BIOCHEMICAL AND MOLECULAR STUDIES ON PESTICIDE-EXPOSED WORKERS OF TEA GARDENS OF NORTH BENGAL

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ZOOLOGY

By

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November, 2018

DECLARATION

I declare that the thesis entitled '**Biochemical and Molecular Studies on Pesticide-Exposed Workers of Tea Gardens of North Bengal**' has been prepared by me under the guidance of Professor Min Bahadur, Department of Zoology, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Tea is cultivated on a large scale in northern part of West Bengal, India. To keep pests populations under control mixed pesticide formulations are regularly applied in the tea gardens. Pesticides are known to cause serious hazards to environment as well as non-target organisms including human beings. The genotoxic effects of pesticides on the tea workers of North Bengal have not yet been thoroughly studied. Therefore, a multidimensional approach has been conducted to determine the extent of toxic effects induced by pesticides on the worker population. A total of 225 individuals (95 pesticide-exposed individuals, 60 controls, 39 smokers and 31 alcohol consumers) were sampled for the study. Since all males and few females of pesticide-exposed worker group smoked cigarettes and all consumed alcohol, smokers and alcohol consumers were included in the study for comparison. The results based on enzyme, micronuclei, comet assay and CYP2C9 gene polymorphism are summarized below.

Acetylcholinesterase and Butyrylcholinesterase activity

Results showed acetylcholinesterase activity of 11.81±3.40 and 6.43±1.85 µmoles/min/ml in controls and tea garden workers (p<0.001), respectively. The activities in smokers and alcohol consumers were 11.04±2.48 and 12.45±2.58, µmoles/min/ml, respectively. The difference with control was not significant.

Butyrylcholinesterase activities were 4.73±1.84 and 3.50±1.89 µmoles/min/ml in control and exposed groups, respectively. The difference in the activity was significant at p<0.001. Butyrylcholinesterase activities of 5.06±1.87 and 5.02±1.41 µmoles/min/ml

observed in smokers and alcoholics, respectively were not significantly different than controls.

In contrast to a slight increase of acetylcholinesterase activity in the females (6.78±2.21 µmoles/min/ml) than males (6.23±1.59 µmoles/min/ml) of the exposed group, the females had significantly higher activity than males in control group (13.47±4.18 versus 11.15±2.83 µmoles/min/ml, $p \leq 0.05$).

The butyrylcholinesterase activities were 4.08±1.66 and 3.15±1.95 µmoles/min/ml in exposed females and males. The activity was significantly high in females ($p \leq 0.05$), whereas, control females and males did not show significant difference (5.14±1.78 versus 4.57±1.86 µmoles/min/ml) in activity.

The effect of gender appears to have very little influence on the acetylcholinesterase and butyrylcholinesterase activity.

Though not significant but reduced acetylcholinesterase and butyrylcholinesterase activities were observed in the age group 15-30 years than age groups 31-45 and 46-62.

Results showed that acetylcholinesterase and butyrylcholinesterase activities were markedly inhibited in the tea garden workers.

Micronucleus Assay

The micronucleus assay showed a significant increase of micronuclei (9.77±2.66, $p \leq 0.001$), nuclear bud (4.39±1.42, $p \leq 0.001$), binucleate (6.12±2.92, $p \leq 0.001$), karyorrhectic (8.01±2.29, $p \leq 0.001$), pyknotic (5.74±1.81, $p \leq 0.05$) and karyolytic (6.89±2.98, $p \leq 0.001$) nuclei in the exposed group than control. Compared to control, the smokers revealed a higher frequency of micronuclei (6.15±2.47, $p \leq 0.001$), nuclear bud

(4.13±2.35, $p \leq 0.05$), binucleate (4.38±2.24, $p \leq 0.01$), karyorrhectic (6.67±2.30, $p \leq 0.001$) and karyolytic (6.18±2.19, $p \leq 0.01$) nuclei except pyknotic cell. Frequency of binucleate (3.97±1.99, $p \leq 0.05$), karyorrhectic (5.55±1.84, $p \leq 0.05$) and karyolytic (6.29±2.64, $p \leq 0.01$) nuclei was higher in the alcoholics than control. An analysis between smokers, smoking <10 cigarettes per day (group I) and those smoking >10 cigarettes per day (group II) showed higher frequency of micronuclei (7.72±1.60, $p \leq 0.001$), nuclear bud (5.61±2.28, $p \leq 0.001$), binucleate (5.83±1.72, $p \leq 0.001$), karyorrhectic (8.00±1.33, $p \leq 0.01$), pyknotic (6.28±2.05, $p \leq 0.01$) and karyolytic (7.72±1.32, $p \leq 0.001$) cells in group II. A higher proportion of micronuclei (10.87±2.38, $p \leq 0.001$), nuclear bud (4.90±1.35, $p \leq 0.001$) and binucleate nuclei (6.98±3.04, $p \leq 0.001$) were detected in the pesticide exposed males than females. The results indicated a synergistic effect of pesticide, smoking and alcohol on cellular damage. Age and duration of exposure have no influence on the micronucleus and other cell death parameters.

Comet assay

The comet assay showed a mean tail length of 45.98±4.25 and 15.14±2.99 μm in the tea garden workers (exposed) and the control individuals, respectively which was about 3 times longer in the worker ($p \leq 0.001$). The smokers and alcohol consumers revealed tail length of 16.83±2.60 and 14.24±1.32 μm, almost similar to that of controls. The mean tail moment and olive tail moment were 8.86±1.18 and 6.41±0.78 in exposed workers and 2.89±0.48 and 2.32±0.36 in controls, respectively indicating significantly higher damage in the workers ($p \leq 0.001$). The smokers and the alcoholics showed a mean tail moment and olive tail moment of 3.96±0.89 and 2.69±0.48 and 3.19±0.55 and 2.60±0.42, respectively which were statistically not different than control.

Age, gender and duration of exposure had no effect on DNA damage.

CYP2C9 polymorphism

The polymorphism of CYP2C9 gene was studied in 94 pesticide-exposed tea garden workers and 130 non-tea garden workers. All the individuals were analysed by PCR-RFLP for CYP2C9 allelic variants compared to wild type.

CYP2C9*1 allele was most predominant both in tea garden workers and non-tea garden workers, present at a frequency of 0.87235 (77.66% of individuals) and 0.87695 (78.46% of individuals), respectively. 2.13% tea garden workers and 2.30% non-tea garden workers were homozygous for CYP2C9*3 allele showing frequency of 0.10105 and 0.09615. No homozygotes for CYP2C9*2 allele were detected in any groups. The CYP2C9*2 allele was least frequent with a frequency of 0.0266 and 0.0269 in the tea garden and the non-tea garden worker population, respectively.

Genotyping revealed that one individual was heterozygous for CYP2C9*2/*3 genotype in each of the worker and non-tea garden worker population with a frequency of 1.06% and 0.77%, respectively. In tea garden worker population, 4.26% and 14.89% were heterozygous for CYP2C9*1/*2 and CYP2C9*1/*3 alleles, while 4.62% and 13.85% were heterozygous for CYP2C9*1/*2 and CYP2C9*1/*3 alleles, respectively in non-tea garden worker population. The allele frequency in pesticide exposed worker population is comparable to those of non-tea garden workers. The observed and expected frequency distribution indicated that the results are in good agreement with that of the expected as calculated by the Hardy-Weinberg law.

PREFACE

The present dissertation entitled "**BIOCHEMICAL AND MOLECULAR STUDIES ON PESTICIDE-EXPOSED WORKERS OF TEA GARDENS OF NORTH BENGAL**" is submitted to fulfil the requirements for the degree of Doctor of Philosophy of the University of North Bengal. This thesis embodies the results of investigations on how pesticides are capable of causing adverse effects on the pesticide-exposed tea garden workers of northern part of West Bengal, India compared to the non-exposed controls, smokers and alcohol consumers. The work presented in this thesis will be a useful guide to detect the level of DNA damage caused by pesticides. All the studies presented in this dissertation have been carried out in Molecular Cell Biology and Genetics Laboratory, Department of Zoology, University of North Bengal under the supervision of Professor Min Bahadur, Department of Zoology, University of North Bengal, Siliguri-734 013. The thesis consists of six chapters. A brief introduction is given in **Chapter 1**. **Chapter 2** describes the level of acetylcholinesterase and butyrylcholinesterase in the pesticide-exposed workers, controls, smokers and alcohol consumers. In **Chapter 3** the use of micronucleus assay in detecting pesticide induced genome damage has been represented. **Chapter 4** focuses on the extent of damage on the peripheral blood lymphocytes of occupationally exposed tea garden workers and other non-exposed groups estimated by comet assay. **Chapter 5** describes the polymorphism of CYP2C9 gene in the tea garden workers and non-tea garden workers. **Chapter 6** sums up the conclusions of this research work. A list of publications and papers presented at symposia and seminars has been listed under **Appendix**.

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ABBREVIATIONS

AChE	:	Acetylcholinesterase
ALAD	:	δ-aminolevulinic acid dehydratase
AP	:	Acephate
APC	:	Aphidicolin
BMI	:	Body mass index
BN	:	Binucleate
BNMN	:	Binucleated cells with micronuclei
BuChE	:	Butyrylcholinesterase
CAT	:	Catalase
CBMN	:	Cytokinesis-block micronucleus
ChEs	:	Cholinesterase
c-PAHs	:	Carcinogenic polycyclic aromatic hydrocarbons
CPFO	:	Chlorpyrifos-oxon
CYP	:	Cytochrome P450
d	:	Day
DMSO	:	Dimethyl Sulfoxide
DPX	:	Distrene plasticizer xylene
DTNB	:	5,5’-dithiobis-2-nitrobenzoic acid
FPG	:	Formamidopyrimidine-DNA glycosylase
GSH-Px	:	Glutathione peroxidise
h	:	Hour
HPV	:	Human papilloma virus
KH	:	Karyorrhectic
KL	:	Karyolytic
LCLs	:	Lymphoblastoid cell line
Ltail	:	Tail length

MANCOVA	:	Multivariate analysis of covariance
MANOVA	:	Multivariate analysis of variance
MAP	:	Methamidophos
MF	:	Medium filter
MH	:	Maleic hydrazide
MN	:	Micronuclei
MNL	:	Micronuclei in binucleate cells
NB	:	Nuclear bud
NER	:	Nucleotide excision repair
NF	:	Non-filtered
NI	:	Nicotine
NPBs	:	Nucleoplasmic bridges
OPs	:	Organophosphates
OSF	:	Oral submucous fibrosis
OTM	:	Olive tail moment
PAHs	:	Polycyclic aromatic hydrocarbons
Pap	:	Papanicolaou
PBMCs	:	Peripheral blood mononuclear cells
PCR	:	Polymerase chain reaction
PPPs	:	Plant protection products
PY	:	Pyknotic
RFLP	:	Restriction fragment length polymorphism
ROS	:	Reactive Oxygen species
s	:	second
SCGE	:	Single cell gel electrophoresis
SD	:	Standard deviation
SEM	:	Standard error of mean
SNP	:	Single Nucleotide Polymorphism
SOD	:	Superoxide dismutase
TAE	:	Tris Acetate EDTA
TM	:	Tail moment

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CHAPTER-1

Introduction

Pesticides, the heterogeneous mixture of compounds, are globally used to keep pests under control and to increase the crop productivity. Due to their toxicity, persistency and biomagnifications the usage of pesticides in agriculture or domestic purpose is a pivotal health concern of each country. Because of poor working conditions and lack of knowledge about potential hazards associated with the manufacturing and application of chemical pesticides, generally users in developing countries are at high risk of chronic exposure to pesticides (Baker et al., 1978; Brunetti et al., 1988; Benedetti et al., 2013; Kausar et al., 2014). Apart from target organisms, pesticides also exert their effects on the environment as well as non-target organisms including humans (Panemangalore et al., 1999; Prakasam et al., 2001; Hernández et al., 2005; Thetkathuek et al., 2005). Besides being the largest producer of pesticides in Asia, India is also the third largest consumer of pesticides in the world (Kumari et al., 2003) and so about 70% of her population depending on agriculture for their livelihood as cultivators, farm owners and laborers (Chakraborty et al., 2009) are at risk of exposure to the toxic pesticides. Workers get exposed to hazardous substances in the form of gases, vapours, fumes or particles that are present in the occupational environment (Çelik et al., 2003; Benites et al., 2006). Though inhalation is the primary route of exposure to these substances, exposure may also take place through oral ingestion or dermal absorption (Benites et al., 2006). Different health hazards which includes a wide range of subclinical and clinical effects (cancer, adverse reproductive outcomes and other chronic illness) are associated with occupational exposure to pesticides (IARC 1991; Arbuckle and Sever, 1998; Lander et al., 2000; Meinert et al., 2000; Priyadarshi et al., 2000; Hagmar et al., 2001; Jenner, 2001; Ji et al., 2001; Alavanja et al., 2004; Hanke and Jurewicz, 2004; Kamel and Hoppin, 2004). Therefore, the evaluation of the toxic effect of the pesticides on the non-target organisms including human beings is the need of the hour.

By using suitable biomarkers the effect of the pesticides can be assessed. A biomarker is a characteristic that can be objectively measured as well as evaluated as an indicator of normal biological or pathogenic processes and pharmacologic responses to therapeutic or other health care interventions (Biomarker Definitions Working Group, 2001). They serve as useful indicators of molecular and cellular events occurring in biological system which may illuminate relationships between hazards, human health and the disease processes (Dusinska and Collins, 2008). Unintended environmental exposure, such as, to chemicals or nutrient can be determined with the help of biomarkers. The response that is measured may be functional, physiological and biochemical at the cellular level or molecular interaction (WHO, 1993). Biomarkers are the internal indicators of environmental or occupational exposures which have the potential to prevent the effects of exposure to carcinogen by early detection (Smith et al., 1993). The growing interest in the use of biomarkers in occupational and environmental medicine parallels the development of human biomonitoring which can be defined as repeated and controlled measurement of chemical(s) or biomarkers in fluids, tissues or other accessible samples from those subjects that are currently exposed or had been exposed in the past or are to be exposed to chemical, physical or biological risk factors in the work place and/or general environment (Manno et al., 2010).

