	0.259
Alcohol drink duration (year)	0.002	0.056	0.031	0.976
Tmoked vegetables	-0.508	0.402	-1.263	0.213
Pickled vegetables and fruits	-0.093	0.502	0.185	0.854
Any smoked meat	0.253	1.673	0.151	0.881
Any salted meat	-0.028	0.480	0.059	0.954
Any pickled meat	-0.657	0.430	-1.527	0.133
Any tinned foods	-0.729	0.509	1.433	0.158
Fermented foods	-0.131	0.452	-0.289	0.774
Soda	-1.847	0.621	-2.972	0.005
Monosodium glutamate	0.757	0.595	1.271	0.210
Familial cancer	0.279	0.387	0.721	0.474

β= Beta coefficient; SEM= Standard error of mean; t= Beta coefficient/Standard error of mean; p= Significant level at 0.05.

Table 4.19: Linear regression showing the effects of different tobacco products on the level of glutathione. R^2: 0.472.

Variables	β	SEM	t	p
Sahdah	0.119	0.097	1.217	0.228
Sahdah: current user	0.016	0.091	0.173	0.863
Sahdah use (Year)	-0.015	0.005	-3.342	0.001
Tuibur	0.352	0.157	-2.243	0.028
Tuibur: current user	-0.165	0.146	1.130	0.263
Tuibur use (Year)	0.015	0.006	2.524	0.014
Smoker	0.220	0.132	-1.666	0.100
Current smoker	0.178	0.081	2.198	0.031
Zozial only	-0.127	0.136	-0.930	0.356
Cigarette only	0.175	0.092	1.888	0.063
Zozial and cigarette	-0.783	0.594	1.318	0.622
Smoke/day	0.013	0.009	1.556	0.124
Duration of smoking (Year)	-0.005	0.004	-1.510	0.136

β= Beta coefficient; SEM= Standard error of mean; t= Beta coefficient/Standard error of mean; p= Significant level at 0.05.

Table 4.20: Linear regression showing the effects of different tobacco products on the activities of glutathione-s-transferase. R^2: 0.783.

Variables	β	SEM	t	p
Sahdah	-0.028	0.029	-0.981	0.330
Sahdah: current user	-0.016	0.027	-0.601	0.550
Sahdah use (Year)	0.004	0.001	2.818	0.006
Tuibur	0.098	0.046	2.134	0.036
Tuibur: current user	-0.010	0.043	-0.230	0.819
Tuibur use (Year)	-0.002	0.002	-1.100	0.275
Smoker	0.162	0.039	-4.185	0.000
Current smoker	-0.068	0.024	-2.856	0.006
Zozial only	-0.027	0.040	-0.665	0.508
Cigarette only	0.015	0.027	0.545	0.587
Zozial and cigarette	-0.086	0.076	-1.132	0.398
Smoke/day	-0.003	0.003	-1.248	0.216
Duration of smoking (Year)	0.007	0.001	6.508	0.000

β= Beta coefficient; SEM= Standard error of mean; t= Beta coefficient/Standard error of mean; p= Significant level at 0.05.

Table 4.21: Linear regression showing the effects of different tobacco products on the activities of catalase. R^2: 0.476.

Variables	β	SEM	t	p
Sahdah	0.139	0.099	1.402	0.165
Sahdah: current user	0.032	0.093	0.347	0.729
Sahdah use (Year)	-0.015	0.005	-3.280	0.002
Tuibur	-0.096	0.160	-0.597	0.552
Tuibur: current user	-0.099	0.149	-0.665	0.508
Tuibur use (Year)	0.010	0.006	1.683	0.097
Smoker	0.032	0.135	-0.240	0.811
Current smoker	0.162	0.082	1.966	0.053
Zozial only	-0.126	0.139	-0.910	0.366
Cigarette only	0.011	0.094	0.119	0.906
Zozial and cigarette	-0.052	0.056	-0.929	0.211
Smoke/day	0.014	0.009	1.603	0.114
Duration of smoking (Year)	-0.005	0.004	-1.378	0.173

β= Beta coefficient; SEM= Standard error of mean; t= Beta coefficient/Standard error of mean; p= Significant level at 0.05.

Table 4.22: Linear regression showing the effects of different tobacco products on the activities of superoxide dismutase. R^2: 0.484.

Variables	β	SEM	t	p
Sahdah	0.133	0.103	1.286	0.203
Sahdah: current user	0.042	0.097	0.432	0.667
Sahdah use (Year)	-0.014	0.005	-2.925	0.005
Tuibur	-0.056	0.167	-0.338	0.736
Tuibur: current user	-0.074	0.156	-0.476	0.636
Tuibur use (Year)	0.010	0.006	1.611	0.112
Smoker	0.040	0.140	0.286	0.776
Current smoker	0.202	0.086	2.357	0.021
Zozial only	-0.087	0.145	-0.603	0.549
Cigarette only	0.009	0.098	0.096	0.924
Zozial and cigarette	0.024	0.043	0.558	0.034
Smoke/day	0.009	0.009	0.948	0.346
Duration of smoking (Year)	-0.007	0.004	-1.747	0.085

β= Beta coefficient; SEM= Standard error of mean; t= Beta coefficient/Standard error of mean; p= Significant level at 0.05.

Table 4.23: Linear regression showing the effects of different tobacco products on the level of lipid peroxidation. R^2: 0.797.

Variables	β	SEM	t	p
Sahdah	-3.060	1.713	-1.786	0.078
Sahdah: current user	-1.465	1.600	-0.916	0.363
Tahdah use (Year)	0.394	0.079	4.968	0.000
Tuibur	6.065	2.759	2.199	0.031
Tuibur: current user	0.436	2.576	0.169	0.866
Tuibur use (Year)	-0.258	0.104	-2.481	0.016
Smoker	9.215	2.326	-3.962	0.000
Current smoker	-5.781	1.422	-4.066	0.000
Zozial only	0.242	2.394	0.101	0.920
Cigarette only	0.809	1.626	0.498	0.620
Zozial and cigarette	-0.572	0.984	0.581	0.094
Smoke/day	-0.145	0.151	-0.961	0.340
Duration of smoking (Year)	0.381	0.064	5.980	0.000

β= Beta coefficient; SEM= Standard error of mean; t= Beta coefficient/Standard error of mean; p= Significant level at 0.05.

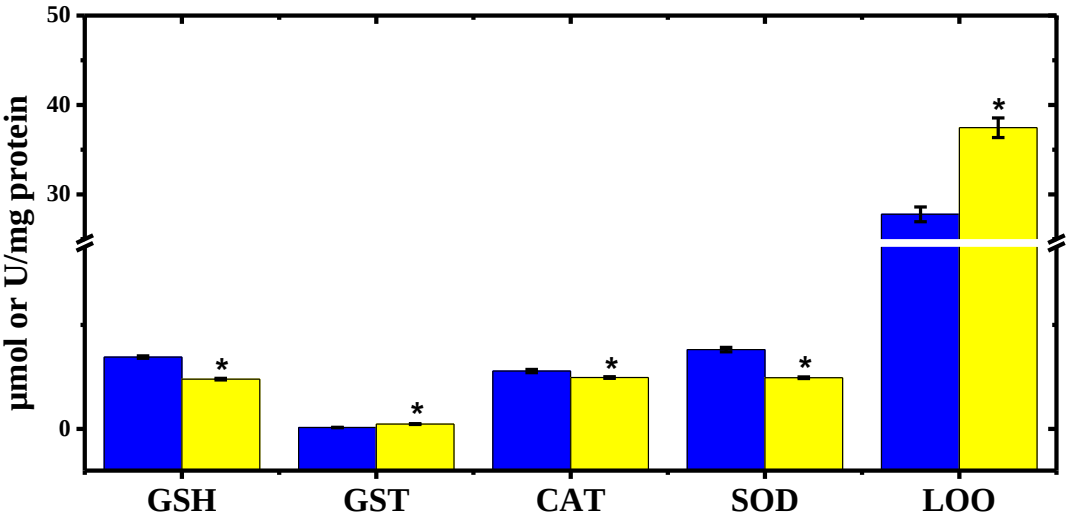

Figure 4.1: Alteration in glutathione concentration and activities of glutathione-s-transferase, catalase and superoxide dismutase and lipid peroxidation in the tobacco consumers.

*Blue bar= Control group; Yellow bar= Tobacco group; GSH= Glutathione; GST= Glutathione-s-transferase; CAT= Catalase; SOD= Superoxide dismutase; LOO= Lipid peroxidation; U= Enzyme unit; *Significant at 0.05 level of significance.*

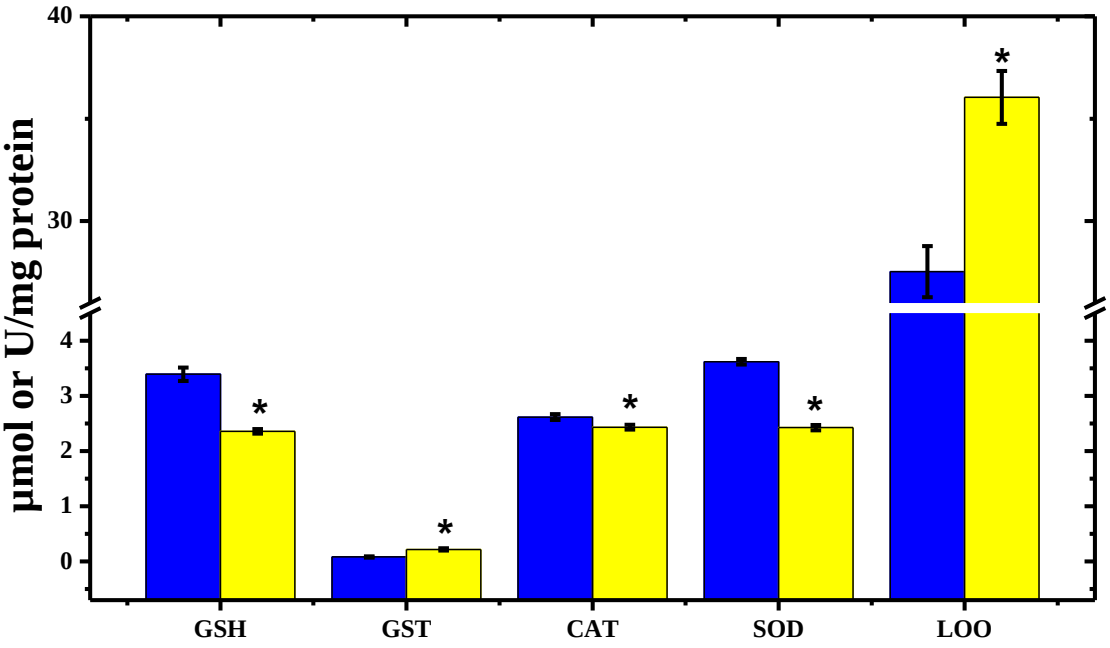

Figure 4.2: Alteration in glutathione concentration and activities of glutathione-s-transferase, catalase and superoxide dismutase and lipid peroxidation among the male tobacco consumers.

Blue bar= Control group; Yellow bar= Tobacco group; GSH= Glutathione; GST= Glutathione-s-transferase; CAT= Catalase; SOD= Superoxide dismutase; LOO= Lipid peroxidation; U= Enzyme unit.

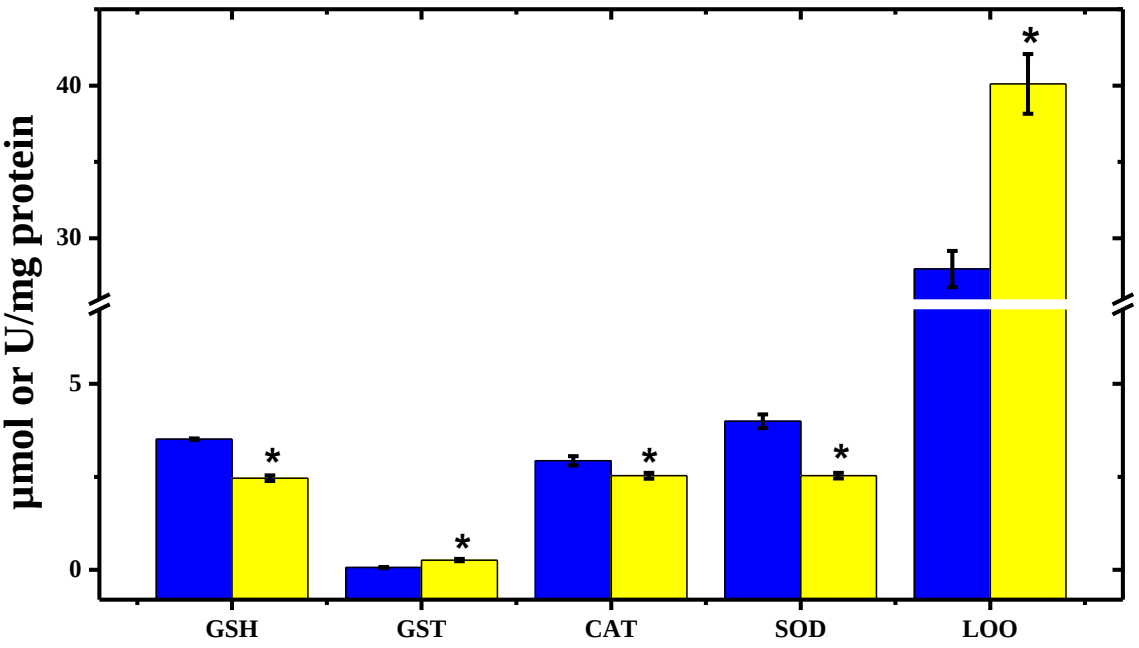

Figure 4.3: Alteration in glutathione concentration and activities of glutathione-s-transferase, catalase and superoxide dismutase and lipid peroxidation in the female tobacco consumers.

Blue bar= Control group; Yellow bar= Tobacco group; GSH= Glutathione; GST= Glutathione-s-transferase; CAT= Catalase; SOD= Superoxide dismutase; LOO= Lipid peroxidation; U= Enzyme unit.

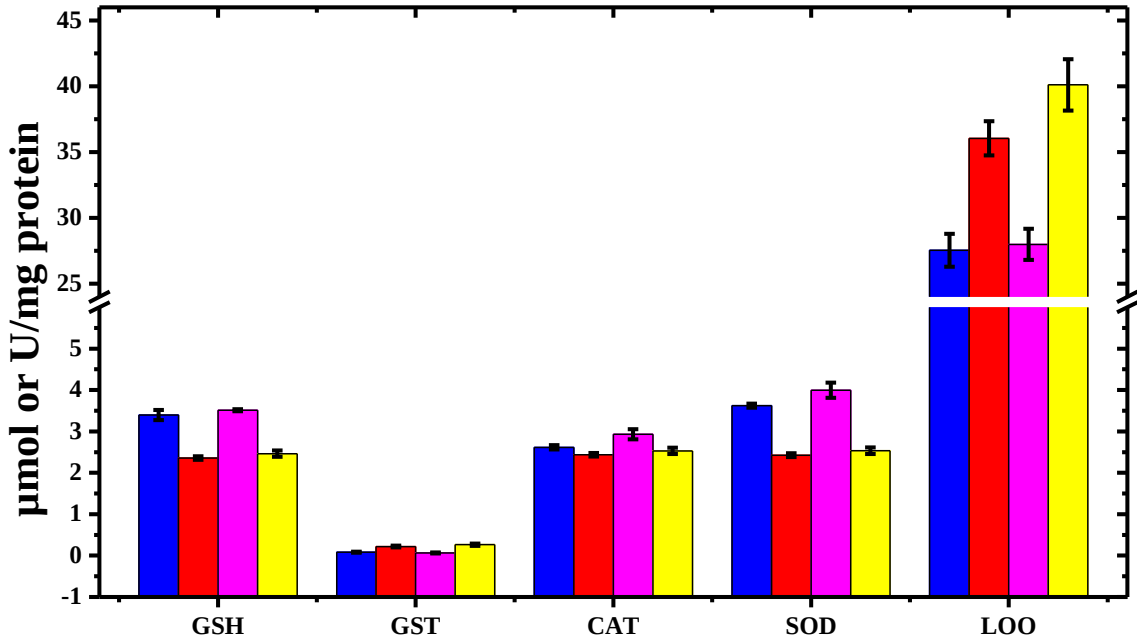

Figure 4.4: Comparison of amount and activities of various biochemical parameters under investigation between male and female control and tobacco groups.

Blue bar= Control male; Red bar= Tobacco male; Magenta bar= Control female; Yellow bar= Tobacco female; GSH= Glutathione; GST= Glutathione-s-transferase; CAT= Catalase; SOD= Superoxide dismutase; LOO= Lipid peroxidation; U= Enzyme unit.

CHAPTER 5

TOBACCO CONSUMPTION EXACERBATES CHROMOSOMAL ABERRATIONS IN MIZO INDIVIDUALS

Abstract

The consumption of tobacco has been linked with several diseases including cancer of different organs. Tobacco affects the genetic and epigenetic components of the cell resulting in the upregulation or downregulation of important genes. It has also been known to cause DNA adducts eventually leading to the formation of nicks in the DNA, which further cause structural damage to the chromosomes. The Mizos residing in the north eastern part of India are prone to cancer of different organs. Many of them have been known to consume several varieties of tobacco and its related products. The effect of tobacco consumption has been studied in cultured peripheral blood lymphocytes of Mizos who are frequently using tobacco in various forms. Blood was collected from tobacco users and non-users of healthy Mizo volunteers and the erythrocytes were allowed to sediment against gravity. The buffy coat containing cells was inoculated into RMPI medium containing phytohemagglutinin and allowed to grow for 44 h followed by the addition of colchicine to arrest the cell at metaphase. Metaphase plates were prepared at 56 h post initiation of the cultures to study chromosomal aberrations. Data analysis revealed that the frequency of chromosomal aberrations increased significantly in the volunteers who regularly consumed tobacco in any form when compared to those volunteers who did not take any form of tobacco. It was found that the individuals who were using smokeless tobacco in the form of tuibur (tobacco brew) and sahdah had higher frequencies of chromosomal aberrations than the users of other forms of tobacco. Our study indicates that the use of tobacco in any form is a risk factor for chromosomal damage.

INTRODUCTION

There are many factors that can alter the genetic components of the human body (Iyama and Wilson, 2013; Acuna-Hidalgo *et al.,* 2016). Such factors are called mutagens and the process and consequence of genetic alteration is called mutation (Chakarov *et al.*, 2014). By definition, mutation is any change in the structure of the genome. Mutation can result in a more detrimental type of genomic event called chromosomal aberrations where the gross structure of the chromosome is altered from the normal types (Mareuca *et al*., 2006). There are many types of chromosomal aberrations such as translocation, inversion, chromosome break, chromatid break, dicentric chromosome, acentric fragments, pulverization, etc. In most cases, chromosomal aberrations lead to devastating consequences including cancer (Christmann *et al.*, 2003; Petrini and Stracker, 2003; Valko *et al.*, 2004; Jeggo *et al.*, 2016).

The lifestyle choice of individuals provides additional exposure to detrimental factors that can result in genomic alteration. The consumption of tobacco and its related products, alcohol, exposure to various forms of radiations such as ultraviolet radiation, electromagnetic and X-ray, etc., can be additional factors which can inflict genomic damage. The effect of tobacco on health has been a topic of interest for quite a long time as tobacco has been linked with many forms of cancers (Boffetta, 2008; Bassiony *et al.*, 2015). Tobacco contains more than eight thousand chemicals, out of which more than 500 are toxic and more than 80 have carcinogenic properties. These chemicals are known to inflict toxic, cytotoxic, genotoxic, mutagenic, teratogenic and carcinogenic insults to cells (Hecht, 2003; Talhout *et al.*, 2011).

The regular consumption of tobacco has been known to be directly involved in the carcinogenesis of the mouth, lungs, esophagus, stomach, liver, pharynx, colon, etc. Besides cancer, several other diseases like bronchitis, asthma, tuberculosis, laryngitis, pharyngitis, cardiovascular, chronic obstructive pulmonary disease, degenerative diseases and dental diseases are directly linked to tobacco use (Musk and De Klerk, 2003; Norman *et al.*, 2011; Elmasry *et al.*, 2015). The consumption of tobacco in combination with the use of alcohol has been reported to increase the risk of colon cancer and other diseases (Slattery *et al.*, 1997; Hart *et al.*, 2010). Tobacco smoking, alcohol consumption and betel quid chewing have also been observed to significantly increase the risk of lung cancer (Phukan *et al.*, 2014; Saikia *et al.*, 2014). Smokeless tobacco has also been attributed to the increase risk of many tobacco related cancers (Gupta and Ray, 2003; *Rose et al.*, 2016).

Therefore, the present study aims to determine the frequency of different types of chromosomal aberrations in cultured peripheral blood lymphocytes of Mizo tobacco users.

METHODOLOGY

Blood collection

The subjects were divided into two groups as described in the previous chapter, i.e. Chapter 2.

Control group: This group consisted of four volunteers in the age group of 18-67 years of either sex who had no known history of any form of tobacco consumption.

Tobacco group: Eighteen volunteers belonging to the age group of 18- 67 years of either sex, who consumed any form of tobacco on regular basis were recruited in this

group. The type of tobacco used may either be smoked or smokeless tobacco or both.

After identifying suitable volunteers for this study, the consent and questionnaire form described in Chapter 2 were distributed to the volunteers for filling and blood was collected from each individuals after their informed consent as described in Chapter 3.

Human peripheral blood lymphocyte (HPBL) culture

HPBLs culture was performed according to standard protocol (Jagetia *et al.*, 2001). The collected blood was allowed to settle for half an hour and the buffy coat was aseptically collected. Usually one million nucleated cells were inoculated into several sterile culture tubes containing 2 ml RPMI-1640 medium supplemented with 25 µl/ml of phytohemagglutinin (PHA) as the mitogen to stimulate the cell division of lymphocytes. The cultures were set in triplicate for each volunteer and transferred into a CO_2 incubator maintained at 37°C.

Metaphase plate preparation

Metaphase plates from the HPBLs were prepared according to standard protocol (Jagetia, 1994). Briefly, after 44 h of initial incubation, 10 µg/ml of colchicine was added into each culture to inhibit the cell division at metaphase and the cells were allowed to grow for another 12 h. The cells were harvested 56 h after the initiation of the culture. The cells were centrifuged at 1000 rpm for 10 min and the supernatant was discarded. The remaining pellet containing lymphocytes was subjected to mild hypotonic treatment (KCl 0.56%) and incubated at 37°C for 30 minutes. After incubation, the tubes were centrifuged at 800 rpm for 5 minutes and the supernatant was discarded. The pellet was fixed with Carnoy's fixative (3:1,

methanol:acetic acid) for 15 minutes and centrifuged at 800 rpm for 5 minutes. After centrifugation, the cells were resuspended in a small volume of fixative and kept overnight in a refrigerator maintained at 4°C. The cells were dropped on to pre-cleaned coded chilled slides. The slides containing cells were stained with Giemsa stain and washed with running tap water and immersed in xylene for a few minutes. The slides were observed under a microscope (DM2500, Leica Mikrosystems GmBH, Wetzlar, Germany) for the presence of various chromosomal aberrations. Metaphase plates with chromatid break (CB), chromosome break (CSB), acentric fragments (AF), dicentric chromosomes (DC), aberrant cells (AC) and total aberrations (TA) were scored and 100 metaphase plates were counted from each culture tube and a total of 300 metaphase plates were scored for each individual.

Statistical analyses

The statistical analyses between the control and tobacco groups were carried out by Mann-Whitney U test for the various parameters used and Pearson's correlation coefficient was calculated to determine the correlation of chromosomal aberrations with age. Linear regression was used to see the effect of different variables on the formation of chromosomal aberrations.

RESULTS

The results of chromosomal aberrations have been expressed as mean±standard error of mean (SEM) and the results of both the control and tobacco groups are shown in Tables 4.1-4.10 and Figure 4.1.

The tobacco group showed significantly higher level of all types of chromosomal aberrations than the control group (Tables 4.1, 4.2 and Figure 4.1). However, the rise in chromosome breaks and acentric fragments did not show statistical significant. Comparison between genders regardless of group exhibited significantly higher acentric fragments and dicentric chromosomes in the male than the female population (Table 4.3). The control group showed strong non-significant correlation of chromatid breaks, acentric fragments, aberrant cells and total aberrations with age. In the control group, chromosome breaks displayed a weak non-significant negative relation, whereas dicentric chromosomes showed weak non-significant positive correlation with increasing age. The tobacco group showed strong positive significant correlation for chromatid break, aberrant cells and total aberrations with age whereas the correlation for chromosome breaks was moderately significant. The correlation of acentric fragments with age was positive, weak and non-significant while for dicentric chromosome it is positive, moderate and non-significant (Table 4.4).

The correlation between different variables and the frequency of chromosomal aberrations was determined by linear regression and the analyses indicated that age, the consumption of betel, betel with tobacco, the frequency of betel consumed every day, alcohol consumption, monosodium glutamate, and pickled foods consumption played an important role in increasing the frequency of

chromatid breaks. Despite this, only age, daily use of alcohol and the consumption of monosodium glutamate showed significant ($p < 0.05$) correlation. The frequency of chromosome breaks has been found to be influenced by age, use of betel, duration of betel use, alcohol use, drinking of branded and local alcohol, daily consumption of alcohol, consumption of smoked and salted meats, tinned foods and sodium bicarbonate. Among these variables, age, duration of betel use and daily alcohol consumption have been found to have significant effects (Table 4.5). For acentric fragment, it was found that the consumption of betel, duration of betel use, local alcohol use, drinking of branded and local alcohol, consumption of sodium bicarbonate and monosodium glutamate and family history of any form of cancer increased the frequency. However, significant correlation was observed only for consumption of betel and duration of betel consumed. Age, betel consumption, dose of betel consumed, alcohol intake, drinking of local and branded alcohol, daily drinking of alcohol, intake of fermented foods and monosodium glutamate and family history of cancer did correlate with the increased frequency of dicentric chromosomes. Among these variables, consumption of betel, monosodium glutamate, daily alcohol drinking and family history of cancer showed significant correlation (Table 4.6).

Variables such as age, betel with tobacco consumption, dose of betel consumed in a day, drinking of local alcohol and daily intake of alcohol showed correlation with aberrant cells, however, only age displayed significant correlation. The factors affecting total aberrations were age, betel with tobacco consumption, duration of betel used, alcohol intake, daily drinking of alcohol, eating of smoked meat, sodium bicarbonate and monosodium glutamate and family history of cancer.

Among these, age, consumption of betel with tobacco, daily drinking of alcohol, consumption of sodium bicarbonate and monosodium glutamate exhibited significant correlation (Table 4.7).

Among the tobacco group, chromatid breaks were found to be influenced by use of sahdah, duration of sahdah use, tuibur intake, smoking, smoking of zozial, zozial smoked per day and duration of zozial smoked. The use of tuibur and duration of smoking showed significant correlation. Sahdah use, duration of sahdah used, tuibur intake, smoking of zozial and both zozial and cigarette showed correlation with increase in chromosome breaks (Table 4.8). The frequency of acentric fragment was affected by the use of sahdah, duration of tuibur used, smoking of zozial and both zozial and cigarette and their amount smoked per day. Among these factors, use of sahdah, duration of tuibur used and frequency of zozial and cigarette smoked per day displayed significant correlation. The use of tuibur, duration of tuibur used, smoking, smoking of zozial and both zozial and cigarette were found to be correlated with increase dicentric chromosomes, however, this correlation is not significant (Table 4.9). The variables affecting increase aberrant cells are use of sahdah, duration of sahdah consumed, tuibur intake, duration of tuibur used, duration of smoking, smoking of zozial and both zozial and cigarette. However, only the use of sahdah exhibited significant correlation. Total aberrations has been found to be correlated with sahdah use, duration of sahdah used, tuibur intake, duration of tuibur intake, smoking, smoking of zozial and both zozial and cigarette and duration of smoking. Among these variables, tuibur intake, smoking and duration of smoking expressed significant correlation (Table 4.10).

DISCUSSION

Mizoram has a high frequency of different types of cancers. Cancer of the stomach, lungs, mouth, esophagus, cervix uteri and breast are especially frequent in this state. There may be many reasons for such high cancer incidence among the Mizos. Some of the dietary habits of the Mizos such as the consumption of smoked meats and vegetables, fermented foods, the use of sodium bicarbonate and monosodium glutamate in the preparation of foods, and the habit of consuming smoked and smokeless tobacco are probable factors that may have put the genome of this population at a higher risk of induction of genetic instability and eventually leading to higher cancer incidence. Epidemiological studies in this population had shown the correlation of tobacco use and cancer of certain sites (Phukan *et al.,* 2005; Lalpawimawha *et al.,* 2015; Lalpawimawha and Lalruatfela, 2016). Therefore the present study was designed to study the genetic instability in the form of chromosomal aberrations among the Mizos who are using any form of tobacco.