Biomarkers that are used to monitor environment and human health can be divided into three classes: biomarkers of exposure, effect and susceptibility (Knudsen and Hansen, 2007). Biomarker of exposure has been defined as an exogenous substance, its metabolite or the product of interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism, while the biomarkers of effect are any biochemical, physiological, behavioral or other alteration within an organism that can be measured and depending on the magnitude can be recognized as associated with an

established or possible health impairment of diseases (WHO, 1993). Biomarker of susceptibility is an indicator of inherent or acquired ability of an organism towards exposure to xenobiotics (WHO, 1993). Human biomonitoring can serve as useful tool in estimation of exposure of selected populations and is currently in use for surveillance programme across the world (Lionetto et al., 2013).

Organophosphates (OPs) inhibit acetylcholinesterase (AChE) in synapses and neuromuscular junctions causing acetylcholine accumulation which results in overstimulation of nervous systems (Kwong, 2002). Butyrylcholinesterase (BuChE) also bind covalently to OPs (Worek et al., 2005). AChE and BuChE are preferentially inhibited by certain OPs, for example, dimethoate predominantly inhibits AChE, while chlorpyrifos and malathion preferentially inhibit BuChE (Simoniello et al., 2008). Both AChE and BuChE can be used as an effective biomarker for exposure to certain pesticides (OPs and carbamates) (Chakraborty et al., 2009; Simoniello et al., 2010; Kapka-Skrzypczak et al., 2011).

Micronuclei are chromosomal fragments or whole chromosome which are not included into daughter nuclei during cell division and are incorporated as small nuclei (Schmid, 1975). Micronucleus (MN) is formed from induction of substances that cause chromosomal breakage (clastogens) and by agents that affect the spindle apparatus (aneugens) (Ghosh et al., 2008). Micronucleus assay is widely used to test the adverse effects of mutagens and carcinogens. It is a commonly used short-term assay in cultured mammalian cells, primary mitogen stimulated lymphocytes (Fenech and Morley, 1985) and in exfoliated epithelial cells like oral, urothelial, nasal etc. (Holland et al., 2008). MN assay is a non-invasive method to study DNA damage, chromosomal instability, cell death and the regenerative potential of buccal mucosal tissue (Çelik et al., 2003; Bonassi

et al., 2011; Thomas and Fenech, 2011). Cytokinesis-block micronucleus (CBMN) technique was developed, which stop the dividing cells from performing cytokinesis by using cytochalasin B. This allows cells which have completed one nuclear division to be recognized as they are binucleate in appearance (Fenech and Morley, 1985, 1986). Umegaki and Fenech (2000) proposed that nucleoplasmic bridges (NPBs) in between the nuclei of binucleated cells should also be scored in CBMN assay since they offer a good measurement of chromosomal rearrangement. NPBs are observed with binucleated cells because cytokinesis is blocked and nuclear membrane is formed around the chromosome. Micronucleus also forms by a unique mechanism called nuclear budding. It can be observed under selective conditions in culture (Toledo et al., 1992; Ma et al., 1993; Shimura et al., 1999) where gene amplification is induced. The amplified DNA is selectively located at the periphery of nucleus. MN forms during the S-phase of cell cycle via elimination of nuclear budding (Shimizu et al., 1998, 2000). **Figure 1.1** explains the various possible fates of cultured cells blocked in cytokinesis following exposure to cytotoxic/genotoxic agents as proposed by Fenech and Crott (2002). Human buccal cell is a valuable source to biomonitor DNA damage by determining frequency of micronuclei (Holland et al., 2008). The oral epithelium maintains itself by continuous cell renewal. New cells that are produced by mitoses in the basal layer migrates to the surface, replacing the ones that are shed (Ten Cate et al., 1998). The basal layer contains stem cells which may express genetic damage as chromosomal breakage or loss in the form of

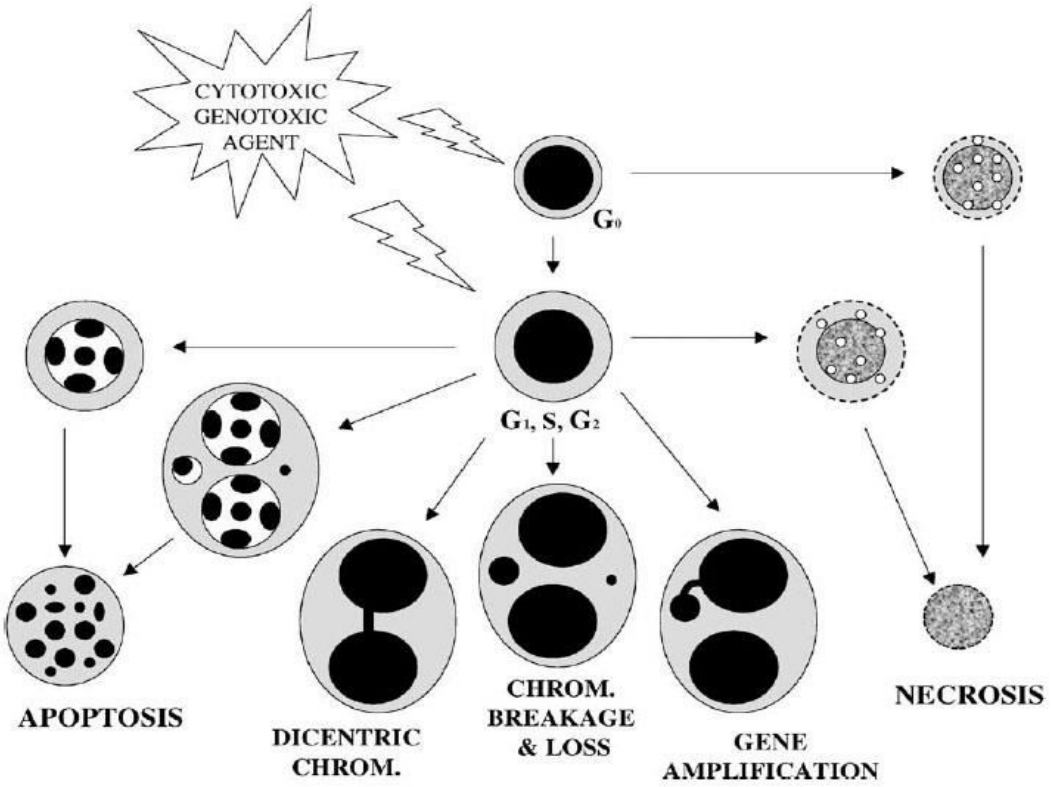


Figure 1.1: Various possible fates of cultured cells blocked in cytokinesis following exposure to cytotoxic/genotoxic agents as proposed by Fenech and Crott (2002).

MN during nuclear division (Rosin, 1992). The daughter cell may or may not contain MN, differentiate into the stratum spinosum cell layer and keratinized superficial layer, and finally exfoliate into the buccal cavity. Some of the cells may degenerate into condensed chromatin, fragmented nuclei, pyknotic nuclei or karyolytic cells which do not have nuclear material. In some cases, cells may be blocked in a binucleated stage or may also exhibit nuclear bud, which is a biomarker of gene amplification, also known as broken eggs (Suspiro and Prista, 2011; Kashyap and Reddy, 2012).

DNA damage and repair can also be detected in individual cells by comet assay, also known as single cell gel electrophoresis (SCGE), which is a rapid and a very sensitive method. Comet assay has important applications in toxicology, which ranges from clinical investigations and mechanistic studies to molecular epidemiology and biomonitoring (Collins, 2002). It was first introduced by Ostling and Johanson (1984) and later modified by Singh et al. (1988). The alkaline version of Comet assay is a useful method to quantify DNA damage (Tice et al., 2000). Comet assay is less time-consuming and can be performed with any type of eukaryotic cells, so is very popular (Hartmann et al., 2003). A number of studies have shown its potential to detect the extent of DNA damage in workers employed in pesticide manufacturing industry (Zeljezic and Garaj-Vrhovac, 2001; Grover et al., 2003; Bhalli et al., 2006; Sailaja et al., 2006; Naravaneni and Jamil, 2007).

The enzymes that belong to the families CYP1, CYP2 and CYP3 are found to catalyze the oxidative biotransformation of exogenous compounds (drugs, pro-carcinogens and alcohols). Metabolism of endogenous compounds (fatty acids, prostaglandins and steroids) is mediated by other CYP450 enzymes (van der and Steijns, 1999). Xenobiotic metabolism is mainly carried out by CYP2C9 and CYP2C19 and genes encoding these

enzymes are polymorphically expressed in the population (Goldstein, 2001; Daly, 2003). Human cytochrome CYP2C9 gene is located on chromosome 10q24 and has an approximate length of 55 kb (Lee et al., 2002). This major enzyme of the CYP2C subfamily in humans constitutes approximately 20% of the hepatic cytochrome P450 enzymes (Takahashi and Echizen, 2001). The biotransformation of a huge variety of xenobiotics including the organophosphates, chlorpyrifos and parathion are found to be associated with CYP2C9 (Foxenberg et al., 2007). Decreased metabolism of substrates have been shown to be associated with CYP2C9*2 and CYP2C9*3 allelic variants (Aynacioglu et al., 1999). Genetic polymorphism can be defined as a mutation which occurs in DNA sequence and is present in at least 1% of the population. The polymorphic alleles contains single nucleotide polymorphisms (SNPs) which results in inversions, deletions or base substitutions. The SNPs may result in a change of amino acid sequence, premature stop codon or a splicing error. The resulting enzyme may have increased, decreased or zero activity as reviewed by Rosemary and Adithan (2007).

Tea is cultivated as a major agro industry in Terai and the Dooars regions of northern part of West Bengal, India. Tea planters of this region use a large number of pesticides to control arthropod pests (Gurusubramanian et al., 2008). This results in the exposure of non-target organisms including humans to pesticides. The female workers of this region are mainly involved in the tea leaf plucking and males are engaged in pesticide spraying and mixing and therefore get occupationally exposed to pesticides (Dutta and Bahadur, 2016). Singh et al. (2015) have reported residual pesticide contamination in water, sediment and fish from the rivers flowing through the tea gardens of Terai region of West Bengal. Residues of ethion, chlorpyrifos, heptachlor, dicofol, alpha-endosulfan, beta-endosulfan, endosulfan sulfate, cypermethrin and deltamethrin have been detected in made tea, fresh tea leaves, soil and water bodies from certain tea gardens in the Dooars

and the hill regions of West Bengal (Bishnu et al., 2009). Owing to increasing pest problem as a result of resistance to pesticides (Sarkar and Mukhopadhyay, 2003, 2006), the planters apply a mixture of pesticides to combat the mixed pest infestation in the tea gardens of this region (Roy et al. 2008; Bishnu et al. 2009; Singh et al. 2015). A number of studies have been conducted related to the pesticide contamination, presence of residues in the water, sediment, fish and development of resistance/tolerance against the pesticides, but studies related to the effect of pesticides on the worker population using biomarkers are lacking. Therefore, to evaluate the effect of pesticides on the occupationally exposed tea-garden workers, the AChE and BuChE activity in the blood, micronuclei frequency, DNA damage by Comet assay and genetic polymorphism of CYP2C9 gene have been attempted. The data generated can be used for proper health risk assessment and effective health care strategy in future.

1.1 Objectives

In the light of the above, following objectives were undertaken for the present study:

1. A survey will be carried out to know the socioeconomic status and living condition of the workers in tea gardens of North Bengal region.
2. To determine the level of acetylcholinesterase and butyrylcholinesterase in pesticide-exposed tea garden workers and control.
3. To study the extent of nuclear DNA damage in the pesticide-exposed tea garden workers.
4. Genetic polymorphism of Cytochrome P450 gene involved in the detoxification of pesticides.
5. Data will be subjected to statistical analysis for association of genomic damages/genotoxicity with appropriate software packages.

CHAPTER 2

Acetylcholinesterase and Butyrylcholinesterase activity

2.1 Review of Literature

Acetylcholinesterase (AChE) (EC 3.1.1.7) belonging to the family cholinesterase (ChE) is a specialized carboxylic ester that hydrolyse the breakdown of acetylcholine into choline and acetate after activation of acetylcholine receptors at postsynaptic membrane (Massoulié et al., 1993). Butyrylcholinesterase (BuChE) (EC 3.1.1.8) which is a pseudocholinesterase also hydrolyses acetylcholine and serves to terminate synaptic transmission thus preventing continuous nerve firing at nerve endings. Hence it is essential for normal functioning of central and peripheral nervous system. Butyrylcholinesterase (BuChE) is found in plasma but its physiological function in blood is unknown (Costa et al., 2005).

Organophosphorus and carbamate pesticides have been known to be specific inhibitors of acetylcholinesterase catalytic activity (Hobbiger, 1961). Erythrocyte AChE inhibition serves as a good biomarker of exposure to organophosphate pesticides in field studies with human population. Organophosphate and carbamate pesticides are widely used for pest control (Meerdink, 1989). However, they exert their effect on non-target organisms as well (Lionetto et al., 2003, 2004; Calisi et al., 2009, 2011). Organophosphate and carbamate compounds are capable of infiltrating through the soil into surface water because of their water solubility (Bondarenko et al., 2004). These are more toxic to human beings compared to organochlorines if misused (Wilson and Tisdell, 2001). The organophosphate and carbamate residues cannot be detected easily by chemical analysis because of their short life but their products of environmental degradation can be very harmful retaining acetylcholinesterase activity (Pehkonen and Zhang, 2002).

2.1.1 Cholinesterase activity in agricultural and farm workers

Workers engaged in agriculture and farms get exposed to synthetic agricultural chemicals while working in the fields. Innes et al. (1990) assayed plasma cholinesterase activity in order to screen 44 farm workers spraying organophosphate pesticides. The entire group had a moderately depressed cholinesterase activity while seven (7) out of 44 farm workers, had reduced cholinesterase activity. The mean body mass of these 7 farm workers was lower than that of the others which also lowered the body mass indices. When these 7 workers were removed from spraying for 6 weeks, the cholinesterase activities were increased significantly. The other individuals of the group had similar values of cholinesterase activity. López-Carillo and López-Cervantes (1993) have reported a significant difference in the median activity levels of serum cholinesterase in agricultural workers compared to age and sex matched control groups. Cholinesterase activity was significantly decreased at the beginning and end of the day's work. They suggested that it can be due to an interaction between the type of work and age of workers. Younger workers had a greater decrease in ChE activity as they were engaged in more dangerous activities. In a study by Ciesielski et al. (1994), the North Carolina migrant farm workers were shown to have significantly lower cholinesterase activity than those of non-farm workers. The effects of acephate (AP), cadmium (Cd), methamidophos (MAP), maleic hydrazide (MH), and nicotine (NI) on the activities of the erythrocyte enzymes δ-aminolevulinic acid dehydratase (ALAD), superoxide dismutase (SOD), and plasma cholinesterase (ChE) was determined on the farm workers since they are exposed to a combination of synthetic agricultural chemicals (Dowla et al., 1996). The studies revealed that the enzyme activities were significantly inhibited by a wide concentration range of the agricultural chemicals acephate, methamidophos, cadmium, maleic hydrazide and nicotine (Dowla et al., 1996). It was also found that erythrocyte superoxide dismutase

was most sensitive to these chemicals, while plasma cholinesterase was not inhibited by maleic hydrazide and nicotine (Dowla et al., 1996). In another study, Tinoco-Ojanguren and Halperin (1998) reported the inhibition of erythrocyte cholinesterase by OP pesticides in three communities and control subjects of Mexico. Adverse health effects existed among the poorest communities. Significantly, lower AChE activity has been reported by Rendógn von Osten et al. (2004) in farmers from 4 rural communities of Campeche (Mexico) compared to the mean activity of control group. Their regression analysis indicated that carbofuran was the dependent variable most related to moderate AChE inhibition.