Tobacco may be the single most lethal legal product in the world, which is consumed in many form by millions of individuals in each country and an estimate by WHO showed that 1.1 billion individuals smoked tobacco in the year 2015. It was estimated that millions of individuals died annually of tobacco related diseases (Britton, 2017). Despite this, the use of tobacco and its related products is not appreciably declining and it can be anticipated that more tobacco related diseases will persist in the near future. The consumption of tobacco has been known to be associated with many forms of diseases like ischemic heart disease, dentine diseases, stroke, myocardial infarction, chronic lung diseases, lung fibrosis, emphysema, oral diseases including staining of dentures, Parkinson's disease, male infertility and

different types of cancers (Miller and Das, 2007; Morse and Rosas, 2014). These diseases are a result of the effect of tobacco on the genetic and epigenetic components of cells. The genetic effects may be in the form of point mutation, deletion, insertion, recombination, transversions, transition, and chromosomal aberrations including aneuploidy and polyploidy (Valko *et al*., 2004).

Among smokers, a significant increase in chromosomal aberrations, micronuclei frequency and sister chromatid exchange have been observed earlier (Zheng *et al.*, 2006; Milic *et al.*, 2008; Chandirasekar *et al.*, 2013; Christobher *et al.*, 2017). A similar effect has been observed in the current investigation where higher occurrence of chromosomal aberrations is reported among tobacco users when compared with non-users. Similarly, the smoking of waterpipe significantly elevated the level of chromosomal aberrations (Alsatari *et al.*, 2012). The consumption of smokeless tobacco has been reported to damage chromosomes (Choudhury *et al.*, 2009; Khanna *et al.*, 2012). A similar result was obtained in the present investigation where the consumption of smokeless tobacco in the form of sahdah and tuibur contributed significantly in increasing different types of chromosomal aberrations in the HPBLs among the study population. Contrastingly, it was reported that unlike smoking tobacco, smokeless tobacco did not affect the level of aberrations in peripheral blood lymphocytes. However, the effect of smokeless tobacco was apparent on the cells of the oral epithelium (Livingston *et al.*, 1990). The in vitro treatment of human papilloma virus-positive (HPV) oral keratinocytes with smokeless tobacco extract indicated that the treatment might elevate the effects of HPV-16 and the risk of DNA aneuploidy thereby increasing the risk to malignant transformation (Merne *et al.*, 2014). Interestingly, a higher risk of chromosomal damage on female was reported

earlier (Milic *et al.*, 2008) which confirms our result indicating that female tobacco users are more prone to DNA damage than male tobacco users.

The exposure to environmental tobacco smoke has also been reported to increase chromosomal aberrations and micronuclei frequency when compared to unexposed control group (Balachandra *et al.*, 2008; Chandirasekar et al., 2011). Among workers engaged in the manufacturing of bidi (an indigenous substitute of cigarette in India), chromosomal aberrations analysis revealed an increased deletion fragments and chromatid gaps when compared to non-exposed group (Mahimkar and Bhisey, 1995). It was suggested that the toxicants of bidi upon entering the human body causes disturbance to the normal form and behavior of chromosomes resulting in chromosomal aberrations and genomic variations (Sundaramoorthy *et al.*, 2013). This suggestion is plausible as the study on HCT116 colorectal cancer cells and RT112 bladder cell line showed that the treatment of these cells with arylamine 4-aminobiphenyl (4-ABP) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) increase chromosomal instability by more than fifty percent (Saletta *et al.*, 2007).

The smoking of tobacco has also been reported to trigger the production of quinone/hydroquinone free radicals that in turn produces superoxide radicals and hydroxyl radicals. (Church and Pryor, 1985; Valavanidis *et al.*, 2009). Free radicals present in tobacco tar had been known to produce nicks in the DNA and increase adducts formation (Pryor, 1997; Sepaniak *et al.*, 2006). Many brands of US cigarettes are known to produce free radicals (Goel *et al.*, 2017). The free radicals induced DNA adducts may be converted into DNA strand breaks and subsequently into different types of chromosomal aberrations. The use of tobacco has been reported to produce DNA and protein adducts (Phillips and Venitt, 2012; Hang,

2010). Tobacco is also known to induce sugar damage, apurinic/apyrimidinic sites, small base damages, bulky DNA adducts, DNA cross links and DNA strand breaks (Hang, 2010). Interestingly, the effect of smoking and smokeless tobacco differs as many new chemicals are formed during the process of pyrolysis in smoking tobacco (Pryor *et al*., 1983; Calafat *et al*., 2004).

Epidemiological studies have shown that the consumption of tobacco causes several mutations in the genome of individuals who have been smoking tobacco and are suffering from cancers related to tobacco consumption (Alexandrov *et al.*, 2016). In a study involving bladder cancer patients, 4-aminobiphenyl-DNA adducts were higher in current smokers than ex-smokers (Martone *et al*., 1998; Faraglia *et al*., 2003). One of the carcinogens present in tobacco, NNK had been found to alter the structure of XRCC1, a DNA repair protein, therefore decreasing the protein's ability to repair damaged DNA and also induce formation of DNA adducts (Abdel-Rahman and El-Zein, 2000; Hang, 2010). Oral cancer patients exhibited microsatellite instability and loss of heterozygosity on 9p21-23 locus of chromosome 9. This genetic alteration was found to be concentrated between markers D9S157 and D9S161 suggesting the possible involvement of p16 (CDNK2) in a subset of chewing tobacco-induced oral cancer (Mahale and Saranath, 2000). Cigarette smoke condensate was also found to inhibit the translation of FANCD2 mRNA in normal airway epithelial cells which was sufficient to induce both genetic instability and apoptosis (Hays *et al.*, 2008). In addition, the use of tobacco had been known to influence the structure and function of important genes like p53, p21, K-ras, H-ras, Bax, IL-6, TNF-α, iNOS, BCl-2, cyclin D1, EGFR, hMLH1, FHIT, hMSH3 and Cox-2 genes (Calvez *et al*., 2005; Krishnan *et al*., 2014;Biswas *et al*., 2015; Tam *et al*., 2016) that may have contributed in various

ways to inhibit DNA repair mechanisms and thereby increase chromosomal aberrations among tobacco consumers. Besides tobacco use, the increase in age has also been reported to increase the level of DNA damage (Bolognesi *et al.*, 1997; Venkatesan *et al.*, 2015). We have observed a similar result in the current study where the level of chromosomal aberrations have direct positive relationship with age.

CONCLUSIONS

The frequency of chromosomal aberrations increased in the tobacco users when compared with the controls. The use of both smoking and smokeless tobacco affected the frequency of chromosomal damage. The consumption of alcohol and betel and age are found to be confounding variables that also increased chromosomal aberration frequency. The high cancer incidence among the Mizos may be the result of increase genomic instability as indicated by increase DNA damage in this study. Increase chromosomal damage in the tobacco consumers may be due to the formation of free radicals and DNA adducts by tobacco and the alteration of different genes.

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Table 5.1: Frequency of different chromosomal parameters under investigation in control group and tobacco group.

ID		Aberrations per 100 cells				Aberrant cells %	Total aberration
		Chromatid break	Chromosome break	Acentric fragment	Dicentric		
CON Group	C4	1.33±0.33	0.00±0.00	1.67±0.88	0.67±0.33	3.33±1.20	3.67±1.33
	C11	0.00±0.00	0.67±0.33	0.33±0.33	0.67±0.33	1.67±0.33	1.67±0.33
	C25	0.33±0.33	0.00±0.00	0.67±0.33	0.00±0.00	1.00±0.00	1.00±0.00
	C37	0.00±0.00	0.00±0.00	0.67±0.33	0.33±0.33	1.00±0.58	1.00±0.58
TOB Group	T5	4.67±0.88	0.00±0.00	2.33±0.33	3.00±0.58	8.67±1.33	10.00±1.53
	T20	2.33±0.88	3.00±0.58	2.67±0.33	3.67±0.33	10.00±0.58	11.67±1.20
	T22	2.67±0.67	0.00±0.00	2.33±0.67	2.00±0.58	5.33±0.67	7.00±0.58
	T32	2.00±0.58	2.33±0.33	0.00±0.00	0.00±0.00	3.33±0.88	4.33±0.33
	T66	2.33±0.33	1.67±0.33	0.00±0.00	1.67±0.67	4.00±1.16	5.67±0.88
	T70	0.00±0.00	0.33±0.33	2.00±0.58	1.33±0.33	2.33±0.33	3.67±0.33
	T82	0.67±0.33	0.00±0.00	1.33±0.33	1.33±0.67	2.67±0.33	3.33±0.33
	T89	0.67±0.33	0.00±0.00	1.67±0.33	0.00±0.00	2.00±0.58	2.33±0.33
	T90	0.33±0.33	0.00±0.00	1.33±0.33	1.33±0.33	2.33±0.33	3.00±0.58
	T101	0.00±0.00	0.00±0.00	1.67±0.66	1.00±0.00	2.33±0.33	2.67±0.67
	T128	0.00±0.00	0.00±0.00	1.33±0.33	1.33±0.88	1.67±0.67	2.67±1.20
	T149	3.00±0.58	2.33±0.67	1.67±0.33	0.00±0.00	6.00±0.58	7.00±0.58
	T176	2.33±0.33	0.00±0.00	1.67±0.33	1.67±0.33	4.67±0.33	5.67±0.33
	T178	1.67±0.33	1.67±0.33	2.33±0.88	2.33±0.67	6.33±1.20	8.00±1.00
	T193	1.67±0.33	0.00±0.00	1.67±0.67	1.67±0.33	4.00±0.58	5.00±0.00
	T198	0.00±0.00	0.00±0.00	2.33±0.67	0.00±0.00	2.00±0.58	2.33±0.67
	T202	1.67±0.67	0.00±0.00	1.67±0.67	1.00±0.00	3.67±0.33	4.33±0.67
	T203	0.00±0.00	0.00±0.00	1.33±0.33	0.00±0.00	1.00±0.00	1.33±0.33

CON= Control group; TOB= Tobacco group; %= Percentage; N=3.

Table 5.2: Summary of the frequency of different chromosomal parameters under investigation of both the study groups.

Variable	Group	N	Mean	SD	SEM	95% CI	
						Lower	Upper
CB	CON	4	0.417	0.631	0.315	-0.587	1.421
	TOB	18	1.444	1.328	0.313	0.784	2.105
CSB	CON	4	0.167	0.333	0.167	-0.364	0.697
	TOB	18	0.630	1.041	0.245	0.112	1.147
AF	CON	4	0.833	0.577	0.289	-0.085	1.752
	TOB	18	1.630	0.722	0.170	1.271	1.989
DC	CON	4	0.417	0.319	0.160	-0.091	0.924
	TOB	18	1.296	1.060	0.250	0.769	1.823
AC	CON	4	1.750	1.101	0.551	-0.002	3.502
	TOB	18	4.019	2.456	0.579	2.797	5.240
TA	CON	4	1.833	1.262	0.631	-0.175	3.841
	TOB	18	5.000	2.821	0.665	3.597	6.403

Con= Control group; Tob= Tobacco group; CB=Chromatid break; CSB= Chromosome break; AF= Acentric fragment; DC= Dicentric chromosome; AC= Aberrant cell; TA= Total aberration; N= Total; SD= Standard deviation; SEM= Standard error of mean;

CI= Confidence interval.

Table 5.3: Mann-Whitney U rank test showing difference in population mean between both gender, both genders of control group and tobacco group.

Test between	Variable	Test between	Variable
All Male & Female	CB	**CON and TOB**	CB*
	CSB		CSB
	AF*		AF
	DC*		DC*
	AC		AC*
	TA		TA*
CON and TOB (Male)	CB	**CON and TOB (Female)**	CB*
	CSB		CSB*
	AF		AF*
	DC*		DC
	AC		AC*
	TA		TA*
CON (Male & Female)	CB	**TOB (Male & Female)**	CB
	CSB*		CSB
	AF*		AF
	DC*		DC
	AC*		AC
	TA*		TA

*CON= Control group; TOB= Tobacco group; CB=Chromatid break; CSB= Chromosome break; AF= Acentric fragment; DC= Dicentric chromosome; AC= Aberrant cell; TA= Total aberration; *Significant at 0.05 level of significant; No symbol= Not significant.*

Table 5.4: Pearson correlation coefficient of different chromosomal aberration parameters with age.

Group	Correlation with age	R-value	Group	Correlation with age	R-value
CON	CB	0.917	**TOB**	CB	0.837*
	CSB	-0.200		CSB	0.569*
	AF	0.784		AF	0.184
	DC	0.271		DC	0.452
	AC	0.828		AC	0.809*
	TA	0.833		TA	0.821*

*CON= Control group; TOB= Tobacco group; CB=Chromatid break; CSB= Chromosome break; AF= Acentric fragment; DC= Dicentric chromosome; AC= Aberrant cell; TA= Total aberration; *Significant at 0.05 level of significant; No symbol= Not significant.*

Table 5.5: Linear regression showing the effects of socio-demographic variables, dietary habits and familial history of cancer on the frequency of CB and CSB.

Variables	Chromosomal aberrations					
	CB (R^2: 0.982)			CSB (R^2: 0.998)		
	β ±SEM	t	p	β ±SEM	t	p
Age	0.252±0.125	2.016	0.032	0.084±0.033	2.545	0.024
Sex	-1.295±0.685	-1.891	0.310	-2.111±0.181	-11.663	0.651
Residence	-0.557±1.148	-0.485	0.712	-1.770±0.304	-5.822	0.239
Betel	5.861±4.768	1.229	0.287	4.018±1.263	3.181	0.194
Betel: Current user	-1.316±1.261	-1.044	0.487	-1.807±0.334	-5.410	0.250
Betel + lime	-0.350±1.639	-0.214	0.866	-1.868±0.434	-4.304	0.145
Betel + tobacco	9.243±4.710	1.962	0.300	-3.054±1.248	-2.447	0.247
Betel frequency/day	3.389±0.171	19.819	0.264	-2.185±0.045	-48.556	0.153
Betel use (year)	-0.374±0.194	-1.928	0.304	1.089±0.051	21.353	0.033
Alcohol	2.148±15.426	0.139	0.051	16.436±5.503	2.987	0.010
Alcohol: Current user	-3.851±8.301	-0.464	0.650	-4.889±2.961	-1.651	0.121
Branded alcohol	-18.276±13.402	-1.364	0.194	-10.796±4.781	-2.258	0.040
Local alcohol	-9.259±5.704	-1.623	0.351	-3.270±1.511	-2.164	0.276
Branded + Local alcohol	-4.402±3.041	-1.448	0.385	1.892±0.806	2.347	0.256
Alcohol: Daily	4.927±3.929	1.254	0.049	0.690±1.041	0.663	0.028
Alcohol: Not daily	-6.472±6.421	-1.008	0.331	-3.129±2.290	-1.366	0.193
Alcohol: age at start	-0.372±0.664	-0.560	0.584	-0.689±0.237	-2.907	0.011
Alcohol (year)	-0.464±0.298	-1.557	0.134	-0.008±0.154	-0.052	0.959
Smoked vegetables	-1.018±1.241	-0.820	0.563	-2.820±0.329	-8.571	0.074
Pickled foods	0.338±1.349	0.251	0.844	-1.583±0.357	-4.434	0.141
Any smoked meat	-0.221±1.457	-0.152	0.510	2.563±0.415	6.176	0.612
Any salted meat	-8.149±5.162	-1.579	0.359	2.514±1.368	1.838	0.317
Any pickled meat	-0.643±0.967	-0.665	0.627	-0.274±0.256	-1.070	0.478
Any tinned foods	-2.367±1.240	-1.909	0.307	1.592±0.328	4.854	0.130
Any fermented foods	-0.044±0.564	-0.078	0.950	-0.880±0.149	-5.906	0.107
Sodium bicarbonate	-1.997±7.868	-0.254	0.803	1.721±2.018	0.852	0.803
Monosodium glutamate	6.850±6.398	1.071	0.037	-6.540±6.008	-1.089	0.302
Relative: Cancer	-2.226±4.114	-0.541	0.597	-1.645±2.514	-0.654	0.597

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05; CB=Chromatid break; CSB= Chromosome break.

Table 5.6: Linear regression showing the effects of socio-demographic variables, dietary habits and familial history of cancer on the frequency of AF and DC.

Variables	Chromosomal aberrations					
	AF (R^2: 0.990)			DC (R^2: 0.904)		
	β ±SEM	t	p	β ±SEM	t	p
Age	-0.046±0.055	-0.836	0.556	0.041±0.230	0.178	0.889
Sex	-1.125±0.298	-3.775	0.165	-0.634±1.261	-0.503	0.703
Residence	-1.987±0.500	-3.974	0.157	-0.693±2.113	-0.328	0.798
Betel	0.564±2.077	0.272	0.031	0.605±8.781	0.069	0.041
Betel: Current user	-1.617±0.550	-2.940	0.209	-1.251±2.323	-0.539	0.686
Betel + lime	-0.746±0.714	-1.045	0.486	-1.136±3.018	-0.376	0.771
Betel + tobacco	-2.065±2.052	-1.006	0.498	-0.616±8.674	-0.071	0.955
Betel frequency/day	-0.048±0.075	-0.640	0.633	0.013±0.315	0.041	0.973
Betel use (year)	0.132±0.084	1.571	0.042	-0.075±0.357	-0.210	0.867
Alcohol	-2.148±15.426	-0.139	0.041	2.148±15.426	0.139	0.041
Alcohol: Current user	-3.851±8.301	-0.464	0.650	-3.851±8.301	-0.464	0.650
Branded alcohol	-18.276±13.402	-1.364	0.194	-18.276±13.402	-1.364	0.194
Local alcohol	1.376±2.485	0.554	0.904	-1.418±10.505	-0.135	0.915
Branded + Local alcohol	2.101±1.325	1.586	0.952	0.862±5.601	0.154	0.903
Alcohol: Daily	-1.188±1.712	-0.694	0.614	0.884±7.236	0.122	0.023
Alcohol: Not daily	-6.472±6.421	-1.008	0.331	-6.472±6.421	-1.008	0.331
Alcohol: age at start	-0.372±0.664	-0.560	0.584	-0.372±0.664	-0.560	0.584
Alcohol (year)	-0.464±0.298	-1.557	0.134	-0.464±0.298	-1.557	0.134
Smoked vegetables	-1.494±0.540	-2.767	0.528	-0.119±2.285	-0.052	0.967
Pickled foods	-0.227±0.588	-0.386	0.766	0.710±2.485	0.286	0.823
Any smoked meat	-1.325±0.286	-4.633	0.366	-0.985±1.354	-0.727	0.842
Any salted meat	-1.306±2.249	-0.581	0.665	-0.088±9.507	-0.009	0.994
Any pickled meat	-1.258±0.421	-2.988	0.206	-0.046±1.782	-0.026	0.983
Any tinned foods	-0.537±0.540	-0.994	0.502	-0.168±2.284	-0.074	0.953
Any fermented foods	-2.176±0.246	-8.846	0.604	0.646±1.039	0.622	0.646
Sodium bicarbonate	1.597±5.821	0.274	0.803	-1.457±3.724	-0.391	0.803
Monosodium glutamate	3.450±3.138	1.099	0.302	2.984±4.375	0.682	0.302
Relative: Cancer	5.522±4.004	1.379	0.597	5.421±4.284	1.265	0.0416

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05; AF= Acentric fragment; DC= Dicentric chromosome.

Table 5.7: Linear regression showing the effects of socio-demographic variables, dietary habits and familial history of cancer on the frequency of AC and TA.

Variables	Chromosomal aberrations					
	AC (R^2: 0.956)			TA (R^2: 0.955)		
	β ±SEM	t	p	β ±SEM	t	p
Age	0.304±0.369	0.824	0.011	0.330±0.443	0.745	0.002
Sex	-2.515±2.018	-1.246	0.430	-3.165±2.424	-1.306	0.416
Residence	-1.996±3.383	-0.590	0.661	-2.894±4.064	-0.712	0.606
Betel	-3.218±14.055	-0.229	0.857	-4.671±16.886	-0.277	0.828
Betel: Current user	-1.234±3.718	-0.332	0.796	-2.360±4.467	-0.528	0.691
Betel + lime	-2.062±4.831	-0.427	0.743	-1.828±5.804	-0.315	0.806
Betel + tobacco	3.757±13.884	0.271	0.832	3.503±16.680	0.210	0.018
Betel frequency/day	0.187±0.504	0.371	0.774	-0.168±0.606	-0.277	0.827
Betel use (year)	-0.238±0.571	-0.417	0.748	0.228±0.686	0.332	0.796
Alcohol	-2.148±15.426	-0.139	0.041	2.148±15.426	0.139	0.141
Alcohol: Current user	-3.851±8.301	-0.464	0.650	-3.851±8.301	-0.464	0.650
Branded alcohol	-18.276±13.402	-1.364	0.194	-18.276±13.402	-1.364	0.194
Local alcohol	5.844±16.815	0.348	0.787	-7.783±20.201	-0.385	0.766
Branded + Local alcohol	-0.539±8.966	-0.060	0.962	-1.546±10.772	-0.144	0.909
Alcohol: Daily	1.688±11.582	0.146	0.908	3.543±13.915	0.255	0.041
Alcohol: Not daily	-6.472±6.421	-1.008	0.331	-6.472±6.421	-1.008	0.331
Alcohol: age at start	-0.372±0.664	-0.560	0.584	-0.372±0.664	-0.560	0.584
Alcohol (year)	-0.464±0.298	-1.557	0.134	-0.464±0.298	-1.557	0.134
Smoked vegetables	-1.597±3.657	-0.437	0.738	-2.178±4.393	-0.496	0.707
Pickled foods	-0.739±3.977	-0.186	0.883	-0.762±4.778	-0.159	0.899
Any smoked meat	-0.452±2.854	-0.158	0.066	0.582±3.574	0.163	0.024
Any salted meat	-5.416±15.217	-0.356	0.782	-7.027±18.282	-0.384	0.766
Any pickled meat	-0.085±2.852	-0.030	0.981	-0.387±3.426	-0.113	0.928
Any tinned foods	-0.186±3.655	-0.051	0.968	-0.069±4.391	-0.016	0.990
Any fermented foods	-0.413±1.663	-0.248	0.845	-0.102±1.998	-0.051	0.968
Sodium bicarbonate	-1.005±7.328	-0.137	0.803	4.547±9.994	0.454	0.033
Monosodium glutamate	-5.350±5.128	-1.043	0.302	3.540±4.008	0.883	0.012
Relative: Cancer	-5.241±2.954	-1.774	0.597	4.122±2.352	1.753	0.597

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05; AC= Aberrant cells; TA= Total aberrations.

Table 5.8: Linear regression showing the effects of different tobacco products on the frequency of CB and CSB among the tobacco group.

Variables	Chromosomal aberrations					
	CB (R^2: 0.801)			CSB (R^2: 0.870)		
	β ±SEM	t	p	β ±SEM	t	p
Sahdah	0.267±2.095	0.127	0.905	3.251±1.190	2.732	0.041
Sahdah: Current user	-1.697±1.890	-0.898	0.420	-4.309±1.112	-3.875	0.012
Sahdah use (Year)	0.049±0.052	0.942	0.406	0.086±0.031	2.774	0.038
Tuibur	1.343±3.010	0.446	0.049	4.337±1.737	2.497	0.055
Tuibur: Current user	-0.044±1.611	-0.027	0.979	-2.316±0.949	-2.440	0.059
Tuibur use (Year)	-0.176±0.273	-0.645	0.554	-0.294±0.158	-1.861	0.122
Smoking	6.711±4.016	1.671	0.060	6.711±4.016	1.671	0.026
Smoking: Current	-1.873±1.201	-1.560	0.194	-2.813±0.701	-4.013	0.010
Zozial	3.954±3.372	1.173	0.306	4.188±1.912	2.190	0.080
Cigarette	-10.247±5.060	-2.025	0.113	4.391±2.847	1.542	0.184
Zozial + cigarette	-2.689±2.691	-0.999	0.374	5.026±1.503	3.344	0.020
Smoke/day	0.142±0.108	1.315	0.258	-0.197±0.063	-3.127	0.027
Smoking (Year)	0.092±0.047	1.957	.0120	-0.027±0.027	-1.000	0.355

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05; CB=Chromatid break; CSB= Chromosome break.

Table 5.9: Linear regression showing the effects of different tobacco products on the frequency of AF and DC among the tobacco group.

Variables	Chromosomal aberrations					
	AF (R^2: 0.893)			DC (R^2: 0.830)		
	β ±SEM	t	p	β ±SEM	t	p
Sahdah	2.081±0.749	2.778	0.039	-1.968±1.386	-1.420	0.215
Sahdah: Current user	-1.192±0.700	-1.703	0.149	-2.284±1.295	-1.764	0.138
Sahdah use (Year)	-0.011±0.019	-0.579	0.581	-0.014±0.036	-0.389	0.710
Tuibur	-0.698±1.093	-0.639	0.551	0.745±2.023	0.368	0.728
Tuibur: Current user	-0.638±0.597	-1.069	0.334	-0.633±1.105	-0.573	0.592
Tuibur use (Year)	0.097±0.099	0.980	0.047	0.122±0.184	0.663	0.538
Smoking	6.711±4.016	1.671	0.026	6.711±4.016	1.671	0.066
Smoking: Current	-1.035±0.441	-2.347	0.066	-2.195±0.817	-2.687	0.043
Zozial	1.584±1.203	1.317	0.245	0.785±2.227	0.352	0.739
Cigarette	-2.115±1.791	-1.181	0.291	-4.178±3.316	-1.260	0.263
Zozial + cigarette	1.411±0.946	1.492	0.196	2.861±1.750	1.635	0.163
Smoke/day	0.016±0.040	0.400	0.045	-0.056±0.074	-0.757	0.482
Smoking (Year)	0.004±0.017	0.235	0.806	-0.036±0.031	-1.161	0.299

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05; AF= Acentric fragment; DC= Dicentric chromosome.

Table 5.10: Linear regression showing the effects of different tobacco products on the frequency of AC and TA among the tobacco group.

Variables	Chromosomal aberrations					
	AC (R^2: 0.893)			TA (R^2: 0.865)		
	β±SEM	t	p	β ±SEM	t	p
Sahdah	7.614±2.551	2.985	0.031	8.348±3.290	2.537	0.052
Sahdah: Current user	-8.330±2.385	-3.493	0.017	-9.580±3.076	-3.114	0.026
Sahdah use (Year)	0.098±0.066	1.485	0.197	0.113±0.085	1.329	0.239
Tuibur	2.713±3.725	0.728	0.499	3.887±4.804	0.809	0.041
Tuibur: Current user	-2.777±2.034	-1.365	0.230	-3.613±2.623	-1.377	0.227
Tuibur use (Year)	0.032±0.339	0.094	0.929	0.031±0.437	0.071	0.946
Smoking	6.711±4.016	1.671	0.026	6.711±4.016	1.671	0.026
Smoking: Current	-7.430±1.504	-4.940	0.004	-8.139±1.940	-4.195	0.009
Zozial	3.885±4.100	0.948	0.387	3.888±5.287	0.735	0.495
Cigarette	-7.889±6.105	-1.292	0.253	-10.050±7.873	-1.277	0.258
Zozial + cigarette	6.983±3.223	2.167	0.082	7.809±4.156	1.879	0.119
Smoke/day	-0.090±0.136	-0.662	0.536	-0.126±0.175	-0.720	0.503
Smoking (Year)	0.058±0.057	1.018	0.354	0.085±0.074	1.149	0.029

β= Beta coefficient; SEM= Standard error of mean; t= .Coefficient/Standard error of mean; p= Significant level at 0.05; AC= Aberrant cells; TA= Total aberrations.