Remor et al. (2009) observed a significant decrease in butyrylcholinesterase (BuChE) activity in farm workers from Rio Grande do Sul (Brazil) compared to the control group. Hematological parameters, MN frequencies and lipid profile did not show any significant difference between the occupationally exposed farm workers and the control, however comet assay revealed higher damage index and damage frequency. Remor et al. (2009) suggested the use of personal protective equipment to prevent contamination.

Similarly, Jintana et al. (2009) measured acetylcholinesterase and butyrylcholinesterase activities in individuals exposed to organophosphate pesticides and control subjects, selecting the high and low exposure periods. The exposed group had significantly lower enzyme activity than control subjects. Also a statistically significant decrease in acetylcholinesterase and butyrylcholinesterase activity was observed in the high exposure period compared to low exposure period. Chakraborty et al. (2009) studied the effect of long term exposure to cholinesterase inhibiting pesticides (organophosphate and carbamate) on the respiratory system of agricultural workers and have shown that the

decreased acetylcholinesterase activity was positively associated with respiratory symptoms.

Recently AChE activity was assayed by Singh et al. (2012) in the blood samples of workers involved in pesticide spraying and control subjects from Delhi, India and reported a significant decrease in AChE activity in workers compared to controls with a positive correlation between the AChE activity and the age and duration of exposure. Similarly, Dhalla and Sharma (2013) showed a positive correlation between serum cholinesterase activity and years of exposure and between body mass index (BMI) and serum cholinesterase inhibition in Bathinda district, Punjab. They observed that the younger population had a significant reduction in serum cholinesterase activity. Crane and workers measured the activity of AChE and BuChE in the blood samples of adolescent agricultural workers exposed to chlorpyrifos and control subjects in Egypt and showed a depressed BuChE activity in both the exposed and controls, but the decrease was significant in exposed group than control. The depression in enzyme activity persisted for 4-7 weeks post pesticide application which may be due to the fact that the non-exposed (control) groups may be receiving environmental chlorpyrifos exposure (Crane et al., 2013).

Tobacco field workers were also investigated for blood enzymes, δ-aminolevulinic acid dehydratase (ALAD), superoxide dismutase (SOD) and cholinesterase (ChE) with respect to different duration of exposure to agricultural chemicals. Panemangalore et al. (1999) showed that the ALAD activity was declined by 30% after 1 day and no decline was observed after 30 days of exposure, SOD activity was declined by 30% after 1 day and 50% after 30 days, while plasma ChE activity was declined by 19% after 1 day and 30 days of exposure. A restoration of the activities of all three enzymes to pre-exposure level

was observed during post-exposure period (no tobacco production). Highest respiratory nicotine level was found after 30 days post-exposure. In order to evaluate the toxicity of pesticides at occupational level, a study was conducted to evaluate the activities of cholinesterase, acid phosphatase, β-glucuronidase and paraoxonase in the plasma of plastic greenhouse workers (Hernández et al., 2004). Cholinesterase and paraoxonase activities were shown to be decreased in the pesticide applicators compared to the ones who were not pesticide applicators. The analysis showed that BuChE was significantly correlated with the level of β-glucuronidase and acid phosphatase. β-glucuronidase and acid phosphatase were related to each other and were associated with pesticide exposure in humans. BuChE was significantly decreased in sprayers compared to non sprayers during maximum exposure period. AChE activity did not differ significantly indicating that BuChE activity is more suitable marker than AChE. A higher inhibition of butyrylcholinesterase activity was found to be associated with the paraoxonase B allele (Hernández et al., 2004). In a study, Thetkathuek and workers investigated the biological effect of chlorpyrifos among 53 Thai fruit farm workers by measuring blood and plasma cholinesterase activities and showed a decrease in plasma cholinesterase activity in exposed workers. They also proposed that plasma cholinesterase activity can be used as a biomarker to detect the toxic effect of the chlorpyrifos insecticides (Thetkathuek et al., 2005).

In the pregnant mothers living in areas of pesticide exposure in Argentina, the placental acetylcholinesterase and catalase activities were shown to be significantly associated with organophosphorus pesticide exposure period, whereas glutathione S-transferase was unaffected (Souza et al., 2005). They also reported a positive correlation between newborn head circumference and the environmental exposure to organophosphorus and carbamate pesticides. The study also showed an association between placental

acetylcholinesterase and catalase activity and prenatal exposure to pesticide (Souza et al., 2005).

2.1.2 Cholinesterase activity in case of occupational exposure to metals

It is clear from the above review of literatures that AChE and BuChE inhibition serves as a useful biomarker for organophosphate and carbamate pesticides. Studies have also been conducted to determine the effect of other environmental pollutants on the AChE and BuChE activity. The inhibitory effects of five metal ions namely, nickel, copper, zinc, cadmium and mercury were assayed and all the metals except nickel inhibited acetylcholinesterase activity (Frasco et al., 2005). The neurotoxic effects of lead have been studied by measuring erythrocyte acetylcholinesterase activity, blood pressure and pulse in the workers exposed to lead and engaged in various works in Abeokuta, Nigeria (Ademuyiwa et al., 2007). In their study, acetylcholinesterase activity was inhibited by 39% and 32%, respectively in male and female petrol station attendants, 31% in welders, 38% in painters and 15% in panel beaters. No significant difference was found between blood pressure and pulse compared to the controls in the same study. An inverse linear relationship was obtained between AChE activity and blood lead levels as calculated by Pearson's method. Thus, acetylcholinesterase activity can be used as a biomarker to detect neurotoxicity induced by lead in occupationally exposed subjects (Ademuyiwa et al., 2007).

Excess of certain metals that are required for physiological functions may cause serious damage. The effect of Fe^{2+} and Fe^{3+} ions on human plasma cholinesterase activity was studied by Karami et al. (2010) and showed the suppression of BuChE activity by Fe^{2+} and Fe^{3+}. In an early study Jett et al. (1999) had reported that some polycyclic aromatic

hydrocarbons (PAHs) enhance the inhibitory effect of chlorpyrifos-oxon (CPFO) on acetylcholinesterase activity.

2.1.3 Cholinesterase activity in earthworm exposed to chemical pollutants

Toxicity assessment studies have also been carried out in animals. Earthworm's biomarker response is studied for a better understanding of pesticide contamination. In a study conducted by Capowiez et al. (2003), the effects of sublethal doses of imidacloprid on the earthworm (*Aporrectodea nocturna* and *Allolobophora icterica*) behavior was tested using AChE as a biomarker. It was observed that AChE activity was not affected by the treatment, which could be due to the fact that imidacloprid blocked the AChE receptor: nicotinic acetylcholine receptor instead of the enzyme itself (Capowiez et al., 2003). Acetylcholinesterase activity in the earthworm *Lumbricus terrestris* exposed to chemical pollutants was assessed in either the whole organism or the pre-clitellar and post-clitellar part of the animal (Calisi et al., 2011). Calisi et al. (2011) noticed a decrease of 70% AChE activity in earthworms following methiocarb (a carbamate pesticide) exposure for 14 days. Two ChEs (E1 and E2) are found in earthworm *Eisenia fetida* (Aamodt et al., 2007). *Eisenia fetida* were exposed to clean soil or soil containing chlorpyrifos (240 mg/kg) for 48h. E1 and E2 and the chlorpyrifos content of earthworms were monitored after transfer to clean soil for 12 weeks. E2 could not recover during 84d in clean soil which indicated that this enzyme was irreversibly inhibited by chlorpyrifos and de novo enzyme synthesis did not occur significantly. Thus E2 can serve as potential biomarker for OP insecticide exposure in *Eisenia fetida* (Aamodt et al., 2007). AChE activity in *Eisenia andrei* was inhibited by carbaryl (carbamate compound) in a dose dependent manner *in vitro*. Pure and co-formulated carbaryl exhibited different time and dose-dependent effects when treated *in vivo*, but they caused persistent and significant

inhibition of AChE. Thus, inhibition of AChE can be used as an indicator of pesticide contamination for soil toxicity monitoring (Gambi et al., 2007). Farrukh (2017), studied the chronic effect of endosulfan (organochlorine pesticide) on the AChE and cellulose enzyme of *Eisenia fetida* and reported that the long term exposure to endosulfan can lead to severe and irreparable effect on earthworms.

2.1.4 Cholinesterase activity in aquatic animals exposed to pesticides

Blood cholinesterase is a useful biomarker in case of organophosphate and carbamate poisoning (Hernández et al., 2005; Safi et al., 2005; Souza et al., 2005; Ng et al., 2009; Simoniello et al., 2010). AChE activity has been used as a complementary tool to chemical analysis in two important fish species *Mullus barbatus* and *Trachurus mediterraneus* and in mussels, *Mytilus galloprovincialis* in the industrialized area of Taranto of Salento peninsula (Lionetto et al., 2004). No significant differences in AChE activity was observed in the two fish species compared to the controls, while AChE activity was inhibited in mussels (Lionetto et al., 2004). The effect induced by exposure to chemical pollutants in native marine organisms from a coastal area, in Salento Peninsula (Italy) was studied by the integrated use of acetylcholinesterase (AChE) and antioxidant enzymes (catalase-CAT, glutathione peroxidise-GSH-Px) (Lionetto et al., 2003). *Mytilus galloprovincialis* (a sessile invertebrate) and *Mullus barbatus* (a benthic teleost fish), the two bioindicator species were included in the study at 8 sampling stations. Four sampling stations (non-urbanized) served as controls and the four other stations were exposed to anthropogenic agents. Lionetto et al. noted a significant difference in AChE activity in *M. galloprovincialis* in the sample sites. The reduction of AChE activity observed in two control stations may be due to leaching of pesticides into the sea from the agricultural lands or because of heavy metals. In *M. barbatus* significant

difference in AChE activity among the sample sites were observed. The activity was found to be inversely correlated with liver GSH-Px activity. Catalase activity did not differ significantly among animals sampled from different stations (Lionetto et al., 2003).

The effect of malathion and cadmium on acetylcholinesterase activity in fish *Seriola dumerilli* was studied by exposing the fish to various concentration of malathion for different periods and cadmium for 2 days (Jebali et al., 2006). In brain, acetylcholinesterase was inhibited after 2 and 7 days of exposure to malathion in a dose-dependent manner, while no inhibition was observed after 13 days of exposure. 50 µg/kg body weight of cadmium showed an increase in acetylcholinesterase activity, whereas higher doses, 100 and 250 µg/kg of cadmium revealed a strong dose-dependent inhibition of AChE activity (Jebali et al., 2006). Following cadmium treatment a rapid increase in malathion concentration was seen in liver, which suggested that the hepatic malathion concentration and brain acetylcholinesterase activity can be used as a biomarker to organophosphate and cadmium toxicity in fishes (Jebali et al., 2006). Vioque-Fernández et al. (2007) conducted a study in *Procambarus clarkii* (a species of cambarid freshwater crayfish) at Doñana National Park to assess the inhibitory effect of pesticides on AChE and carboxylesterase activities. The activities were significantly reduced in *P. clarkii* from affected sites compared to the ones from reference sites. It was also proposed that the metals inhibited the esterases too in combination with the pesticides, since high metal concentration was found at rice-growing sites compared to the other affected and reference sites (Vioque-Ferńandez et al., 2007).

2.1.5 Cholinesterase activity in rats and mice.

Tomokuni and Hasegawa (1985), determined the erythrocyte, plasma and brain ChE activities in rats and mice exposed to diazinon and observed that the ChE was most

remarkably inhibited in plasma of mice. Cocaine toxicity is supposed to cause low plasma cholinesterase activity. Cahill-Morasco et al. (1998) tested the cocaine toxicity in Swiss albino mice reared on high protein diet, low protein isocaloric diet and a protein with calorie deficit diet for 3 weeks. After acclimatization for 3 weeks the animals were allowed to feed on these diets for 3 additional weeks. The mice were treated intraperitoneally with a single dose of 75 mg/kg body weight cocaine and were kept on observation for 4 hours to record seizures and death. After 4 hours, ChE activity was measured. The cocaine toxicity was shown to be associated with reduced plasma cholinesterase activity by 4% and 10% in low protein isocaloric diet and protein with calorie deficit diet, respectively whereas the plasma cholinesterase activity remained stable for high protein diet group of mice. Recently, Santos et al. (2013), reported that the activity of plasmatic and erythrocyte ChEs in rats decreased from 29% to 0.5% and from 35.9% to 33% on increasing disulfoton (an organophosphate insecticide) dose from 0-6.6 mg/kg body weight.

2.1.6 Cholinesterase activity and clinical management of patients

Serum AChE level can be a helpful parameter to determine acute OP poisoning. Eddleston et al. (2008) measured BuChE activity and OP pesticide concentrations in the blood samples of patients related to either chlorpyrifos or dimethoate poisoning. Out of 91 patients, 25 died of dimethoate poisoning, whereas 11 out of 208 patients died of chlorpyrifos poisoning. Eddleston et al. found greater OP concentration in deceased patients compared to the ones who survived and suggested that BuChE activity should be interpreted correctly based on the type of the ingested OP.