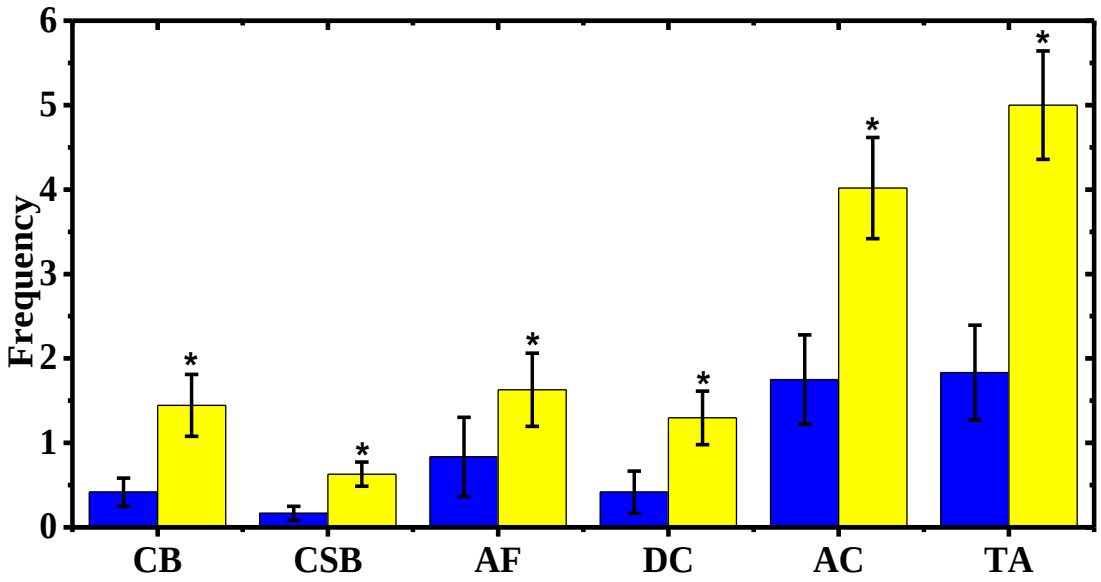


Figure 5.1: Comparison of frequency of different chromosomal aberrations between control and tobacco groups.

*Blue bar= Control group; Yellow bar= Tobacco group; CB= Chromatid break; CSB= Chromosome break; AF= Acentric fragment; DC= Dicentric chromosome; AC= Aberrant cells; TA= Total aberrations; *Significant difference at 0.05 level of significance; No symbol= Not significant.*

PHOTOPLATES

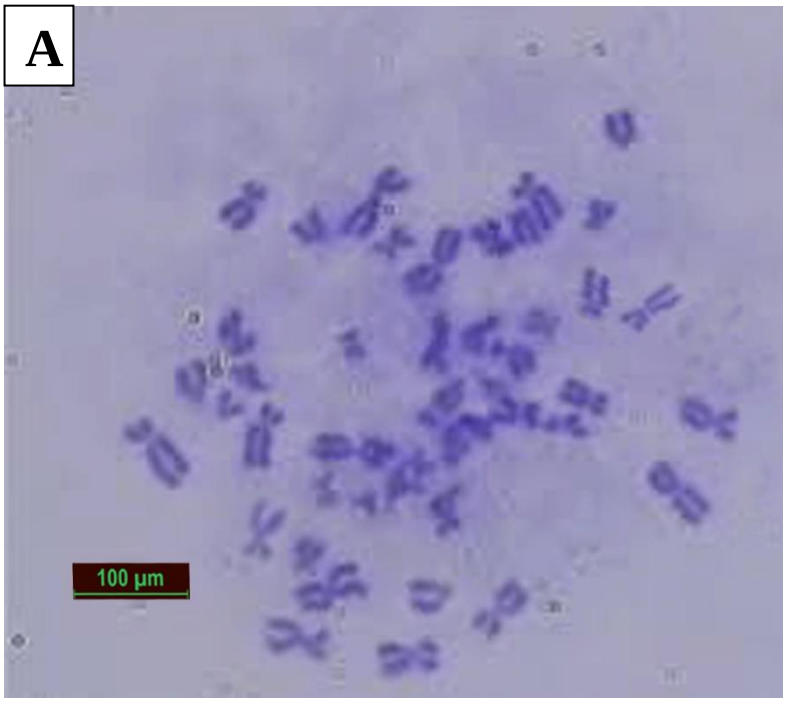
Plate A: Normal metaphase plate.

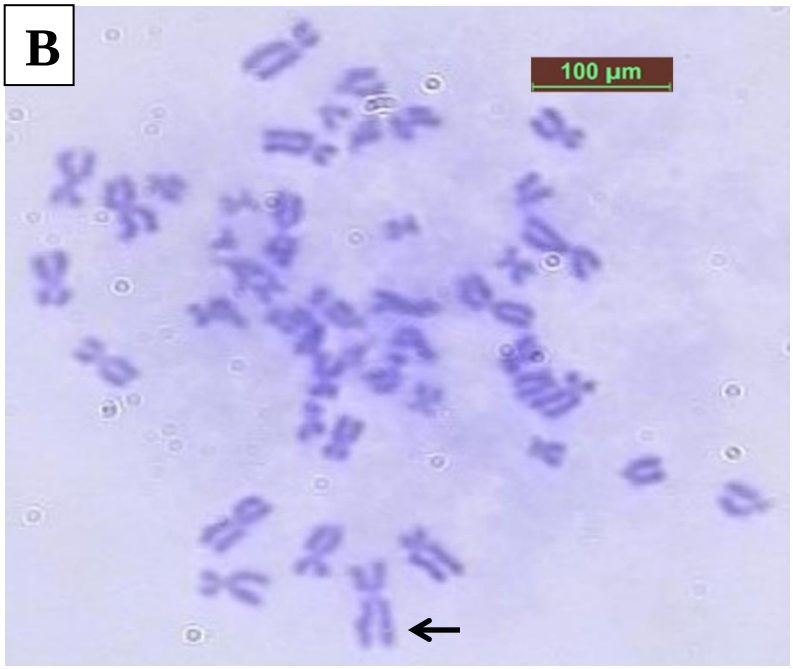
Plate B: Chromatid break.

Plate C: Chromosome break.

Plate D: Acentric fragment.

Plate E: Dicentric chromosome.

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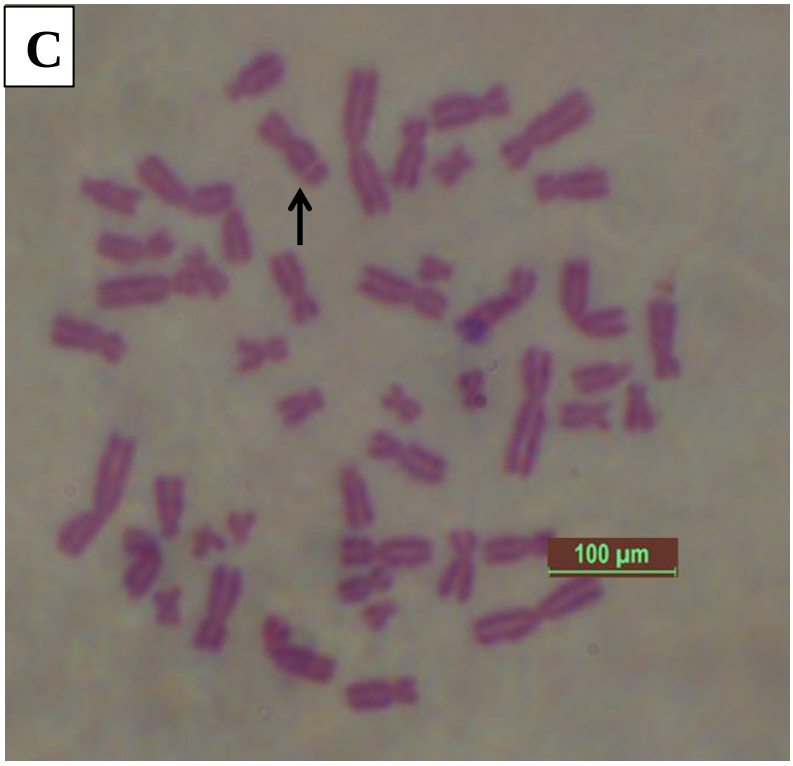
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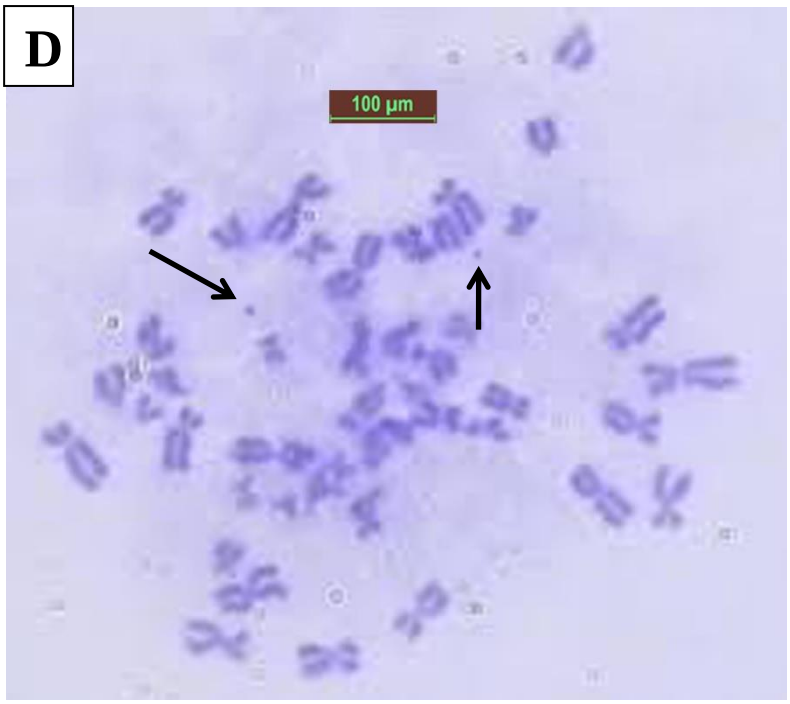
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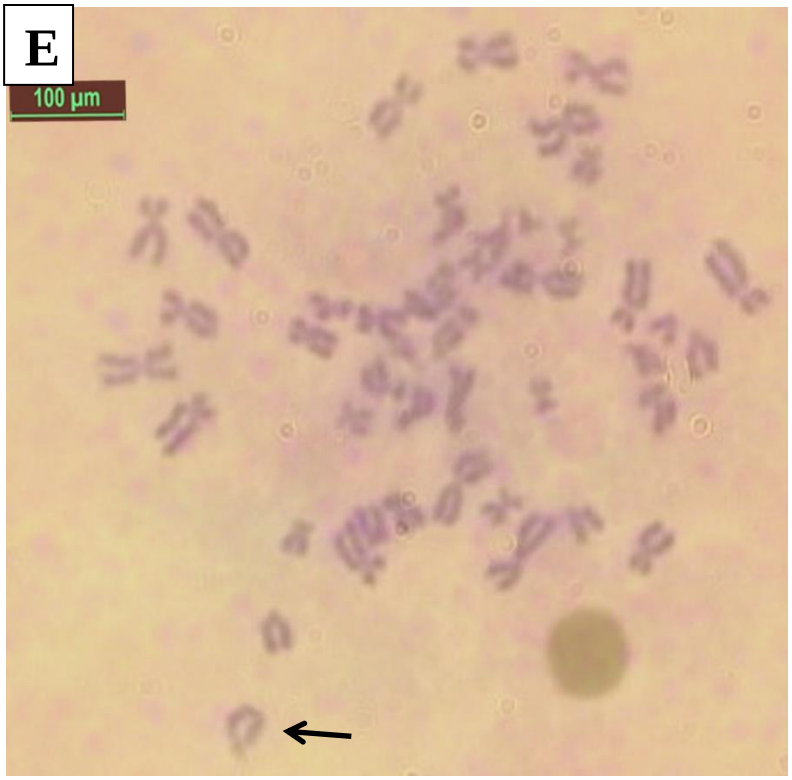
E
100 µm

CHAPTER-6

TUIBUR TREATMENT ABATES THE VIABILITY OF CULTURED HUMAN PERIPHERAL BLOOD LYMPHOCYTES

Abstract

The consumption of tobacco and tobacco products is indicated in many ailments including cancer. Tuibur (brewed tobacco), a form of smokeless tobacco is in frequent use in Northeast India and Mizoram is no exception, where tuibur use is highly prevalent. Therefore the impact of two grades of tuibur was studied on the survival of human peripheral blood lymphocytes cultured *in vitro* along with nicotine. Separate treatment of human peripheral blood lymphocytes for 24 h with two grades of commercial tuibur and nicotine showed a concentration dependent decrease in cell viability studied by trypan blue dye exclusion assay. MTT assay also revealed a concentration dependent increase in cell death. Our results indicate that constant consumption of tuibur has a deleterious effect on cultured human peripheral blood lymphocytes.

INTRODUCTION

Tobacco is known to contain more than eight thousand chemicals, out of which roughly more than eighty are probable carcinogens (IARC, 2004; Arimilli *et al.*, 2012; Ding *et al.*, 2008; Cooper, 2006). Some of the common toxic chemicals include benzo[a]pyrene (B[a]P), N'-nitrosonornicotine (NNN), N'-nitrosoanatabine (NAT), N'-nitrosoanabasine (NAB), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-Nitrosodimethylamine (NDMA), nitrite, cadmium, lead, arsenic, nickel, chromium, etc. (Stepanov and Hecht, 2006; Borgerding *et al.*, 2012). The consumption of both smoked and smokeless tobacco is popular throughout the world and its detrimental effect could be observed from many medical records. Besides its deleterious consequence upon the pulmonary system, it has been linked with many

forms of cancer. In fact, many studies suggested that almost all known cancer could be linked to tobacco use (Musk and De Klerk, 2003; Elmasry *et al.*, 2015).

The form of tobacco used may vary considerably in different places and according to the choice of the user. Some individuals prefer smoking tobacco while others are inclined to use smokeless tobacco, or both. However, there is no denying the fact that more than half of the tobacco users prefer smoking tobacco (WHO global report, 2015). Tobacco smokers and those who use smokeless tobacco are exposed to thousands of chemicals present in the tobacco as well as tobacco smoke. The smoking or combustion of tobacco generates additional chemicals which are carcinogens and therefore smokers as well as passive smokers are also at the risk of developing cancer (Johnson *et al.*, 2009; Baker *et al.*, 2004).

From times immemorial, the Mizos have been using smokeless tobacco locally called tuibur (tobacco brew). It is used popularly throughout the state and is commercially available in the local market. Although there is no standard parameter for tuibur quality, it is usually categorized into two grades. The grading is mostly dependent on the amount of tobacco used in tuibur production. Tuibur is usually kept in the mouth by the individuals for roughly 5-10 minutes and then spitted out. The duration to keep tuibur in mouth depends on its alkalinity and it is spitted out when it is no longer alkaline (Lalpawimawha *et al.*, 2015; Lalmuanpuii and Muthukumaran, 2016). The frequent use of tuibur in Mizoram stimulated us to investigate its effect on the survival of human peripheral blood lymphocytes *in vitro*.