OP pesticides act as an inhibitor of AChE which results in accumulation of neurotransmitter acetylcholine and continuous nerve firing. This could be treated with an

oxime antidote which reactivates the inhibited acetylcholinesterase and the biochemical effect of acetylcholine can be reversed with atropine (Kwong, 2002). However, certain studies reported that the oxime treatment was dependent on the concentration of OP in plasma and that the effects were minimal at high levels of OP in blood (Finkelstein et al., 1989).

Different clinical symptoms (vomiting, respiratory distress, lacrimation, abdominal pain) were reported to be associated with different level of reduction of plasma cholinesterase in human. Deceased patients had lowest plasma cholinesterase level and respiratory problems (Prasad et al., 2013). No significant difference was found in the serum acetylcholinesterase activity in patients with severe and mild organophosphate poisoning on the first day, but a significant difference was found in serum acetylcholinesterase activity in patients with non intermediate symptoms on days 1 and 3 than those with intermediate symptoms. No increase in serum acetylcholinesterase was noted on first and last day in patients who died compared to the patients survived (Aygun et al., 2002). In another study related to organophosphate poisoning, Chen et al. (2009) proposed that high mortality rates were associated with cases where the serum cholinesterase activity was not elevated and therefore, serum cholinesterase can play a role in management of patient within 48 hours of OP poisoning.

2.2 Materials and methods

2.2.1 Sampling area

The study was carried out in the tea gardens located in the Terai region of Darjeeling foothill specifically, Upper Bagdogra (26°68'16.73" N, 88°25'83.11" E) and Matigara (26°74'00.82" N, 88°37'83.69" E).

2.2.2 Characteristics of the participants

A total of 225 individuals consisting of 95 (60 males and 35 females) pesticide exposed tea garden workers between 15 and 62 years of age (mean age 35.37 ± 9.48), 60 (43 males and 17 females) pesticide non-exposed, non-smoker and non-alcoholic controls between 22 and 63 years of age (mean age 31.05 ± 9.93), 39 smokers (males) who smoked 6-30 cigarettes/day (different from the exposed ones) between 23 and 60 years of age (mean age 35.36 ± 11.47) and 31 alcoholic males consuming 60-120 ml alcohol/day (different from the exposed ones) between 24 and 55 years of age (mean age 39.94 ± 7.12) were analyzed. The pesticide exposed individuals were involved in either pesticide spraying/mixing (males) or tea leaf plucking (females) for at least 8h/day for 6d/week for at least a year and the work duration ranged from 1 to 30 years, hence were exposed to a mixture of pesticides simultaneously. The workers involved in the study were not found to use any kind of protective measures (gloves, breathing masks, protective goggles, impermeable boots, etc.). Most of the workers had self-reported symptoms like headache, abdominal pain, nausea, watery eyes and vomiting. The male individuals involved in pesticide spraying smoked cigarettes or bidi (a bidi is thin, Indian cigarette filled with tobacco flake) and consumed alcohol too. Few female individuals involved in tea leaf plucking were also found to smoke and all consumed alcohol. The control subjects (non-exposed, non-smokers and non-alcoholic) were from different walks of life who voluntarily participated in the study. Since the pesticide exposed workers smoked cigarettes and consumed alcohol, we have included 39 smokers and 31 alcoholics in our study. Control, smokers and alcoholics with apparently no exposure to genotoxic agents were randomly collected away from the target area (**Table 2.1**).

Personal information such as exposure period, smoking habit, alcohol consumption, drug intake, X-ray exposure, health status and any particular disease was obtained in the form

of a structured questionnaire. The individuals considered as 'smokers' were non-alcoholic and the 'alcoholics' consumed alcohol but did not smoke cigarettes. Individuals under any sort of medication or exposure to X-ray in the past 1 year were excluded from the study.

Table 2.1: Characteristics of the study population.

Variables	Control (N=60)	Tea garden workers (N=95)	Smokers (N=39)	Alcoholics (N=31)
Male	43	60	39	31
Female	17	35	0	0
Height(ft)	5.42 ± 0.26	5.08 ± 0.49	5.49 ± 0.25	5.46 ± 0.19
Weight(kg)	58.36 ± 5.38	47.81 ± 11.25	65.03 ± 9.82	66.37 ± 8.29
Age in years (mean ± SD)	31.05 ± 9.93	35.37 ± 9.48	35.36 ± 11.47	39.94 ± 7.12
Work Duration in years (mean ± SD)	0	14.27 ± 9.48	0	0
Smoking	No	Yes	Yes	No
Alcohol	No	Yes	No	Yes

2.2.3 Sampling procedure

Prior consent was obtained from each individual before the collection of blood. The venous blood was collected by venepuncture using 5ml sterile disposable syringe, transferred immediately in tubes containing EDTA as an anticoagulant and brought to the laboratory. Blood was processed for separation of plasma and erythrocytes for enzyme activity.

2.2.4 Plasma separation

1 ml whole blood was taken in 1.5 ml microcentrifuge tube and centrifuged at 5000 rpm for 3 minutes at 4^{o}C to remove plasma from blood. Supernatant plasma was removed carefully and kept on ice for further assay.

2.2.5 Erythrocyte separation

The erythrocyte pellet was washed twice with 0.9% saline by centrifugation at 1000 rpm for 2 minutes. Erythrocytes were suspended in 0.9% saline corresponding to the initial volume of whole blood for lysis. Hemolysate was prepared by diluting the cell suspension 600 times with 0.1M phosphate buffer, pH 8.0 (0.1M sodium phosphate monobasic, 0.1M sodium phosphate dibasic) and kept for 10 minutes at room temperature.

2.2.6 Measurement of enzyme activities of erythrocyte acetylcholinesterase

Enzyme activities were determined from blood samples following the protocol of Ellman et al. (1961) with slight modification. For the measurement of activity of AChE, 3 ml of hemolysate was pipetted into a quartz cuvette. To it 0.10 ml of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) or Ellman's Reagent (10 mM DTNB, 17.85 mM $NaHCO_3$ in 100 mM phosphate buffer pH 7.0) was added. The cuvette was placed in the spectrophotometer (Rayleigh UV-2601). 0.02 ml of acetylthiocholine solution (75 mmol acetylthiocholine iodide) was added to the cuvette. The blank consisted of hemolysate and DTNB. Change in absorbance was recorded at 60 s interval for 3 minutes at 412 nm. The activities were expressed as micromoles of acetylthiocholine iodide hydrolyzed per min per ml using an extinction coefficient of 13,600/cm/M.

2.2.7 Measurements of enzyme activities of plasma butyrylcholinesterase

BuChE activity was measured according to the protocol of Ellman et al. (1961) with slight modification. Plasma obtained above was diluted 150 times with phosphate buffer (0.1M pH 8.0) (0.1M sodium phosphate monobasic, 0.1M sodium phosphate dibasic) and kept for 10 minutes at room temperature. 3 ml of diluted plasma was pipetted into a quartz cuvette. To it 0.10 ml of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) or Ellman's Reagent (10 mM DTNB, 17.85 mM $NaHCO_3$ in 100 mM phosphate buffer pH 7.0) was added. The cuvette was placed in the spectrophotometer (Rayleigh UV-2601). 0.02 ml of acetylthiocholine solution (75 mM acetylthiocholine iodide) was added to the cuvette. The blank consisted of diluted plasma and DTNB. Change in absorbance was recorded at 60 s interval for 3 minutes at 412 nm. The activities were expressed as micromoles of acetylthiocholine iodide hydrolyzed per min per ml of plasma using an extinction coefficient of 13,600/cm/M.

2.2.8 Ethical consideration

The study was approved by the Institutional Human Ethics Committee of the University of North Bengal, Siliguri, District- Darjeeling, West Bengal, India [Ref No. Zoo/9114(i)/2015, dated 22 September 2015].

2.2.9 Statistical analysis

A descriptive analysis was performed and data were expressed as mean ± SD. Crosstable of groups versus gender along with Chi-square (χ^2) test for homogeneity has been done. Pearsonian product moment correlation coefficient and test for normality (Shapiro-Wilks test) of the parameters (four groups, sex) have also been done. The departure from the normality has been observed in very few cases. The problems of confounding factor due

to gender in case of control and exposed were resolved by multiple comparisons using the software SPSS version 21 (SPSS Inc., Chicago, IL). The level of significance was considered at 0.001 and 0.05. For testing the hypothesis of differences of means of parameters for AChE and BuChE across the groups and gender were carried out with the help of multivariate analysis of variance (MANOVA), multivariate analysis of covariance (MANCOVA) along with post-hoc (Tukey's test) for multiple comparison through generalised linear model (in case of normality of parameters, equivalent non-parametric Kruskal Wallis test was performed but the results of MANOVA and Kruskal Wallis were almost the same).

2.3 Results

Results showed an AChE activity of 11.81 ± 3.40 (2.74-12.40) µmoles/min/ml and 6.43 ± 1.85 (4.51-21.25) µmoles/min/ml in controls and tea garden workers, respectively. The difference was highly significant at $p \leq 0.001$. The measured AChE activities in smokers and alcohol consumers were 11.04 ± 2.48 (4.32-15.61) µmoles/min/ml and 12.45 ± 2.58 (7.19-17.63), µmoles/min/ml, respectively. The comparison with control did not reveal any significant difference. Similarly, BuChE activity of 4.73 ± 1.84 (1.52-9.58) and 3.50 ± 1.89 (0.08-7.28) µmoles/min/ml were recorded in control and exposed groups, respectively. The difference in the activity was significant at $p \leq 0.001$ (**Table 2.2**). BuChE activity recorded in controls, smokers and alcohol consumers were 4.73 ± 1.84 (1.52-9.58) µmoles/min/ml, 5.06 ± 1.87 (1.86-10.82) µmoles/min/ml and 5.02 ± 1.41 (2.41-7.23) µmoles/min/ml, respectively. The activities were not significantly different.

The activities of AChE and BuChE were also compared among the workers, smokers and alcohol consumers. Almost two fold higher activity of AChE in smokers (11.04 ± 2.48 µmoles/min/ml) and alcohol consumers (12.45 ± 2.58 µmoles/min/ml) than the exposed

workers (6.43 ± 1.85 µmoles/min/ml) was recorded. The difference between AChE activity was found to be highly significant ($p \leq 0.001$). The results showed a BuChE activity of 5.06 ± 1.87 µmoles/min/ml and 5.02 ± 1.41 µmoles/min/ml in smokers and alcohol consumers, respectively which was approximately 1.5 times higher than the exposed workers (3.50 ± 1.89 µmoles/min/ml). The difference was significant ($p \leq 0.001$) (**Table 2.3**).

Since the control and worker population consisted of both males and females, the activity of AChE and BuChE was also analyzed separately in the males as well as in the females. The exposed males showed almost two-fold lower activity of AChE (6.23 ± 1.59 µmoles/min/ml) which is significantly lower than the control males (11.15 ± 2.83 µmoles/min/ml) at $p \leq 0.001$. A two-fold decrease in the AChE activity was recorded in the exposed females (6.78 ± 2.21 µmoles/min/ml) than females in control group (13.47 ± 4.18 µmoles/min/ml). The difference was highly significant ($p \leq 0.001$). When BuChE activity was compared separately in males and females, the activities in the exposed males and females were found to be (3.15 ± 1.95 µmoles/min/ml) and (4.08 ± 1.66 µmoles/min/ml) which were slightly lower than their non-exposed control males (4.57 ± 1.86) and control females (5.14 ± 1.78), respectively however, the differences observed in both the groups were found to be significant at $p \leq 0.001$ (**Table 2.4**).

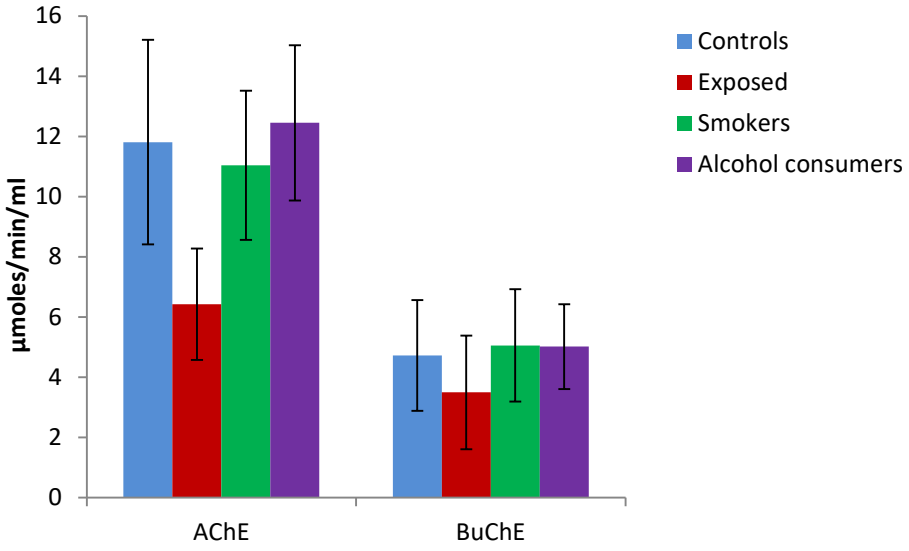
In order to test the effect of gender (sex) in the exposed and control groups, an analysis using MANOVA was also performed. Significant difference existed in the AChE activity when females and males of the controls were compared (13.47 ± 4.18 versus 11.15 ± 2.83 µmoles/min/ml, $p \leq 0.05$). A slight increase in the AChE activity was observed in the exposed females (6.78 ± 2.21 µmoles/min/ml) than the exposed males (6.23 ± 1.59 µmoles/min/ml) however, the difference was non-significant. When BuChE was analysed

gender wise in control females and control males, the activities were 5.14 ± 1.78 and 4.57 ± 1.86 µmoles/min/ml, respectively. The slight difference observed was not significant. Similarly, the BuChE activities were 4.08 ± 1.66 and 3.15 ± 1.95 µmoles/min/ml in exposed females and males, respectively. The analysis showed that the difference was significant at $p \leq 0.05$. The effect of gender (sex) observed in the present study appears to have very little influence on the AChE and BuChE activity, however, a wide range of individual variation exists (**Table 2.4**).

In an attempt to check whether age can be correlated with the activity, the exposed subjects were categorized into 3 groups of 15-30, 31-45 and 46-62 years of age. AChE activity was recorded to be 6.35 ± 1.87, 6.44 ± 1.68 and 6.60 ± 2.11 µmoles/min/ml in the age groups 15-30, 31-45 and 46-62 years, whereas BuChE activities were 3.31 ± 2.06, 3.65 ± 1.53 and 3.68 ± 2.06 in the age groups 15-30, 31-45 and 46-62 years, respectively (**Table 2.5**). Though not significant but reduced cholinesterase (AChE and BuChE) activities was found in the age group 15-30 years.