MATERIALS AND METHODS

Chemicals

Two grades of locally produced tuibur-A (special grade) and tuibur-B (ordinary grade), were procured commercially form Aizawl market. Pure nicotine was purchased from Cayman Chemical Company, whereas MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], sodium dodecyl sulphate, RPMI-1640 media and trypan blue were purchased from Sigma Aldrich Chemical Co., Kolkata, India. Hydrochloric acid and isobutanol were obtained from SD Fine Chemicals, Mumbai, India.

Human peripheral blood lymphocyte culture and treatment

Human peripheral blood lymphocyte (HPBLs) culture was performed using standard protocol (Jagetia *et al.,* 2001). Briefly, peripheral blood was collected by venipuncture in a heparinized vacutainer from a 27 year old healthy male volunteer, who had no known history of tobacco consumption. The blood was allowed to stand for half an hour and the buffy coat containing lymphocytes was separated and used for culture.

Experimental

Although there is no standard protocol to prepare the tuibur, the manufacturers graded the *tuibur* depending on the quantity of tobacco used in its production, henceforth it will be referred as tuibur-A (prepared using more tobacco) and B (prepared using lesser tobacco than A).

The viability and cytotoxicity of different treatments were tested by dividing the lymphocyte culture in to the following groups:

Tuibur-A group: The cultures of this group were immediately treated with different doses of tuibur-A.

Tuibur-B group: This group of cultures was immediately treated with different doses of tuibur-B.

Nicotine group: The cultures of this group were immediately exposed to different doses of nicotine.

Cell viability and cytotoxicity were carried out as described below.

Trypan blue dye exclusion assay

An experiment was conducted to determine the viability of lymphocytes where grouping and other conditions were described above. The cultures were treated with 0, 2.5, 5, 10, 20, 30, 40, and 50 µl/ml of tuibur-A or B or 0, 2.5, 10, 20, 30, 40, and 50 µg/ml of nicotine for 24 h. The cultures were terminated at the end of 24 h at 37 °C and the viability of cells was tested by aspirating 20 µl of lymphocyte suspension, which was mixed with 20 µl of 0.08% trypan blue dye and allowed to stand for 10 minutes. A fixed amount of this mixture was transferred to hemocytometer and the cells were counted using transmitted light microscope (Leica, Wetzlar, Germany). The viable cells did not take up the stain whereas dead cells stained blue. Usually triplicate cultures were setup for each concentration for each group and the results were confirmed by repetition of the experiment. The percentage of viable cells was calculated as follows:

Cell viability (%) = Live cells counted X100/Total cells counted.

MTT assay

In a separate experiment, the cytotoxicity of the treatment was evaluated, where grouping and other conditions were exactly similar to that described above except that the 5000 HPBLs were seeded into several wells of a 96 well microplates and tuibur-A and B groups were exposed to 0, 10, 20, 40, 60, 80, and 100 µl/ml

tuibur-A and B, respectively whereas nicotine group was treated with 0, 10, 20, 40, 60, 80, and 100 µg/ml of nicotine. The MTT assay was performed according to standard protocol (Mosmann, 1983). The HPBLs were immediately exposed to tuibur-A or B or nicotine as the case may be. The cultures were incubated at 37°C for 48 h in a CO_2 incubator in an atmosphere of 5% CO_2 in air and 95% humidity. After 48 h of incubation, 20 µl of MTT was added to each well and the cells were further incubated for 2-4 h. After the formation of formazan crystal, 100 µl of MTT lysis buffer was added to each well to dissolve the crystals. The cultures were further incubated overnight and the OD was taken at 570 nm in a microplate spectrophotometer (SpectraMax M2). The survival of the cells has been expressed as percentage. Usually four wells were used for each concentration in each group and the experiment was repeated for confirmation.

The cytotoxicity was calculated by the formula: Treatment/ControlX100.

Statistical analyses

All statistical analyses were performed using OriginPro-8 (OriginLab Corporation, Northampton, USA) and Microsoft excel 2013. Student's t-test was employed to determine significant difference among the treatment groups. Correlation coefficient was performed to determine relationship between different treatment concentrations and viability within a group. The test of homogeneity was applied between the repeated experiment and no statistical difference was reported between the two experiments.

RESULTS

The results are shown in table and figures as mean±standard error of the mean (SEM).

Trypan blue dye exclusion assay

The pH of tuibur-A and tuibur-B was determined and it was found to be 9.81 and 10.09, respectively. The HPBLs of control group (0 µg/ml) showed 100% viability in the trypan blue dye exclusion assay. The HPBLs viability declined in a concentration dependent manner in all the three i.e. tuibur-A and B and nicotine groups as indicated by the trypan blue dye exclusion assay. The cell viability reached a nadir at the highest concentration of 50 µl for tuibur-A and B and 50 µg/ml for nicotine treated group (Figure 6.1), where the viability of HPBLs reduced by 34.43%, 23.86% and 17.29% for tuibur-A, B and nicotine respectively (Table 6.1). The statistical analysis indicated that HPBLs viability declined significantly in all groups in comparison to control (0 dose). However, comparison of tuibur-A and tuibur-B, tuibur-A and nicotine, tuibur-B and nicotine showed no significant difference in viable cells (Table 6.2). A strong negative correlation was observed between cell viability and concentration in all the treatment groups (Table 6.3).

MTT assay

The evaluation of cytotoxic effects by MTT revealed that the cytotoxic effect of tuibur-A and B and nicotine increased in a concentration dependent manner and the maximum cytotoxic effect was observed at 100 µl/ml tuibur-A and tuibur-B or µg/ml nicotine treatment (Figure 6.2). The cytotoxic effect was approximately 75.03%, 74.72% and 53.61% in HPBLs treated with a maximum concentration of 100 µl/ml of tuibur-A or tuibur-B or 100 µg/ml nicotine, respectively (Table 6.4). The treatment of HPBLs with tuibur-A or tuibur-B or nicotine resulted in a significant rise in the cytotoxicity when compared to control (0 dose), whereas comparison among these groups did not show any significant difference despite the

fact that tuibur-A was most cytotoxic (Table 6.2). Correlation coefficient showed a strong negative correlation between cell viability and concentration in all the MTT assay treatment groups (Table 6.3) indicating that cytotoxic effect was concentration dependent.

DISCUSSION

Tobacco has been used by humans since a long time however its adverse effects came to light in the last century. Tobacco is known to contain numerous chemicals and many of which have been reported to induce cancer and other diseases in the tobacco consumers (Stepanov *et al.*, 2005; Perfetti et al., 2011; Arimilli *et al.*, 2012). Most studies, if not all, reported the use of tobacco in any form only has negative health impact on the users. There has been only a handful of literature on the scientific investigation of tuibur. Therefore, the present study was designed to study the viability and cytotoxic effect of different grades of tuibur in cultured human peripheral blood lymphocytes.

A preliminary report on the chemical composition of tuibur showed the presence of polyaromatic hydrocarbons and carbonyl compounds in the tar phase (Lalmuanpuii and Muthukumaran, 2016). An epidemiological study among the Mizos showed that tuibur users are at a higher risk of developing gastric cancer. The combined use of tuibur along with smoking, betel (paan), and sahdah have been reported to increase the risk of gastric cancer (Phukan *et al.*, 2005; Lalpawimawha *et al.*, 2015). Besides gastric cancer patients in Mizoram, tuibur consumers were found to have a variety of mtDNA D-loop region mutations and polymorphisms (Lalmuanpuii *et al.*, 2015). Individuals with Arg/Pro genotype, GSTM1 null genotype and GSTT1 non-null genotype were also suggested to have a higher risk of

gastric cancer if they have habits of using tuibur and smoking tobacco (Malakar *et al.*, 2012; Malakar *et al.*, 2014). The present study indicates that tuibur treatment reduced the viability of HPBLs and the cytotoxicity of tuibur increased in a concentration dependent manner and it was found that tuibur was more toxic than nicotine treatment alone. Further tuibur-A was more toxic than tuibur-B. Similarly, cytological studies have reported that nicotine inhibited cell proliferation and decreased protein synthesis in a dose dependent manner in cultured periodontal ligament fibroblast (Chang *et al.*, 2002). Nicotine has been reported to stimulate endothelial cell DNA synthesis and proliferation at concentrations lower than $<10^{-8}$ M and it was cytotoxic at a concentration $>10^{-6}$ M (Villablanca, 1998). Cigarette smoke extract has been reported to induce cytotoxicity in orbital fibroblasts (Kau *et al*., 2016). Our study indicates that tuibur has the ability to induce cellular death and that it may lead to increased risk of cancer development among the frequent consumers of tobacco in any form. Onion bulbs treated with tuibur showed reduced root growth, reduced mitotic index, and formation of micronuclei, lagging chromosomes, and c-mitosis (Ra, 2012). A study on seven smokeless tobacco aqueous extracts showed concentration-dependent inhibition on the growth and viability of oral bacteria cultured under anaerobic conditions (Liu *et al.*, 2016).

The exact mechanism of action of tuibur to reduce cell viability and increase the cytotoxicity in HPBLs is not known. It may have used multiple pathways to exert its effect. Since one of the major components of tobacco is nicotine, the effects observed in the present study may be correlated to the effects of nicotine. Tobacco contains NNN, and NNK apart from nicotine that have been found to have cytotoxicity (Moghbel *et al.*, 2016). Nicotine has been reported to reduce antioxidant

enzymes including superoxide dismutase, catalase, glutathione-s-transferase, and glutathione reductase, and increase lipid peroxidation (Cooper, 2006). Therefore increased oxidative stress may be another reason for the cytotoxic effect in the present study. Cigarette smoke extract has been reported to induce reactive oxygen species (ROS) in cultured fibroblast (Kau *et al.*, 2016). Chewing tobacco has been reported to induce, lipid peroxidation, DNA fragmentation and DNA ladders (Bagchi *et al.*, 2012), which may have contributed to death of HPBLs. The induction of DNA damage by tuibur seems to be one of the important mechanisms of HPBLs death. Nicotine has been reported to induce DNA damage in human tonsillar tissue, lymphocytes and respiratory tract cells (Kleinsasser et al., 2005; Ginzkey et al., 2012).

CONCLUSIONS

Our study demonstrates that tuibur attenuated cell viability and increased cytotoxic effect in a concentration dependent manner in the HPBLs. Tuibur-A was found to be more toxic than tuibur-B and nicotine. The effect of tuibur may be mediated by increased ROS production and lipid peroxidation, and reduction in the antioxidant enzymes. The cytotoxicity may also be due to rise in DNA damage by tuibur. The change in pH by tuibur may have also increased the cytoxic effect in HPBLs. Therefore, the consumption of tuibur might have potential side effects on the health of its users and increased frequency of cancer in Mizoram may be due to the use of tobacco products.

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Table 6.1: Viability of human peripheral blood lymphocytes treated with various concentration of tuibur and nicotine estimated by trypan blue dye exclusion assay.

Concentration (µl/ml or µg/ml)	Percentage of viable cells (Mean ±SEM)		
	Tuibur-A	Tuibur-B	Nicotine
0	100.00±0.00	100.00±0.00	100.00±0.00
2.5	100.00±0.00	100.00±0.00	98.25±0.06*
5	96.39±0.58*	95.06±0.40*	96.55±0.25*
10	90.34±1.86*	90.35±0.97*	92.32±0.59*
20	86.41±0.62*	87.03±0.29*	91.1±0.23*
30	79.43±2.22*	85.65±1.20*	87.9±0.50*
40	71.07±1.97*	79.15±0.58*	85.57±0.14*
50	65.57±0.62*	76.14±1.11*	82.71±2.31*

*Standard error of mean (SEM); *Significant at 0.05 level of significance; No symbol= Not significant; N: 3.*

Table 6.2: Cytotoxicity test showing viable human peripheral blood lymphocytes treated with different concentration of tuibur-A, tuibur-B and nicotine by MTT assay.

Concentration (µl/ml or µg/ml)	Percentage of viable cells (Mean ±SEM)		
	Tuibur-A	Tuibur-B	Nicotine
0	100.00±0.00	100.00±0.00	100.00±0.00
10	94.55±1.93*	92.28±2.51*	89.63±3.26*
20	76.38±4.38*	83.38±1.46*	79.51±2.68*
40	52.29±3.69*	77.76±1.38*	69.98±6.68*
60	46.69±2.75*	59.09±0.93*	54.61±2.28*
80	34.37±1.35*	41.21±0.80*	50.44±1.51*
100	24.97±1.35*	25.27±1.82*	46.39±0.95*

*Standard error of mean (SEM); *Significant at 0.05 level of significance; N: 4.*

Table 6.3: Student's t-test between mean percent viability of different treatment groups at 95% confidence interval.

Student's t-test between	P-value at 95% CI	
	Trypan blue exclusion assay	MTT assay
Tuibur-A & Tuibur-B	=0.60	=0.59
Tuibur-A & Nicotine	=0.27	=0.44
Tuibur-B & Nicotine	=0.45	=0.88
Control & Tuibur-A	≤0.00*	≤0.00*
Control & Tuibur-B	≤0.00*	≤0.00*
Control & Nicotine	≤0.00*	≤0.00*

**Significant difference at 0.05 significant level; No symbol= Not significant; ≤ Approximate value; = Actual value.*

Table 6.4: Correlation between cell viability and different treatment concentrations of treatment groups.