Table 2.2: Comparison of AChE and BuChE activity between controls, exposed, smokers and alcohol consumers.

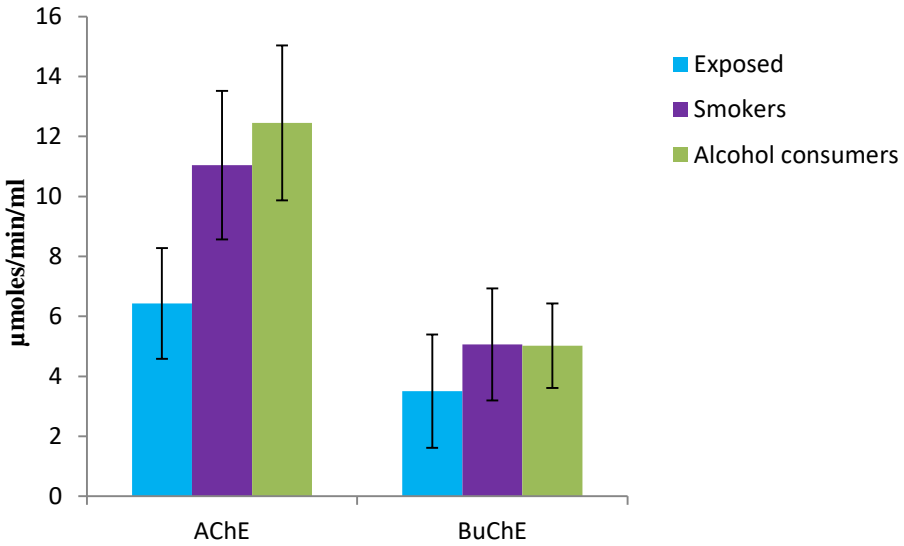
Groups	Enzyme activity	
	AChE	**BuChE**
Controls (N=60)	11.81 ± 3.40	4.73 ± 1.84
Exposed (N=95)	6.43 ± 1.85***	3.50 ± 1.89***
Smokers (N=39)	11.04 ± 2.48	5.06 ± 1.87
Alcohol consumers (N=31)	12.45 ± 2.58	5.02 ± 1.41



Results are expressed as mean ± standard deviation
Activities are measured in µmoles/min/ml
***$p \leq 0.001$ compared with control using MANOVA

Table 2.3: Comparison of AChE and BuChE activity between exposed, smokers and alcohol consumers.

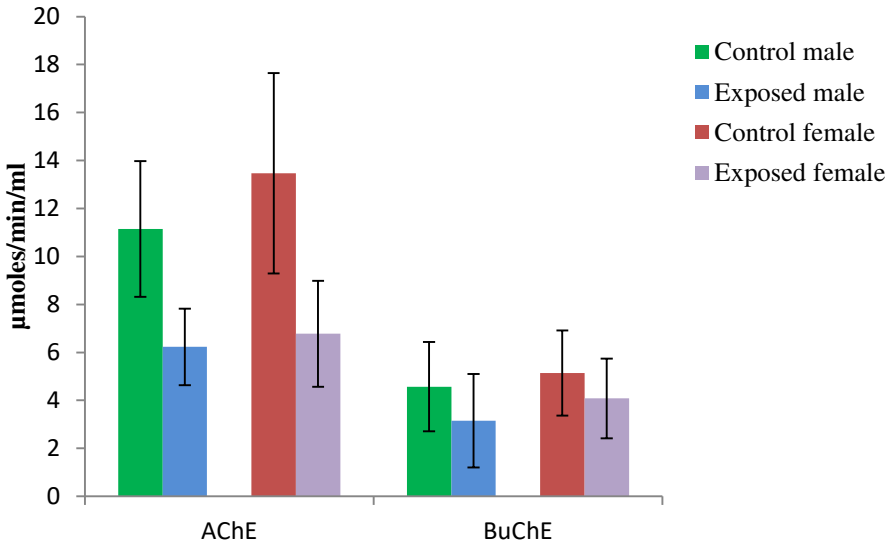
Groups	Enzyme activity	
	AChE	**BuChE**
Exposed (N=95)	6.43 ± 1.85	3.50 ± 1.89
Smokers (N=39)	11.04 ± 2.48***	5.06 ± 1.87***
Alcohol consumers (N=31)	12.45 ± 2.58***	5.02 ± 1.41***



Results are expressed as mean ± standard deviation
Activities are measured in µmoles/min/ml
***$p \leq 0.001$ compared with exposed using MANOVA

Table 2.4: Comparison of AChE and BuChE activity between males and females of control and exposed groups.

Groups	Enzyme activity	
	AChE	**BuChE**
Controls		
Male (N=43)	11.15 ± 2.83	4.57 ± 1.86
Female (N=17)	13.47 ± 4.18	5.14 ± 1.78
Exposed		
Male (N=60)	6.23 ± 1.59***	3.15 ± 1.95***
Female (N=35)	6.78 ± 2.21***	4.08 ± 1.66***



Results are expressed as mean ± standard deviation
Activities are measured in µmoles/min/ml
***$p \leq 0.001$ compared with control males and females using MANOVA

Table 2.5: AChE and BuChE activity of the exposed group classified according to age.

Age	Enzyme activity	
	AChE	**BuChE**
15-30 (N=45)	6.35 ± 1.87	3.31 ± 2.06
31-45 (N=30)	6.44 ± 1.68	3.65 ± 1.53
46-62 (N=20)	6.60 ± 2.11	3.68 ± 2.06

Results are expressed as mean ± standard deviation
Activities are measured in µmoles/min/ml
compared with individuals of age 15-30 years using MANOVA

2.4 Discussion

Health damages like carcinogenesis (Hagmar et al., 2001), neurotoxicity (Priyadarshi et al., 2000; Alavanja et al., 2004; Kamel and Hoppin, 2004), reproductive and developmental alterations (Hanke and Jurewicz, 2004) and immunological effects are associated with chronic exposure to pesticides (McCauley et al., 2006). The pesticides used in the tea plantations of Terai and Dooars of West Bengal comprise of a heterogeneous mixture of compounds belonging to organophosphates (OP), organochlorines and pyrethroids (Roy et al., 2008; Bishnu et al., 2009; Singh et al., 2015). OP and carbamate pesticides inhibit acetylcholinesterase (AChE), causing accumulation of acetylcholine at peripheral and central cholinergic receptors, leading to overstimulation of the cholinergic system (Altuntas et al., 2002) and subsequent paralysis. The butyrylcholinesterase (BuChE), also known as pseudocholinesterase, is abundant in plasma, liver, smooth muscle and fat cells (Simoniello et al. 2010) and can bind covalently to OPs and carbamates. Tea garden workers are at high risk of exposure to complex mixtures of pesticides. Under these circumstances, it becomes difficult to determine the effect of a single pesticide separately, however the measurement of AChE and BuChE activity can serve as a good biomarker of monitoring exposure to a mixture of pesticides which includes organophosphorus and the carbamates too, and to assess the health risk in the pesticide exposed human populations in the tea gardens of the Terai regions of Darjeeling foothills.

In the present study a population of tea garden workers (sprayers and tea leaf pluckers), non-exposed controls, smokers and alcohol consumers were analyzed. A significantly lower activity of AChE and BuChE in the exposed group indicated the inhibitory effect of organophosphate pesticides present in pesticide mixture (**Table 2.2**) (Tinoco-Ojanguren

and Halperin, 1998; Ali et al., 2008; Remor et al., 2009). Tinoco-Ojanguren and Halperin (1998) have reported the inhibition of erythrocyte cholinesterase by OP pesticides in three communities and control subjects of Mexico. Adverse health effects existed among the poorest communities. A study conducted by Ali et al. (2008) in female Pakistani agricultural workers exposed to pesticides revealed that serum cholinesterase activity was significantly lower in exposed than the unexposed females ($p < 0.001$). Remor et al. (2009) also reported a significant decrease in butyrylcholinesterase (BuChE) activity in farm workers from Rio Grande do Sul (Brazil) compared to the control group. In India, Singh et al. (2007) reported that the organophosphate and pyrethroid pesticides significantly inhibited the AChE and BuChE activities in the sprayer group of mango orchards of Malihabad, Lucknow and showed that these pesticides were neurotoxic. In a study, Vidyasagar et al. (2004) had reported decreased AChE activity with enhanced lipid peroxidation in OP exposed subjects. Similar results have also been shown by other workers (Panemangalore et al., 1999; Abu Mourad, 2005; Hernández et al., 2005; Jintana et al., 2009). Our result is consistent with the above findings. The inhibition may be due to the fact that organophosphate pesticides are capable of disrupting the cholinesterase activity resulting in acetylcholine accumulation in the synapses (Ali et al., 2008).

Moreover, the tea garden workers involved in spraying used knapsack sprayers to apply pesticides without any protective measures (like nose or mouth cover, gloves, impermeable boots nor washing hands or taking bath immediately after pesticide handling), therefore are at high risk of exposure to pesticide. This may enhance the exposure and subsequently decrease the enzyme activities. Various studies have indicated that the amount of exposure is influenced by use of protective equipments (Sivayoganathan et al., 1995; Gomes et al., 1999). In a study, Jintana et al. (2009) have shown a positive association between the use of personal protective equipment and the

AChE activity. Those who did not use personal protective equipment had comparatively lower AChE activity than those using personal protective equipment. However, Ntow et al. (2009) showed that practices such as protective cover, method of pesticide application, direction of pesticide spraying and farmer retry period had no significant association with the ChE activity. It was also shown by Nerilo et al. (2014) that merely washing hands and face after direct pesticide exposure was not effective for minimizing the exposure and the workers who used these procedures were intoxicated more than those who took bath after exposure.

As all the males and few females were found to smoke and consume alcohol, smokers (non-alcoholic and non-exposed) and alcoholics (non-smoker and non-exposed) were included along with the exposed and control group. Nicotine found in tobacco has been shown to exert numerous toxic effects on the central and peripheral nervous system (Nakayama et al., 1993). However, Dowla et al. (1996) have shown that nicotine has no inhibitory effect on plasma cholinesterase activity. Recently, Jintana et al. (2009) have also reported that smoking and alcohol consumption have no effect on the AChE and BuChE activity. In the present study a comparison with smokers and alcohol consumers showed no significant difference in the activity of AChE and BuChE compared to the control group (**Table 2.2**) which corroborates the findings of Dowla et al. (1996) and Jintana et al. (2009). A marked decrease of AChE and BuChE activity in the exposed group compared to the smokers and alcohol consumers strongly suggests that the low enzyme activities in the exposed group were mainly due to pesticide(s) exposure, which includes OPs too (**Table 2.3**).

Inspite of slightly high activities of AChE and BuChE in females compared to males, the sex biased activity differences was significant ($p \leq 0.05$) in the control groups for AChE

and in exposed group for BuChE only ($p \leq 0.05$) (**Table 2.4**). Despite of slight activity differences, a positive correlation between the sexes and enzyme activity was lacking which is also in agreement with the results of Maroni et al. (2000) showing no correlation between sex and the enzyme activity. Maroni and workers had also stated that AChE and BuChE activities may vary due to inter individual differences or due to specific physiopathological conditions in healthy people also. No effect of sex was observed on the mean whole blood ChE activity in a study conducted by Ntow et al. (2009). Moreover, in the present study, the females were only involved in plucking of tea leaves so were exposed to pesticides mainly through skin contact, whereas the males involved in spraying and mixing were more exposed to pesticides both dermally as well as through inhalation. But our results showing higher value of cholinesterase in females compared to males (though not significant in all cases) are in good agreement with the findings of Jintana et al. (2009) who have shown a higher activity of AChE and BuChE in females over high- and low- exposure periods. It has also been suggested that the hormonal and other physiological factors may modify or act as confounding factors on the effect of pesticide(s) exposure in females (Maroni et al., 2000). Moreover, the overall enzyme activity (**Table 2.2**) in a particular group and the gender wise activity (**Table 2.4**) were not different i.e., the enzyme activities were not influenced by a particular sex.

Younger workers had reduced cholinesterase activity in cases where they were engaged in more dangerous activities like they were assigned tasks that bear greater risk of pesticide exposure (López-Carillo and López-Cervantes, 1993; Singhaseni, 1999; Dhalla and Sharma, 2013). In the present study, AChE and BuChE activities were not significantly reduced in any particular age group, however slightly lower ChE activities observed in the 15-30 years age group reflect that these individuals might be engaged in the activities with greater risk (**Table 2.5**). López-Carillo and López-Cervantes (1993) reported the

serum cholinesterase activity to be 4.87, 4.74 and 4.28 KU/l in the age groups <20, 20-29 and 30+, respectively. Our results showing AChE activity of 6.35 ± 1.87, 6.44 ± 1.68, 6.60 ± 2.11 µmoles/min/ml and BuChE activity of 3.31 ± 2.06, 3.65 ± 1.53, 3.68 ± 2.06 µmoles/min/ml in the age groups 15-30, 31-45, 46-62, respectively are in accordance with López-Carillo and López-Cervantes (1993). However, Ntow et al. (2009) in their study reported that work practices that caused high exposures had no significant effect on the ChE activity of the exposed group.

Dose and duration of exposure has been hypothesized to be associated with changes in ChE activity (López-Carillo and López-Cervantes, 1993; Jintana et al., 2009; Dhalla and Sharma, 2013). Also, pesticide toxicity is related to its ability to inhibit acetylcholinesterase (Ecobichon, 2001). AChE is inhibited by OP pesticides in the order of chlorpyrifos > monocrotophos > profenofos > acephate, whereas BuChE is strongly inhibited by malathion, diazinon, chlorpyrifos and dichlorvos (Das et al., 2006; Jintana et al., 2009) suggesting that the sensitivity of the biomarkers can be assessed as per the chemical nature and mode of action of pesticides. The dose and duration of exposure and the sensitivity of biomarkers to the individual pesticide can be assessed only in controlled experiments. It was found that pesticides were applied throughout the year in the form of mixed formulations in the tea gardens; therefore a similar analysis was not possible in the present study.