Treatment groups	Pearson's Correlation	
	Trypan blue exclusion assay	MTT assay
Tuibur-A	-.099*	-0.96*
Tuibur-B	-0.96*	-0.99*
Nicotine	-0.97*	-0.97*

**Correlation is significant at 0.05 level (2-tailed).*

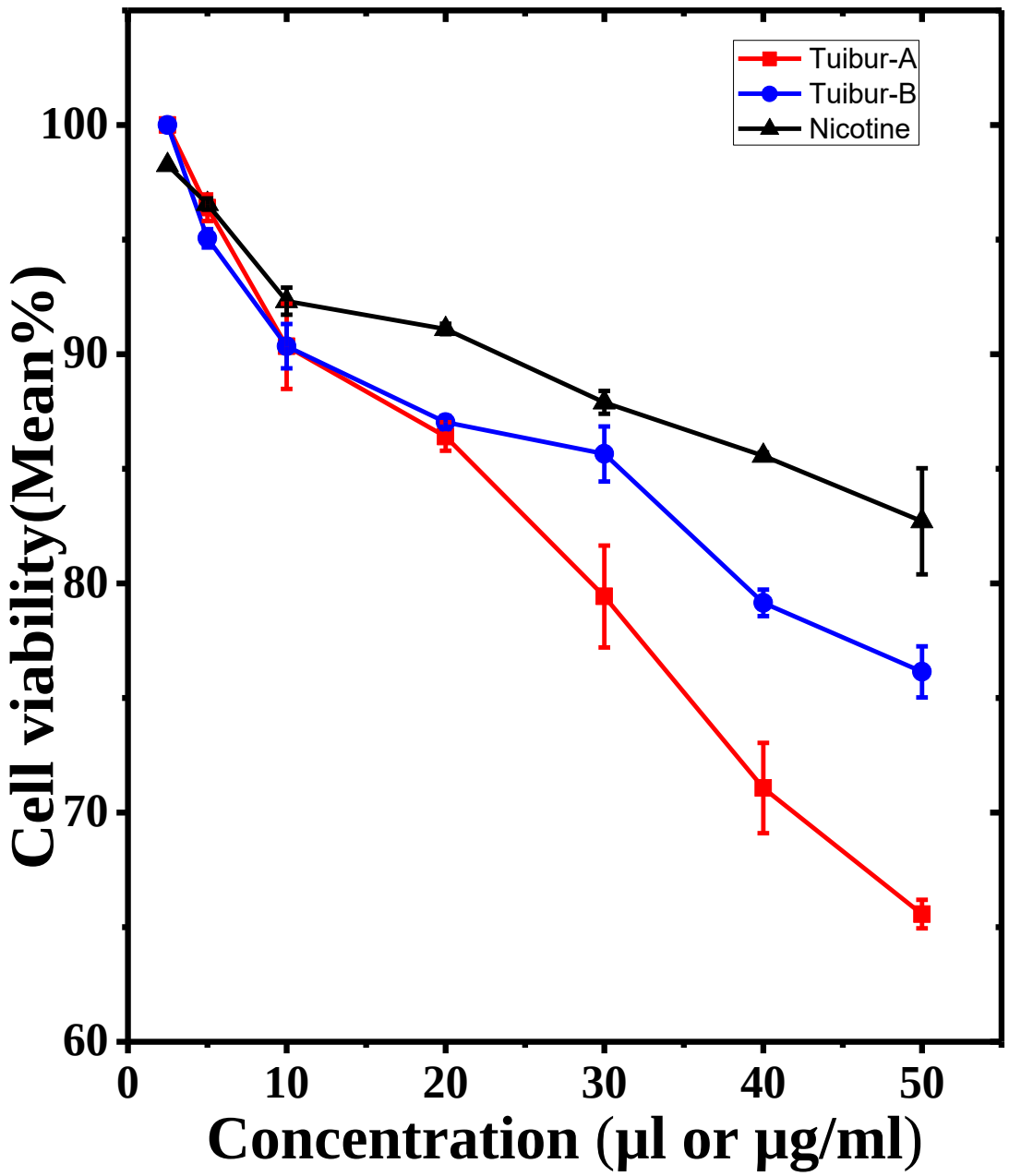

Figure 6.1: Effect of different concentrations of tuibur on the viablity of human peripheral blood lymphocyte by trypan blue exclusion assay.

Squares= Tuibur-A; Circles= Tuibur-B; Trangles= Nicotine.

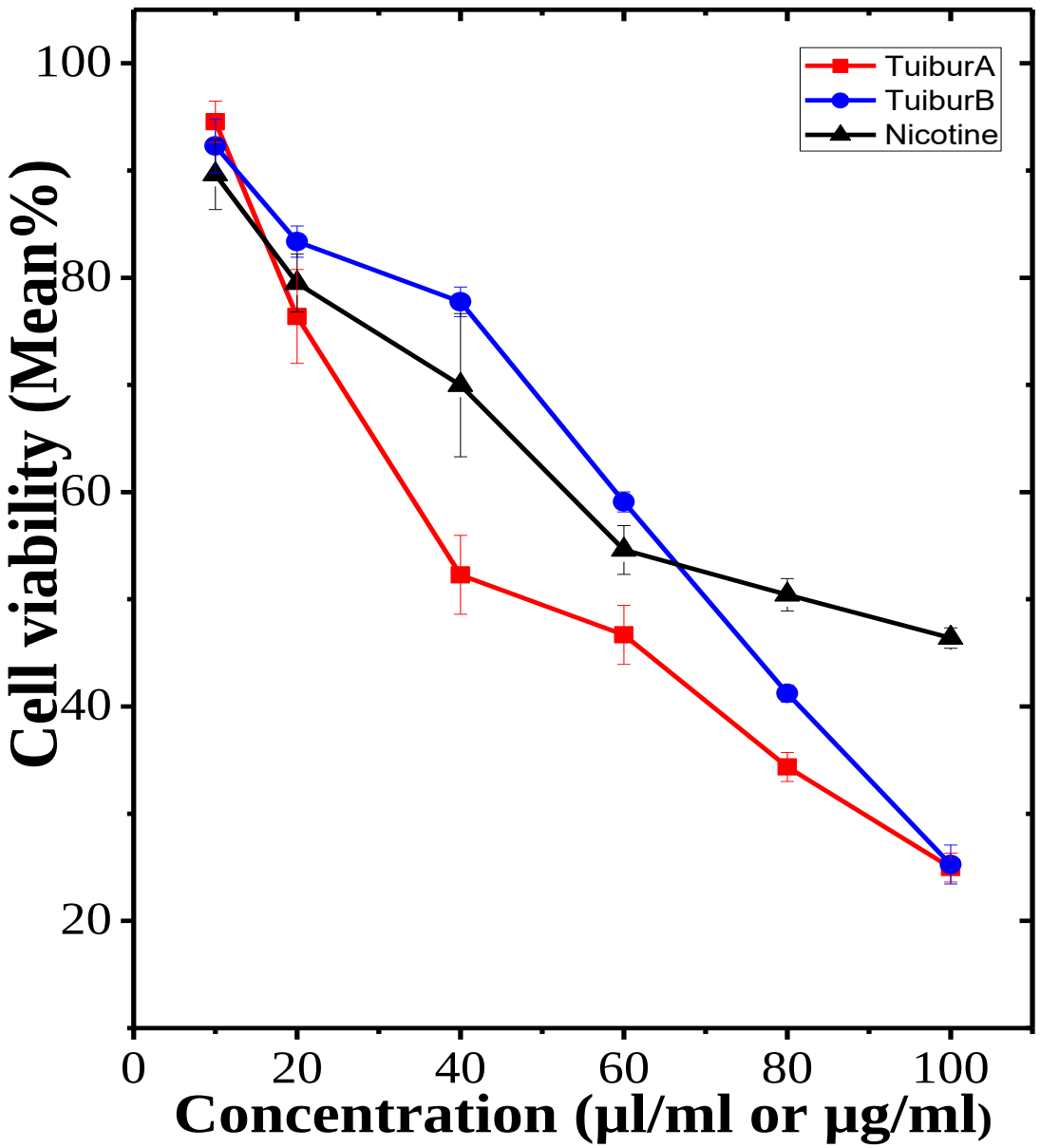

Figure 6.2: Effect of different concentrations of tuibur on the viability of human peripheral blood lymphocyte by MTT assay.

Squares= Tuibur-A; Circles= Tuibur-B; Trangles= Nicotine.

CHAPTER 7

SUMMARY AND CONCLUSIONS

INTRODUCTION

Tobacco is a crop plant product that is popularly used all over the world (Kishore, 2014). The deleterious effects of tobacco on health is well documented, however, its consumption has not been declining especially in underdeveloped and developing countries (WHO, 2015). This maybe the result of the addictive substances present in tobacco (Benowitz, 1998). Tobacco contains many different types of chemicals, some of which are known to exert detrimental health problems to its consumers (IARC, 2004; Arimilli *et al.*, 2012; Bassiony *et al.*, 2015). The molecular mechanism of action of the chemicals of tobacco is not fully understood, however, these chemicals are known to act on the genetic and epigenetic components of the cell resulting in the upregulation or downregulation of many genes (Jenuwein and Allis, 2001; Esteller, 2011). Besides this, different cellular components are also affected by free radicals of tobacco or free radicals produced by the metabolism of tobacco chemicals inside the cell (Ezzati and Lopez, 2003; Sepaniak *et al.*, 2006).

The Mizos inhabiting the state of Mizoram in the north eastern part of India are different in lifestyle and dietary habits when compared with other parts of India. The indigenous foods of the Mizos contain smoked and fermented meats and vegetables, and the use of sodium bicarbonate for the preparation of local dish called “Bai” (Phukan *et al.,* 2006; Lalthanpuii *et al.,* 2015). The use of tobacco and its related products is also fairly common among this population (Phukan *et al.,* 2005). These tobacco products includes smoked and smokeless tobaccos. The local smoked tobacco comprised of a local cigarette called “Zozial” and pipe. A form of locally manufactured smokeless

tobacco called “Tuibur” (tobacco brew) and “Sahdah” (shredded tobacco) are also prevalent among the Mizos (Lalruatfela *et al*., 2017). The state of Mizoram has one of the highest incidences of cancer in India (NCRP, 2010; Malakar *et al.,* 2014). Epidemiological studies in this population have shown that smoke and smokeless tobacco increased the risk of gastric and lung cancers among the Mizos (Phukan *et al.*, 2005; Lalpawimawha *et al*., 2015; Lalpawimawha and Lalruatfela, 2016).

AIM OF THE STUDY

As Mizoram has one of the highest record of cancer in the country despite its low population density, many researchers have recently focused on this field of study. However, data regarding this is still scarce as many studies are underway. In fact, only a handful of literatures on epidemiological and molecular studies are currently available. Therefore, the aim of the present study was to determine the effect of tobacco consumption on the level of DNA damage in tobacco users and non-users among the Mizo ethnic group.

CHAPTER 1

This chapter provides information on tobacco plant, a brief history of tobacco consumption, the global consumption status and types of tobacco used. It also highlights the chemical composition of tobacco and its detrimental effects on the health of its users. An overview of the effects of tobacco on the genetic and epigenetic components of the cell is also given along with the involvement of free radicals in increasing the level of oxidative stress. Information regarding the subjects of this investigation, the

Mizos, their dietary habits, tobacco consumptions and previous epidemiological data and the primary aim of the study are also mentioned in this chapter.

CHAPTER 2

Chapter 2 deals with the methods of subject selection and the design of consent form and structured questionnaire. Bilingual (English and Mizo language) consent and structured questionnaire consisting of lifestyle and dietary habits, family history of cancer, and other relevant information, approved by the Human Ethics Committee of the Mizoram University, Aizawl, India, were filled by the volunteers. The volunteers were divided into Control (CON) group which consisted of any male or female volunteers who had no known history of any form of tobacco consumption and tobacco (TOB) group comprising of habitual tobacco users of either sex. A total of 245 volunteers, 42 individuals from the CON group and 203 from the TOB group participated in this study. The CON group consisted of 23 males and 29 females. The TOB group included 129 males and 74 females.

CHAPTER 3

This chapter describes the effect of tobacco on the induction of DNA damage which is analyzed in the form of micronucleus from cultured human peripheral blood lymphocytes (HPBLs). For this assay, cytokinesis blocked micronucleus assay was employed which is an excellent tool to assess any DNA damage precisely. Lymphocyte by virtue of their longevity and nonproliferation are able to retain the signature of DNA damage over the years and express it in the form of micronuclei once they are stimulated to divide. Blood from tobacco users and non-users was collected from healthy Mizo

volunteers and cultured in the laboratory. The buffy coat of cells was inoculated into RMPI medium containing phytohemagglutinin and allowed to grow for 44 h. Cytochalasin-B was added to block cytokinesis and the micronuclei were study at 72 h post culture. The data analyses revealed that the frequency of micronuclei increased significantly in the individuals who were regularly consuming tobacco in any form when compared to those individuals who did not take any form of tobacco. It was found that the individuals who were using smokeless tobacco in the form of tuibur (tobacco brew) had higher frequency of micronuclei than the other forms of tobacco. Our study clearly indicates that use of tobacco in any form is a risk factor for DNA damage.

CHAPTER 4

In this chapter an account on the alteration in the antioxidant status in the tobacco users of Mizo ethnic population is given. The human body is subjected to many types of biological, chemical and physical factors that threaten the integrity of the genome. These factors often result in the production of highly reactive species called free radicals. Free radicals cause oxidative stress in the human body, which is often detrimental to the cells. The human body synthesizes antioxidants to defend against the free radical induced oxidative stress. The antioxidant molecules remove the unwanted free radicals from the body. Therefore, the increase or decrease in the amount and activities of enzymatic and non-enzymatic antioxidants is a reflection of increased or reduced oxidative stress. In the present study, the level glutathione, glutathione-s-transferase, catalase, superoxide dismutase and lipid peroxidation was estimated in the serum of Mizo tobacco users and controls. Tobacco consumption has resulted in a

significant depletion of glutathione concentration and the activities of catalase and superoxide dismutase accompanied by a rise in the activity of glutathione-s-transferase and lipid peroxidation significantly than the control group. The use of tobacco in any form was also found to greatly enhance the level of oxidative stress as indicated by the increased lipid peroxidation and reduction in the glutathione concentration and the activities of catalase and superoxide dismutase.

CHAPTER 5

This chapter analyzed the effect of the consumption of smoke and smokeless tobacco among the Mizo population on chromosome damage. Tobacco affects the genetic and epigenetic components of the cell resulting in the upregulation or downregulation of important genes. It has also been known to cause DNA adducts eventually leading to the formation of nicks in the DNA, which further cause structural damage to the chromosomes. The Mizos residing in the north eastern part of India are prone to cancer of different sites. Many of them have been known to consume several varieties of tobacco and its related products. The effect of tobacco consumption has been studied in cultured peripheral blood lymphocytes of Mizos who are frequently using tobacco in various forms. Blood was collected from tobacco users and non-users of healthy Mizo volunteers and erythrocytes were allowed to sediment against gravity. The buffy coat of cells was inoculated into RMPI medium containing phytohemagglutinin and allowed to grow for 44 h followed by the addition of colchicine to arrest the cell at metaphase. Metaphase plates were prepared at 56 h post initiation of

the cultures to study the chromosomal aberrations. The data analysis revealed that the frequency of chromosomal aberrations increased significantly in the volunteers who regularly consumed tobacco in any form when compared to those volunteers who did not take any form of tobacco. It was found that the individuals who were using smokeless tobacco in the form of tuibur (tobacco brew) and sahdah had higher frequency of chromosomal aberrations than the other forms of tobacco. Our study indicates that the use of tobacco in any form is a risk factor for chromosomal damage.