Chakraborty et al. (2009) reported a 47% decline of AChE activity in the regular sprayers than control and a 25% decline in the occasional sprayers. Jintana et al. (2009) reported 30% and 26% inhibition of AChE and BuChE activity, respectively in individuals exposed to OP pesticides in Thailand. Agricultural workers in Kenya were reported to have 35% inhibition of AChE activity (Ohayo-Mitoko et al., 2000). Our results showing

47% and 26% inhibition of AChE and BuChE activity, respectively in the tea garden workers exposed to pesticide(s) are in concurrence with Ohayo-Mitoko et al. (2000) and Chakraborty et al. (2009). 50.6% lesser activity of ChE in the whole blood was observed in the farmers at Akumadan when they were compared to farmers (controls) from Tono Irrigation project, Ghana (Ntow et al., 2009). The guidelines of WHO for interpretation of erythrocyte AChE measurement state that 20-30% inhibition is an indicator of exposure, 30-50% inhibition is the indicator of hazard and 50% or greater is an indicator of poisoning (WHO, 1986). The percentage of AChE and BuChE inhibition in the exposed worker in the present study indicates exposure to pesticides (including OPs) and health hazards.

CHAPTER 3

Micronucleus Assay

3.1 Review of Literature

Micronuclei, also known as Howell-Jolly bodies, were first identified by an American, William Howell and a Frenchman, Justin Jolly in red cell precursors at the end of nineteenth century (Sears and Udden, 2012). Micronucleus are the acentric chromatid and chromosome fragments which lag behind when the centric elements move towards the spindle poles and are incorporated into the daughter nuclei (Schmid, 1975). The micronucleus (MN) assay is extensively used as a biomarker in human biomonitoring studies to evaluate the extent of chromosomal damage due to genotoxic agents or having susceptible genotype and instability of genome (Corvi et al., 2008; Glaviano et al., 2009; Weng and Morimoto, 2009). MN assay has greater accuracy as a large number of cells can be analysed in minimum time. The assay is popular over other biomonitoring studies as it is less invasive in terms of cell collection, low-cost, cells can be easily scored and ease of slide preparation with buccal epithelial cells makes it ideal for cytotoxic studies (Stich et al., 1982; Fenech et al., 2011; da Silva et al., 2012). It can be used to express the genotoxic effects of inhaled or ingested genotoxic agents and chemical metabolites being in immediate contact (Tolbert et al., 1991; Fenech et al., 1999). The basal layer of the buccal epithelia contains stem cells that may express genetic damage during nuclear division. Some of the exfoliated cells degenerate into cells with condensed chromatin, fragmented nuclei, pyknotic nuclei, karyolytic cells and some cells are blocked in binucleated stage or exhibit nuclear bud known as broken egg, a biomarker of gene amplification. These provide an assessment of genome damage (Suspiro and Prista, 2011; Kashyap and Reddy, 2012). Micronucleus can be identified from its round or oval shape. It is $1/16^{th}$ to $1/3^{rd}$ times the diameter of the main nucleus. It is non-refractile having the same or slightly darker staining intensity as the main nucleus (Fenech et al., 2003).

Micronucleus assay has been widely used in occupational and dietary studies, and also to study the effects of various carcinogenic agents on cells (Rosin, 1992; Nersesian, 1996; Majer et al., 2001; Kimura et al., 2004; Bonassi et al., 2005). Not only the micronuclei but also the nuclear buds, broken egg, karyolysis, karyorrhectic, pyknotic cells provide an important mechanistic insights into cytotoxic and genotoxic effects (Fenech, 2007). Any dividing tissue such as cervix epithelia, oesophagus, bladder, bronchial, nasal including buccal epithelium can be used for micronucleus evaluation (Kassie et al., 2001; Yadav and Sharma, 2008). The peripheral blood lymphocytes can also be used for MN assay to determine the effect of long-term exposure to a complex mixture of pesticides (Pastor et al., 2001).

3.1.1 Micronucleus assay in occupationally exposed pesticide workers

A number of studies have been conducted to demonstrate the efficiency of MN assay for detecting pesticide induced DNA damage (da Silva Augusto et al., 1997; Gómez-Arroyo et al., 2000). Croatian workers occupationally exposed to pesticides (containing atrazine, alachlor, cyanazine and 2,4-dichlorophenoxyacetic acid and malathion) were analysed for MN frequencies during the 8 months exposure to pesticides and after 8 months post exposure. It was observed that the frequency of MN was significantly higher in the exposed group compared to the control. Garaj-Vrhovac and Zeljezic (2001) showed that the MN frequencies were slightly reduced 8 months after the exposure but the frequency was still higher than control. Gómez-Arroyo et al. (2000) reported the MN frequencies in the pesticide exposed floriculture workers which was three times higher than the non-exposed subjects.

In a study female Pakistani agricultural workers exposed to pesticides were assayed for binucleated cells with micronuclei (BNMN) and total number of micronuclei in

binucleate cells (MNL) compared to control. Micronuclei were found to increase with age, though the increase was greater in the exposed group compared to the control (Ali et al., 2008). Brazilian workers in soybean fields exposed to pesticides showed significantly higher number of cells with micronucleus in exposed than non-exposed control. Age, smoking habit, smoking time, number of cigarettes/day, alcohol consumption and years of pesticide exposure was not found to influence MN frequency (Moura de Bortoli et al., 2009). A study carried out in Assam, India revealed a significant increase in cell death parameters in female tea garden workers compared to control. A strong positive correlation was found between the duration of occupation and micronucleus frequency and cell death parameters (Kausar et al., 2014). Recently, Gaikwad et al. (2015) reported a significant rise in the micronucleated cells of buccal mucosa among the pesticide sprayers of grape garden than the control group. In the same study a significant decrease of WBC count and a significant increase of uric acid and malondialdehyde level in urine supported the genotoxic effect of the pesticides. Studies have shown that exposure to pesticides also cause increased miscarriages (Pastor et al., 2001). A number of other studies have positively correlated the increase in MN and pesticide exposure (Costa et al., 2007; Ergene et al., 2007; Benedetti et al., 2013).

3.1.2 No effect of pesticides on micronucleus formation

Whereas there are many studies which prove that pesticide exposure cause increase in MN frequencies, others show that there is no such relationship between the two (Scarpato et al., 1996; Lucero et al., 2000; Remor et al., 2009). Micronucleus assay performed with the epithelial cells of oral mucosa to assess genotoxic effects on floriculturist in Brazil showed no statistical difference in the frequencies of micronuclei and other nuclear anomalies (Wilhelm et al., 2015).

There was no statistically significant difference in the MN frequencies conducted on buccal cells and peripheral blood lymphocytes between the Polish farmers exposed to pesticides and the control subjects. In case of lymphocytes multiple linear regression analysis indicated that alcohol inversely influenced the studied cytogenetic endpoints while in case of buccal cells, a negative binomial regression indicated that the MN values were directly influenced by the ingestion of red meat. An inverse negative relationship existed between the cytokinesis-block proliferation index and age due to the exposure to pesticides. In the same study an inverse negative relationship was found between MN frequency and age (Pastor et al., 2001). Age, gender, personal protective equipment, mode of application of pesticides and alcohol consumption had no significant effect on genetic damage (Sailaja et al., 2006; Benedetti et al., 2013).

3.1.3 Micronucleus assay in individuals exposed to environmental toxic pollutants

Besides the monitoring of the effects of pesticides and other toxic substances, the MN assay can also be used to determine the effects of occupational hazard in humans. Cytokinesis-block micronucleus assay (CBMN) in the peripheral blood lymphocytes of underground coal miners for assessment of DNA damage revealed significant increase in the frequency of binucleated cells with micronuclei, nucleoplasmic bridges and protrusions compared to the non-exposed control groups (Sinitsky et al., 2016). However age, smoking and the length of service were not correlated to the level of chromosomal damage (Sinitsky et al., 2016). Thus, the CBMN can be useful in studies related to genotoxicity, to monitor hygiene and to adopt preventive measures in occupational environment.

Cytogenetic damage due to occupational exposure to the petrol and petroleum derivatives such as benzene was analyzed by micronucleus assay in exfoliated buccal cells. The results revealed a significantly higher MN frequencies and nuclear abnormalities (binucleated cells, karyorrhexis and karyolysis) in the petrol station workers compared to the control subjects. Smoking too had a significant effect on the damage parameters (Çelik et al., 2003). In a similar study in Indore (India), Singaraju et al. (2012) showed higher MN and nuclear abnormalities ($p \leq 0.01$) in the petrol station workers suggesting genotoxic effect of the petrol. Statistically significant increase in micronucleated cells was observed in the car painters than non-exposed individuals; however, other nuclear alterations (karyorrhexis, pyknosis and karyolysis) did not reveal any significant correlation. The findings from this study indicated that the car painters are at higher risk of damage as paints are capable of inducing genotoxic and mutagenic effects in oral mucosa cells (da Silva et al., 2012). Micronucleus assay has also been extended to the exfoliated buccal epithelial cells of occupational workers exposed to ashes from burning sugarcane that generates polycyclic aromatic hydrocarbons (PAH) (Martínez-Valenzuela et al., 2015). Significantly higher frequencies of MN and other nuclear abnormalities, like binucleated cells, pyknosis, karyolysis, condensed chromatin and nuclear buds indicate that the PAHs generated by sugarcane burning have genotoxic effects and the workers are at high risk of genetic damage (Martínez-Valenzuela et al., 2015).

3.1.4 Micronucleus assay in smokers/ tobacco chewers/ alcohol consumers

There are number of MN studies in the smokers, tobacco chewers and alcohol consumers. Wu et al. (2004) performed micronucleus assay to detect clastogenic effect of areca quid chewing and cigarette smoking and showed a significant relationship between smoking (>20 cigarettes/day) and micronucleus formation. A non-significant relationship was

found for the interaction between smoking and areca quid use. Clastogenic effect for areca quid chewing was not detected (Wu et al., 2004). Sadagura, a smokeless tobacco preparation contains the sun dried and roasted tobacco leaves in combination with fenugreek seed and aniseed for flavor is used by the people of southern Assam province of North-East India (Kausar et al., 2009). Kausar et al. (2009) reported a significant increase in the frequency of MN in sadagura chewers, smokers, betel quid with sadagura and smokers chewing betel quid with sadagura compared to the control subjects which suggests that sadagura chewed alone or in combination with betel quid resulted in genome damage. Other studies have also shown significantly high frequency of micronuclei in peripheral blood lymphocytes in smokers than non-smokers along with high frequency of apoptotic cells. The damage was also influenced by the age and duration of smoking (Haveric et al., 2010; Naderi et al., 2012).

The impact of tar and nicotine contents of cigarettes on chromosomal damage was studied in exfoliated buccal cells by evaluating the effect of smoking different types of cigarettes (i.e., ultralight filter, light filter, medium filter and unfiltered cigarettes) on induction of nuclear anomalies like micronuclei, broken egg, binucleates, condensed chromatin, karyorrhexis, karyolysis and pyknosis. The significant increase in micronuclei was noticed in individuals smoking non-filtered (NF) cigarettes. Broken eggs and binucleates were also shown to increase in medium filter (MF) smoking groups. Karyolysis and karyorrhexis were increased in both NF and MF groups but not in smokers of light cigarettes (Nersesyan et al., 2011). MN assay was performed in buccal epithelial cells and blood lymphocytes of cigarette smokers (heavy and light) compared to non-smokers in Tamil Nadu (India). It was observed that the MN frequency was higher in smokers particularly in heavy smokers (Christobher et al., 2017).

The frequencies of MN and other metanucleated anomalies in the buccal epithelial cells with oral and oropharyngeal carcinoma were studied in alcoholics compared to control subjects (Ramirez and Saldanha, 2002). A seven-fold increase in MN frequency in area opposite to the lesion, three-fold increase around the lesion and two-fold increase (non-significant) in the upper gingival-labile gutter were recorded. Contradictory results were obtained from regression analysis which showed that MN frequency decreased with age and alcohol consumption (Ramirez and Saldanha, 2002). Markers of genome instability, commonly seen in cancer such as binucleated cells with micronuclei, nucleoplasmic bridges and nuclear buds were shown to be induced in case of chronic exposure to alcohol (Benassi-Evans and Fenech, 2011). In a study conducted by dos Santos Rocha et al. (2014), individuals who used both mouthwashes and alcoholic drinks had highest frequency of micronuclei compared to those who used only mouthwashes, though the difference was not significant. Alcohol consumption is a common practice throughout the world. Recently the genotoxic effect of alcohol was reported by Jeeva Priya et al. (2015) by performing micronucleus test on the alcohol consumers and control subjects and showed a higher frequency of micronuclei in alcohol consumers than the control subjects. Singh et al. (2015) have also found increased MN and other nuclear anomalies due to alcohol consumption.

3.1.5 Micronucleus and ionizing radiation

In a study conducted by Angelini et al. (2005) showed significantly higher frequency of micronuclei in the workers exposed to low level of ionizing radiation compared to controls. Workers with allelic variants of *XRCC*1 or *XRCC*3 or wild type allele *XPD* exon 23 or 10 polymorphisms had higher MN frequency compared to controls having similar genotypes. In the same study smoking was not found to influence MN frequency,

however age and sex were positively correlated. Females had higher MN than males (Angelini et al., 2005). Human fibroblast was exposed *in vitro* to neutron beams at Los Alamos Nuclear Science Center and the MN induction was analyzed. In order to study the effect of shielding on the protection of neutron induced damages, the cells were placed behind water column. A linear dose response in the MN frequency was observed for the samples with and without the shielding. The slope of yield of MN was reduced by a factor of 3.5 behind shielding. When the MN induction in human fibroblast was exposed to similar low dose of γ source, the relative biological effectiveness of neutrons was found to be 16.7 and 10.0 with and without the water shielding, respectively (Gersey et al., 2007). Automated MN scoring was used to distinguish severely exposed individuals of large-scale radiation accidents. The results confirmed that automated MN assay can be used efficiently for fast population triage (Willems et al., 2010). Ribeiro and Angelieri (2008) also studied the MN formation in exfoliated buccal cells from healthy individuals following dental X-ray exposure and showed that dental panoramic radiography did not induce chromosomal damage but cytotoxicity was promoted. They suggested that dental X-ray should only be used on necessity (Ribeiro and Angelieri, 2008). Recenly, Qian et al. (2016) designed a study to investigate the effects of ionizing radiation on micronuclei using peripheral blood lymphocytes from 1392 radiation workers and 143 controls and reported significantly higher frequency of MN in the exposed group than controls. The results also indicated that chronic-exposure to low level ionizing radiation may have harmful effect on the health of radiation workers (Qian et al., 2016).