CHAPTER 6

The use of tuibur (tobacco brew) is popular in Mizoram and is usually available in two grades depending on the amount of tobacco used in its production. This chapter described the cytotoxic properties of commercially available tuibur on cultured HPBLs, where whole blood was collected from a 27 year old male Mizo volunteer who had no known history of tobacco consumption. The collected blood was allowed to sediment and the buffy coat was collected for culture. The cultures were separated into four groups, tuibur-A group, tuibur-B group, nicotine group and control group. For trypan blue dye exclusion assay, approximately one million cells were inoculated in different culture tubes while for MTT assay, approximately 5000 cells were inoculated into each wells of a 96 welled microtiter plate. Immediately after inoculating the lymphocytes into RPMI-1640 medium, each group was separately treated with different doses of tuibur-A, tuibur-B, and nicotine respectively. The control group, however, did not receive any treatment. The cells were kept in a CO_2 incubator maintained at 37°C for 24 h and trypan blue dye exclusion assay and MTT assay were performed. The data analyses

showed that the two grades of commercial tuibur and nicotine showed concentration dependent decrease in cell viability studied by trypan blue dye exclusion assay. The MTT assay also revealed a concentration dependent increase in cell death. Our results indicate that tuibur has a cytotoxic effect which may cause different types of cellular damages and may be harmful for the health of its consumers.

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In vitro effect of tuibur (tobacco brew) on the viability of human blood lymphocytes

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The use of tobacco and its products are known to cause many illnesses including cancer. A smokeless tobacco locally manufactured called *tuibur* (tobacco brew) has been consumed by the Mizos from a very long time. In this experiment we aim to determine the cytotoxicity of *tuibur* by an *in vitro* study on *tuibur*-treated human peripheral blood lymphocytes. We have found that 24 h treatment of human lymphocytes with two grades of commercial *tuibur* and nicotine showed a concentration dependent decrease in cell viability. We, therefore, concluded that as the *in vitro* use of *tuibur* has an adverse effect on cell survival, its consumption might have potential side effects on the health of the users.

Key words: Cell viability, lymphocytes, tobacco, tuibur.

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Introduction

Tobacco is linked with many diseases and has been known to contain more than eight thousand chemicals, out of which roughly 68 are probable carcinogens.[1-3] Some of the common toxic chemicals include benzo[a]pyrene (B[a]P), N'-nitrosonornicotine (NNN), N'-nitrosoanatabine (NAT), N'-nitrosoanabasine (NAB), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosodimethylamine (NDMA), nitrite, cadmium, lead, arsenic, nickel, chromium, etc.[4,5] The consumption of both smoking and smokeless tobacco is popular throughout the world and its detrimental effect could be observed from many medical records. Besides its deleterious consequence upon the pulmonary system, it has been linked with many forms of cancer. In fact, many studies suggested that almost all known cancer could be linked to tobacco use.[6,7]

It would be safe to say that every nation throughout the globe has tobacco users in its population.[8] The form of tobacco used may vary considerably. Some prefer smoking tobacco while others prefer smokeless tobacco, or both. But, it may be acceptable to say that more than half of the tobacco users used it in the form of smoking tobacco.[9] The Mizo tribes living in the northeastern part of India use both smoke and smokeless tobacco.[10] A form of smokeless tobacco locally called *tuibur* (tobacco brew) is used popularly and is commercially available in the local market, generally in two grades, which largely depend on

the amount of tobacco used in its production. The method of practice is the users of *tuibur* put the product in the mouth for roughly 5-10 minutes which is then spitted out. The duration is determined when the alkalinity of the *tuibur* is depleted.[11]

In this experiment, we aimed to determine the effect of two grades of commercial *tuibur* on the viability of *tuibur*-treated human peripheral blood lymphocytes *in vitro*.

Materials and Methods

Chemicals

A small quantity of two grades of commercial *tuibur*, labelled as *tuibur*-A (special grade) and *tuibur*-B (ordinary grade), produced in a local industry were purchased from the market. Although there is no standard protocol, the manufacturers graded the *tuibur* depending on the quantity of tobacco used in its production. Pure nicotine (Cayman Chemical Company) and trypan blue (Sigma) were purchased from local supplier. RPMI-1640 media (HiMedia) was obtained from local supplier and prepared in the laboratory using standard protocol.

Lymphocyte culture and treatment

Lymphocyte culture were performed using the protocol described by Jagetia *et al.*[12] Briefly, peripheral blood lymphocytes were collected by venipuncture in a heparinized vacutainer from a 27-year-old healthy male volunteer who has no known history of tobacco consumption. The collected blood was allowed to stand for roughly half an hour and the upper translucent layer containing lymphocytes was taken for culture. Approximately two million lymphocytes were cultured in different test tubes containing 2 ml RPMI-1640 culture media without the addition of any growth factor.

The tubes were separated into four groups (I, II, III & IV) and different volumes of *tuibur*-A and *tuibur*-B were added to group I & II (2.5, 5, 10, 20, 40, and 50 µl/ml) respectively. To group III, 2.5, 10, 20, 40, and 50 µg/ml of nicotine was added and this served as positive control. Group IV or blank acted as negative control and did not contain any chemical other than the cells and the media. All cultures were performed in triplicate. These tubes were incubated at 37°C for 24 h. After 24 h, the survival of the cells was checked by modified trypan blue exclusion assay.[13] The number of living and dead cells were counted in a hemocytometer and the mean percentage of surviving cells was taken as viability.

Statistical analysis

All statistical analysis were performed using Microsoft Excel 2013 and OriginPro-8. Correla-

Table1 | Mean percentage of viable human peripheral blood lymphocytes for blank and treatment with different concentration of *tuibur*-A, *tuibur*-B and nicotine.

Concentration (µl/ml or µg/ml)	Mean % of viable cells±SEM			
	Tuibur-A	*Tuibur*-B	Nicotine	Blank
0	-	-	-	100.00±0.00
2.5	100.00±0.00	100.00±0.00	98.25±0.06	-
5	96.39±0.58	95.06±0.40	96.55±0.25	-
10	90.34±1.86	90.35±0.97	92.32±0.59	-
20	86.41±0.62	87.03±0.29	91.1±0.23	-
30	79.43±2.22	85.65±1.20	87.9±0.50	-
40	71.07±1.97	79.15±0.58	85.57±0.14	-
50	65.57±0.62	76.14±1.11	82.71±2.31	-

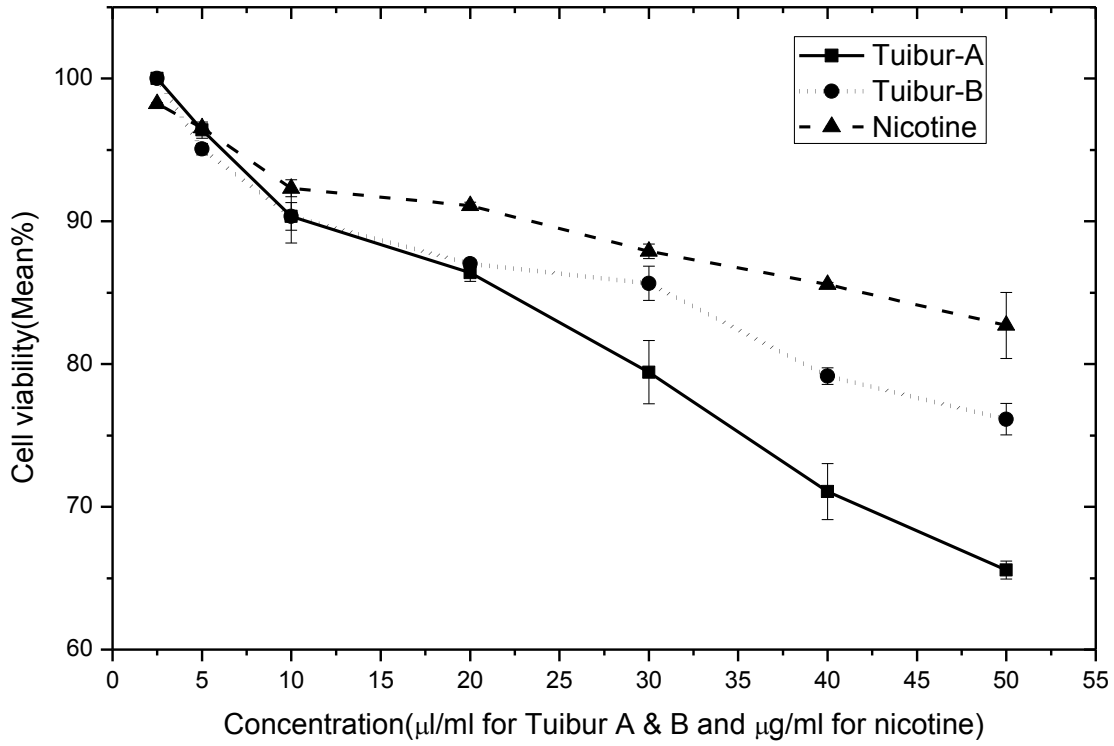

Figure 1 | Graph showing mean percentage of viable human peripheral blood lymphocytes treated with different concentration of *tuibur*-A, *tuibur*-B and nicotine.

tion coefficient was performed to determine relationship between different treatment concentrations and viability within a group. Student's *t*-test was employed to determine significant difference between the treatment groups.

Table 2 | Student's *t*-test between different treatment groups at 95% confidence interval.

Student's t-test between	p-value at 95% CI	Inference
Tuibur-A & *Tuibur*-B	0.60	No significant difference
Tuibur-A & Nicotine	0.27	No significant difference
Tuibur-B & Nicotine	0.45	No significant difference
Control & *Tuibur*-A	≤0.00	Significant difference
Control & *Tuibur*-B	≤0.00	Significant difference
Control & Nicotine	≤0.00	Significant difference

Results

The pH of *tuibur*-A and *tuibur*-B were found to be 9.81 and 10.09 respectively. Table 1 and Figure 1 showed the mean percentage of viable cells for the different treatment groups. The negative control showed 100% viability while *tuibur*-A, *tuibur*-B and nicotine showed a concentration dependent viability. Lymphocytes treated with a maximum concentration of 50 µl/mlof *tuibur*-A and *tuibur*-B showed 65.57% and 76.14% viability respectively while a minimum concentration of 2.5 µl/ml of both the two *tuibur* grades resulted in 100% viability in both the groups. A maximum concentration of 50 µg/ml and a minimum concentration of 2.5 µg/ml of nicotine showed 82.71% and 98.25% viability respectively. A strong negative correlation was observed between cell viability and concentration of *tuibur*-A (-0.994), *tuibur*-B (-0.969) and nicotine (-0.979). This

means higher the concentration of the chemicals, lower the viability and vice versa.

Statistical analysis by t-test at 95% CI (Table 2) between mean percentage of viable cells for blank and *tuibur*-A, blank and *tuibur*-B, blank and nicotine showed a significant difference (p-value≤0.00). However, comparison of *tuibur*-A and *tuibur*-B (p-value=0.60), *tuibur*-A and nicotine (p-value=0.27), *tuibur*-B and nicotine (p-value=0.45) showed that there is no significant difference in mean percentage of viable cells between these groups.

Discussion

Tobacco is known to contain enormous amount of different chemicals, many of which have been reported to have carcinogenic and cytotoxic properties.[1,14] Most studies, if not all, reported the use of tobacco in any form only have negative impact on the physiological wellbeing of the users. There have been only a handful of literatures on the scientific investigation of *tuibur*. A preliminary report on the chemical composition of *tuibur* showed the presence of polyaromatic hydrocarbons and carbonyl compounds in the tar phase.[11]

An epidemiological study among the Mizos showed that *tuibur* users have a higher risk of developing gastric cancer and the combine use and frequency of smoking, betel, *tuibur* and *sahdah* were reported to have a significant influence on the risk of gastric cancer.[10] Phukan *et al.*[15] have also reported *tuibur* use as a risk factor for gastric cancer. Besides gastric cancer patients in Mizoram, *tuibur* consumers were found to have a variety of mtDNA D-loop region mutations and polymorphisms.[16] Individuals with Arg/Pro genotype,GSTM1 null genotype and GSTT1 non -null genotype were also suggested to have a higher risk of gastric cancer if they have habits of using *tuibur* and smoking tobacco.[17,18]

The damaging effect of tobacco may be attributed to its vast array of chemical compositions. Heavy metals like cadmium and lead present in tobacco have also been found to cause glomerular dysfunction. Many of these effects may be because of nicotine's ability to affect certain antioxidant enzymes like lipid peroxidase, superoxide dismutase, catalase, glutathione-s-transferase, glutathione reductase, etc.[3] Cytological studies have reported nicotine to inhibited cell proliferation and decreased protein synthesis in a dose dependent manner in cultured periodontal ligament fibroblast,[19] while it was also reported to stimulate endothelial cell DNA synthesis and proliferation at concentrations lower than $<10^{-8}$ M. The cytotoxicity of nicotine was reported to be at a higher concentration, i.e. $>10^{-6}$.[20]

Onion bulbs treated with *tuibur* showed a reduced root growth, reduced mitotic index, formation of micronuclei, lagging chromosomes, and c-mitosis.[21] A study on seven smokeless tobacco aqueous extracts showed a concentration-dependent effects on the growth and viability of oral bacteria cultured under anaerobic conditions.[22] These effects may be a result of increase superoxide anion production, lipid peroxidation, DNA fragmentation and DNA ladders caused by the use of chewing tobaccos.[23]

Our result showed concentration dependent cell viability for the *tuibur* and nicotine treatment groups while the untreated negative control group showed 100% viability. We are uncertain as to what chemical(s) in the tobacco brew would cause the cells to die. But from the nicotine treatment group, we may be able to say, although carefully, that the nicotine might contributed significantly in this result. However, one study suggested other biologically active compounds like NNN, NNK, etc., other than nicotine present in tobacco leave extract to be the source of cytotoxicity.[24]

Another probable factor for the decrease in viability of the *tuibur* treatment groups would be the change in pH of the culture media. As we have shown in our result, the pH of both the two grades of *tuibur* are alkaline in nature, a slight rise in pH of the culture media was observed after the addition of both the *tuibur* (data not shown). This change in pH may be a factor that leads to decrease cell viability. In conclusion, our result showed that 24 h treatment of human lymphocytes with *tuibur* and nicotine may have an ad-

verse effect on their survival and hence these chemicals might have cytotoxic properties. Therefore, the consumption of *tuibur* might have potential side effects on the health of the users.

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