3.1.6 Micronucleus assay for assessing environmental exposure

Micronucleus is a good biomarker used to assess the genotoxic effect of environmental pollutants/toxicants. Ghosh et al. (2008) reviewed cytogenetic damage in arsenic exposed

populations by evaluating different cell types and concluded that for studying arsenic-induced micronuclei, the lymphocytes may be superior to other epithelial cells. Fenech and Crott (2002) analyzed folic acid deficit lymphocytes by cytokinesis block micronucleus assay and showed that folic acid concentration was significantly as well as negatively correlated with chromosomal damage that indicated genome instability and gene amplification due to folic acid deficiency.

Chromosomal damage due to environmental mutagen exposure was studied by measuring MN by automated image analysis in human peripheral blood lymphocytes. The results demonstrated the ability of carcinogenic polycyclic aromatic hydrocarbons (c-PAHs) to increase MN frequency even when the exposure occurred up to 60 days prior to blood collection (Rossnerova et al., 2009).

Assessment of air pollution during pregnancy and its association with MN frequency was studied in mothers and newborns. An association was found between particulate matter of diameter below 2.5 μm ($PM_{2.5}$) and MN frequency in mothers, especially among those mothers who did not fulfil recommended vitamin C allowances during pregnancy. Smoking was associated positively with higher level of air pollution as these caused an increased MN frequency (O'Callaghan-Gordo et al., 2015). Cytokinesis-block micronucleus (CBMN) assay was performed to see the effect of road traffic exposure in Danish school children and their mothers (Mørck et al., 2016). The studies showed a significant correlation between mothers and children with respect to the frequency of micronuclei. 2.5 times higher micronuclei in mononucleated T lymphocytes were found in children living within the 50m of busy road (Mørck et al., 2016).

3.1.7 Micronucleus assay on rats and mice

Increased genome damage has been associated with exposure to mixture of pesticides in the occupational environment (Bolognesi, 2003). MN test carried out in the bone marrow of mice to see the genotoxic effect of 1-nitropropane, 2-nitropropane (Nitroparaffins used as solvents in chemical and pharmaceutical industries) and cisplatin (chemotherapeutic compound to treat human malignancies) revealed negative results for the first two compounds, but a dose-dependent increase in the number of polychromatic erythrocytes with micronuclei was noticed for cisplatin (Kliesch and Adler, 1987). Micronucleus test can also be performed in cultured human lymphocytes and rat bone-marrow to assess the pesticide induced genotoxicity (Demsia et al., 2007). Two widely used pesticides imidacloprid (a systemic chloronicotinyl insecticide) and metalaxyl (a systemic benzenoid fungicide) were tested separately as well as in mixed concentrations for three different doses on cultured lymphocytes. In spite of increase in the frequency of MN in a test with single or mixed pesticides, the results were non-significant. The micronucleated polychromatic erythrocytes in rat bone-marrow were significant for a dose up to 300 mg/kg body weight of imidacloprid and metalaxyl when treated separately, whereas a dosage up to 200 mg and 400 mg per kg body weight were significant for compounds, respectively when treated in combination (Demsia et al., 2007). The rat and mouse models are commonly used to check the effect of single pesticide. The genotoxic effect of lindane and endosulfan (organochlorine pesticides) and chlorpyrifos and monocrotophos (organophosphate pesticides) was studied by Yaduvanshi et al. (2012) *in vivo* in mouse bone marrow cells. The results showed that these pesticides either alone or in combination caused significantly higher damage evidenced from the concomitant increase of MN with respect to increase of pesticide concentration. Dichlorophene, an organochlorine pesticide was found to have significant cytogenetic damage on bone

marrow cells of *Rattus norvegicus* causing an increase in MN and chromosomal aberration (Lone et al., 2013). Micronucleus assay and Comet assay were carried out on male Sprague-Dawley rats to evaluate the genotoxic effect of six isomers of Dinitrotoluene (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, 3,5-DNT), which are found to contaminate the soil and water near ammunition production plants. The results revealed 2,6-DNT caused DNA damage in liver tissue while the frequency of peripheral nucleated reticulocytes was unaffected at all treated doses (Lent et al., 2012). Therefore, MN assay is an easy and quick technique in pharmaceutical and chemotherapeutic assessment.

3.1.8 Micronucleus assay on fish

MN test in fish can also be used as a suitable biomarker for *in situ* biomonitoring of genotoxic pollution in marine environment (de Moraes Pantaleão et al., 2006; Corredor-Santamaría et al., 2016). A study was conducted on fish to monitor the genotoxic effects of industrial waste products in the Baltic sea (Sweden) at three sites, located at 2 km (station 1), 4.5 km (station 2) and 8 km (station 3) from the industrial waste discharge point. MN test for this investigation showed mostly a decrease of micronuclei with the increase in sampling distance from the waste discharge points (Al-Sabti and Hardig, 1990). Çavaş and Ergene-Gözükara (2005) performed MN test in peripheral blood erythrocytes and gill cells of the grey mullet (*Mugil cephalus*) from three sampling stations off the southern Mediterranean coast of Turkey on the basis of pollution level and showed that the frequencies of micronuclei and the other nuclear abnormalities were significantly higher in mullets captured from polluted sites compared to the mullets captured from relatively unpolluted sites i.e. micronuclei induction decreased with increasing sampling distance from the waste discharge point. Two fresh water fish species *Astyanax bimaculatus* and *Hoplias malabaricus* have been used as *in situ* biological

indicator of chemical contamination of Japaratuba river, Brazil. The results indicated that micronucleus test in fish can be a useful biomarker to detect chemical contamination of aquatic environment. The test also showed that *A. bimaculatus* and *H. malabaricus* are differentially sensitive to MN induction (de Moraes Pantaleão et al., 2006).

3.1.9 Artificial induction of micronucleus

Presence of micronuclei has been studied in various types of cancer (Arora et al., 2010; Dey et al., 2012). N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide (captan), a fungicide, which inhibits the growth of fungi on plants used as food stuffs. Two amphibian larva (*Xenopus laevis* and *Pleurodeles waltl*) were used to evaluate the genotoxic potential of captan using the MN test and comet assay. MN test revealed the genotoxic nature of captan at 62.5 µg/L for *Xenopus laevis* but not for *Pleurodeles waltl*. The differential behavior could be due to the influence of other factors on the generation of MN (Mouchet et al., 2006).

The review of literature suggests that MN assay is an easy, cheap and quick method for assessment of genotoxic effect of various compounds/chemicals and have immense clinical as well as pharmaceutical applications.

3.2 Materials and methods

3.2.1 Sampling area

Sampling area was the same as mentioned under **section 2.2.1**

3.2.2 Characteristics of the participants

As the individuals used for enzyme assay were also used in micronucleus test, the characteristics of the participants were the same as mentioned under **section 2.2.2 Characteristics of the participants.**

3.2.3 Sampling procedure

After obtaining consent, the buccal epithelial cells were collected from healthy volunteers who were not under any medication or were not exposed to the X-ray during the past 6 months. The individuals were asked to rinse their mouth with 0.9% saline water. Then with the help of a sterile stainless steel spatula the buccal epithelial cells were obtained from inner cheeks, suspended in 0.9% saline, brought to the laboratory, washed twice in normal saline through centrifugation at 2000 rpm at room temperature for 5 min. Supernatant was discarded. The cells (pellet) were fixed in 1:3 aceto-methanol. Slides were prepared by air-drying method.

3.2.4 Pap staining and scoring

The slides were stained by Papanicolaou (Pap) method as described by Mondal et al. (2011). The slides were passed through graded ethanol (100-50%) for 2 minutes in each step and stained with Harris' Hematoxylin for 2 minutes. The slides were rinsed in distilled water and treated in Scott's tap water ($MgSO_4.7H_2O$:$NaHCO_3$=10:1) substitute for bluing, washed in running tap water and dehydrated through 50%, 70%, 90% and absolute ethanol for 2 minutes in each step. The slides were stained in Orange-G6 for 20 min and differentiated in 95% alcohol. Then the slides were incubated in EA-50 solution for 20 minutes at room temperature, dehydrated in absolute ethanol (100%), cleared in xylene, and mounted with distrene plasticizer xylene (DPX). The slides were coded and

examined under a light microscope (Nikon Eclipse E200, Nikon, Tokyo, Japan) with the 100x oil immersion objective for the detection of micronuclei (MN) and the other nuclear anomalies like nuclear bud (NB), binucleate (BN), karyorrhectic (KH), pyknotic (PY), and karyolytic (KL) cells.

3.2.5 Scoring criteria

Two slides were used for each individual. For each individual, 1000 non-overlapping, well-differentiated, uniformly stained nuclei of buccal epithelial cells were analyzed. The cells, which were considered for MN assay, have (i) round or oval shape (ii) diameter ranged between 1/3rd and 1/16th of the main nucleus (iii) same staining intensity and texture as the main nucleus (iv) MN located within the cytoplasm of the cells and (v) usually have one MN per cell, but the number could be two or more following severity of genotoxic insult. Besides MN, other nuclear anomalies like "broken egg" (cells with nuclear buds), binucleate cells, karyorrhectic (nuclear disintegration), pyknotic (shrunken nuclei), and karyolytic (dissolution of the nucleus) were evaluated following the established criteria (Tolbert et al., 1991; Thomas et al., 2009).

3.2.6 Ethical consideration

Ethical consideration was the same as mentioned under **section 2.2.8.**

3.2.7 Statistical analysis

To represent the micronuclei data a descriptive statistical analysis was performed and data were expressed as mean ± SD. Crosstables along with Chi-square (χ^2) test for homogeneity of (a) four groups versus age group and (b) four groups versus gender have been done. Pearsonian product moment correlation coefficients and Spearmen rank

correlation coefficient of the parameters with age along with t-test for testing significance of correlation coefficients were also performed. Only few cases showed significance of correlation coefficients. Test for normality (Shapiro-Wilks test) of the parameters (four groups, sex) showed departure from the normality only in some cases. The testing of hypothesis of the differences of means across the groups and gender were carried out with the help of multivariate analysis of variance (MANOVA), multivariate analysis of covariance (MANCOVA) along with post-hoc (Tukey's test) (for multiple comparison) through generalized linear model (in case of non-normality of parameters, equivalent non-parametric Kruskal-Wallis test was performed but the results of MANOVA and Kruskal-Wallis were almost same). Except few sporadic cases, no significant effect of age on the parameters was noticed however, effect of gender has been observed. The problems of confounding factors due to gender, cigarette smoking and alcohol consumption were solved by multiple comparisons using the software SPSS version 21 (SPSS Inc., Chicago, IL). The level of significance was considered at 0.001, 0.01, and 0.05.

3.3 Results

1000 buccal epithelial cells were scored to evaluate the number of cells with micronuclei, nuclear bud, binucleate, karyorrhectic, pyknotic and karyolytic nuclei. Evaluation of epithelial cells revealed a mean frequency of 9.77 ± 2.66 micronuclei in the tea garden workers which was almost two-fold higher than the control (4.37 ± 2.04) highly significant at $p \leq 0.001$. The other nuclear abnormalities such as nuclear bud (4.39 ± 1.42, $p \leq 0.001$), binucleate (6.12 ± 2.92, $p \leq 0.001$), karyorrhectic (8.01 ± 2.29, $p \leq 0.001$), pyknotic (5.74 ± 1.81, $p \leq 0.05$) as well as karyolytic (6.89 ± 2.98, $p \leq 0.001$) nuclei were also found to increase 1.38, 2.23, 1.79, 1.21, 1.48 times in comparison to the non-exposed group (control) (**Table 3.1**). Buccal epithelial cells showing normal nuclei, micronuclei,

nuclear bud, binucleated nuclei, karyorrhectic, pyknotic and karyolytic nuclei are shown in **Figures: 3.1, 3.2, 3.3, 3.4, 3.5, 3.6 and 3.7.**

Comparison of the smokers and control subjects revealed a higher frequency of micronuclei (6.15 ± 2.47, $p \leq 0.001$), and other cell death parameters like nuclear bud (4.13 ± 2.35, $p \leq 0.05$), binucleate (4.38 ± 2.24, $p \leq 0.01$), karyorrhectic (6.67 ± 2.30, $p \leq 0.001$) and karyolytic (6.18 ± 2.19, $p \leq 0.01$) cells in the smoker group. Though the frequency of pyknotic cells were slightly elevated in the smokers (5.03 ± 2.31) than controls (4.75 ± 2.49), the difference was non-significant (**Table 3.1**).

Results also showed a higher frequency of binucleate (3.97 ± 1.99, $p \leq 0.05$), karyorrhectic (5.55 ± 1.84, $p \leq 0.05$) and karyolytic (6.29 ± 2.64, $p \leq 0.01$) nuclei in the alcoholics compared to the control (2.75 ± 2.33, 4.48 ± 1.80, 4.67 ± 2.01) (non-alcoholics). A non-significant relation was found between micronucleus, nuclear bud and pyknotic cells when compared with the control subjects (**Table 3.1**).

The male workers involved in the study were found to mix pesticides and were involved in the task of pesticide spraying. The frequencies of micronuclei, nuclear buds and binucleate were 10.87 ± 2.38, 4.90 ± 1.35 and 6.98 ± 3.04 in males and 7.89 ± 1.98, 3.51 ± 1.07 and 4.63 ± 1.97 in females (leaf harvester), respectively. The frequencies in males were significantly higher ($p \leq 0.001$). On the other hand the frequency of the karyorrhectic, pyknotic and karyolytic cells in males and females were not significantly different (**Table 3.1**). No significant differences were observed in the damage parameters between males and females of the control group (**Table 3.1**).

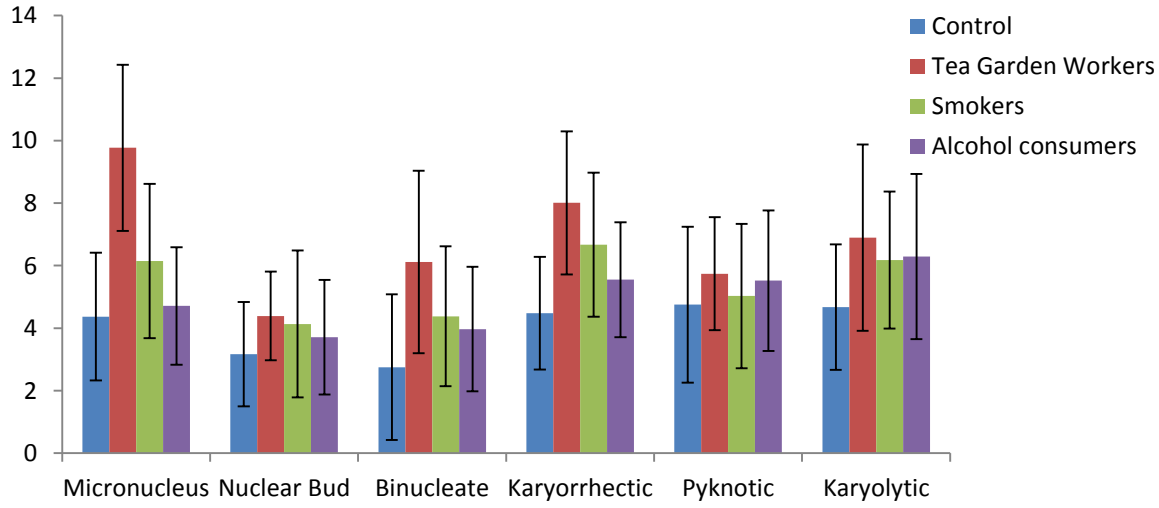
A comparison was also made between smokers, smoking less than 10 cigarettes per day (group I) and those smoking more than 10 cigarettes per day (group II). The group II showed a higher number of cells with micronuclei (7.72 ± 1.60, $p \leq 0.001$), nuclear bud (5.61 ± 2.28, $p \leq 0.001$), binucleate (5.83 ± 1.72, $p \leq 0.001$), karyorrhectic (8.00 ± 1.33, p

≤ 0.01), pyknotic (6.28 ± 2.05, $p \leq 0.01$) as well as karyolytic (7.72 ± 1.32, $p \leq 0.001$) cells compared to the group I (**Table 3.2**).

The male tea garden workers in the present study smoked cigarettes and consumed alcohol too. Only few female workers were found to smoke, while all the female workers under study consumed alcohol. So a comparison was made between the tea garden workers with the smokers and alcohol consumers. The analysis revealed that the frequency of micronucleus, binucleate and karyorrhectic nuclei in the smokers were 6.15 ± 2.47, 4.38 ± 2.24 and 6.67 ± 2.30, respectively, comparatively lower than the tea garden workers significant at $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$, respectively. When the tea garden workers were compared with the alcohol consumers, the cells with micronucleus (4.71 ± 1.88, $p \leq 0.001$), nuclear bud (3.71 ± 1.83, $p \leq 0.05$), binucleate (3.97 ± 1.99, $p \leq 0.001$) and karyorrhectic (5.55 ± 1.84, $p \leq 0.001$) nucleus were significantly lower in the alcohol consumers compared to the tea garden workers (**Table 3.3**). The pyknotic and karyolytic nuclei in the tea garden workers, smokers and alcohol consumers were not significantly different (**Table 3.3**). Though cells with nuclear bud were slightly higher in the tea garden workers compared to the smokers, the difference was non-significant.

Table 3.1: Frequency of micronucleus and other nuclear anomalies in the exfoliated buccal epithelial cells of control, tea garden workers, smokers and alcoholics, and comparison of nuclear biomarkers between males and females in pesticide exposed workers and control. For each participant, 1000 cells were scored.

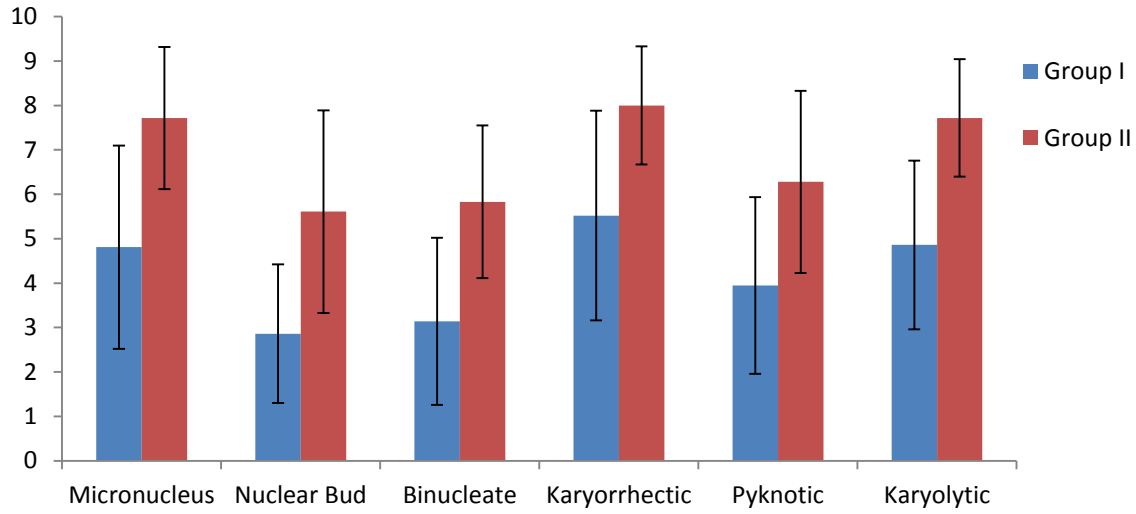
	Micronucleus	Nuclear Bud	Binucleate	Karyorrhectic	Pyknotic	Karyolytic
Control(N=60)	4.37± 2.04	3.17±1.67	2.75±2.33	4.48±1.80	4.75±2.49	4.67±2.01
Male(N=43)	4.40±2.16	3.21±1.60	2.67±2.24	4.67±1.91	4.84±2.62	4.40±2.13
Female(N=17)	4.29±1.76	3.06±1.89	2.94±2.61	4.00±1.41	4.53±2.21	5.35±1.50
Tea Garden Workers(N=95)	9.77±2.66***	4.39±1.42***	6.12±2.92***	8.01±2.29***	5.74±1.81*	6.89±2.98***
Male(N=60)	10.87±2.38***	4.90±1.35***	6.98±3.04***	8.12±2.29	5.92±1.79	7.27±2.93
Female(N=35)	7.89±1.98	3.51±1.07	4.63±1.97	7.83±2.33	5.43±1.82	6.26±3.00
Smokers(N=39)	6.15±2.47***	4.13±2.35*	4.38±2.24**	6.67±2.30***	5.03±2.31	6.18±2.19**
Alcohol consumers(N=31)	4.71±1.88	3.71±1.83	3.97±1.99*	5.55±1.84*	5.52±2.25	6.29±2.64**



Results are expressed as mean ± standard deviation
* $p \leq 0.05$ compared with control using MANOVA
** $p \leq 0.01$ compared with control using MANOVA
*** $p \leq 0.001$ compared with control using MANOVA

Table 3.2: Comparison of nuclear biomarkers between smokers Group I (smoking less than 10 cigarettes/day) and Group II (smoking more than 10 cigarettes/day). For each participant, 1000 cells were scored.

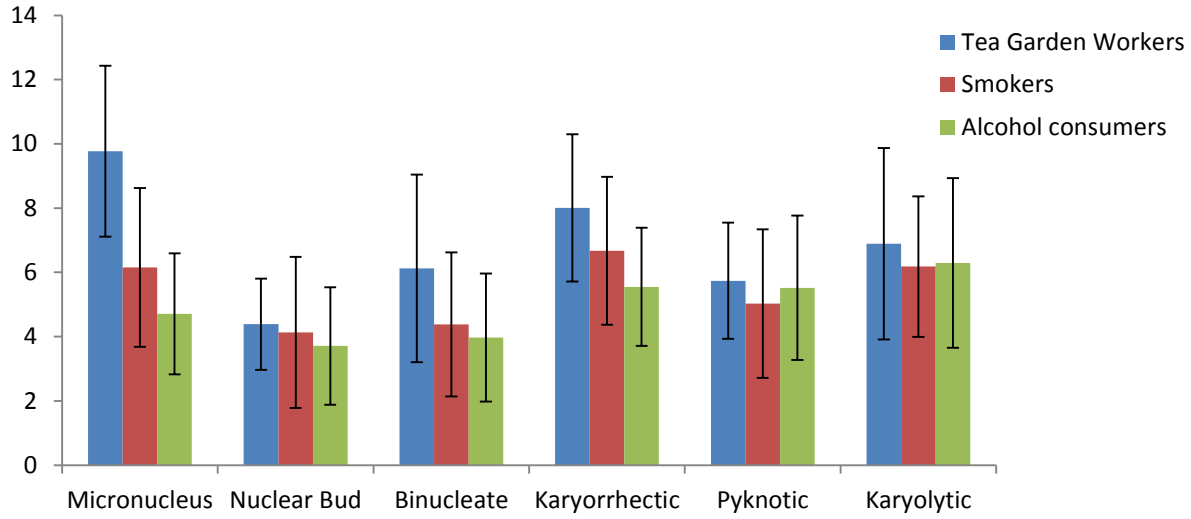
	Comparison between two groups of smokers	
	<10 cigarettes per day (Group I)(N=21)	**>10 cigarettes per day (Group II)(N=18)**
Micronucleus	4.81 ±2.29	7.72±1.60***
Nuclear bud	2.86±1.56	5.61±2.28***
Binucleate	3.14 ±1.88	5.83±1.72***
Karyorrhectic	5.52±2.36	8.00±1.33**
Pyknotic	3.95±1.99	6.28±2.05**
Karyolytic	4.86±1.90	7.72±1.32***



Results are expressed as mean ± standard deviation
** $p \leq 0.01$ compared with Group I smokers
*** $p \leq 0.001$ compared with Group I smokers

Table 3.3: Comparison of nuclear biomarkers between tea garden workers, smokers and alcohol consumers. For each participant, 1000 cells were scored.

	Tea garden workers (N=95)	**Smokers (N=39)**	**Alcohol consumers(N=31)**
Micronucleus	9.77±2.66	6.15±2.47***	4.71±1.88***
Nuclear bud	4.39±1.42	4.13±2.35	3.71±1.83*
Binucleate	6.12±2.92	4.38±2.24**	3.97±1.99***
Karyorrhectic	8.01±2.29	6.67±2.30*	5.55±1.84***
Pyknotic	5.74±1.81	5.03±2.31	5.52±2.25
Karyolytic	6.89±2.98	6.18±2.19	6.29±2.64



Results are expressed as mean ± standard deviation
* $p \leq 0.05$ compared with tea garden workers using MANOVA
** $p \leq 0.01$ compared with tea garden workers using MANOVA
*** $p \leq 0.001$ compared with tea garden workers using MANOVA

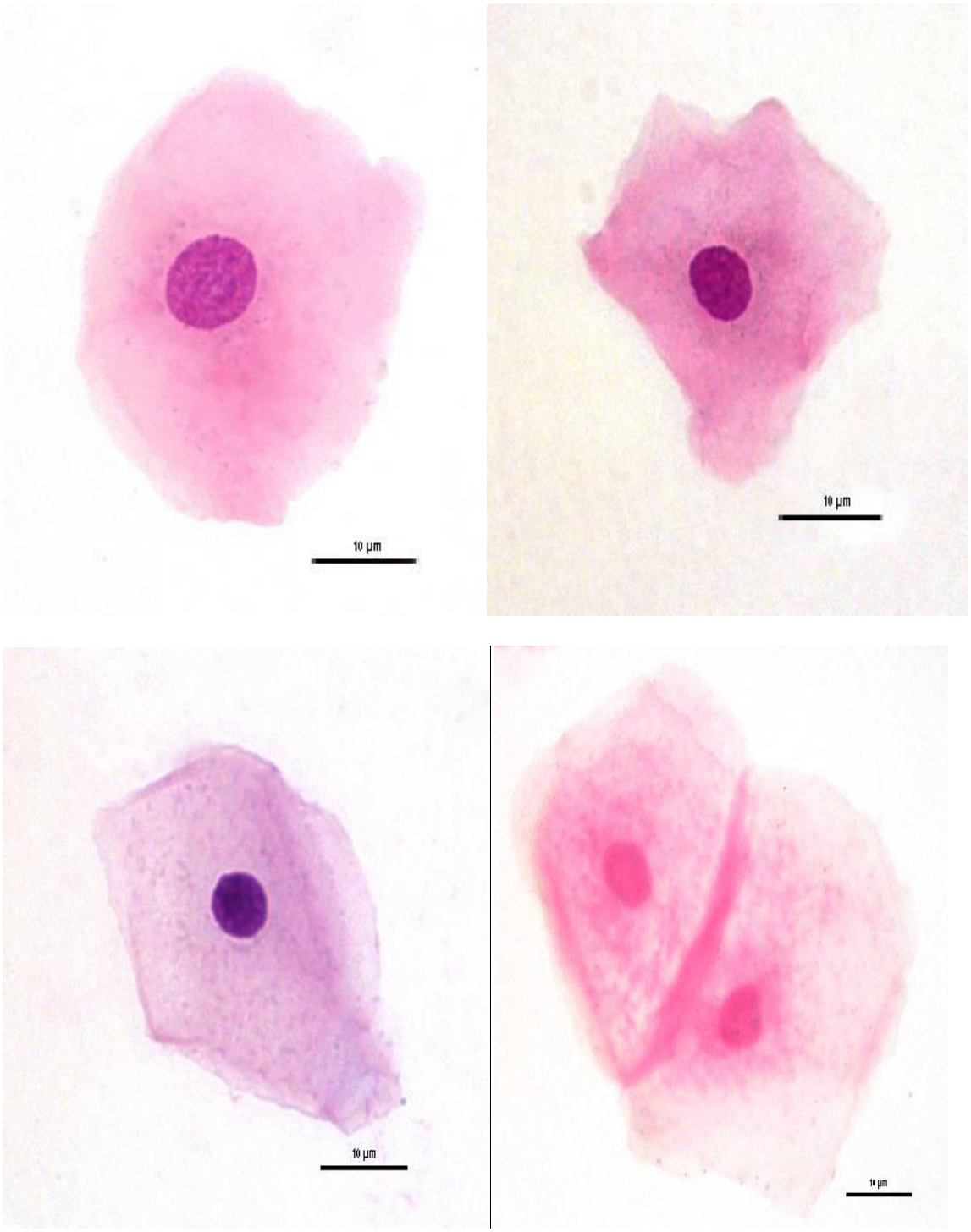


Figure 3.1: Buccal epithelial cells showing normal nucleus. Bar = 10 µm.

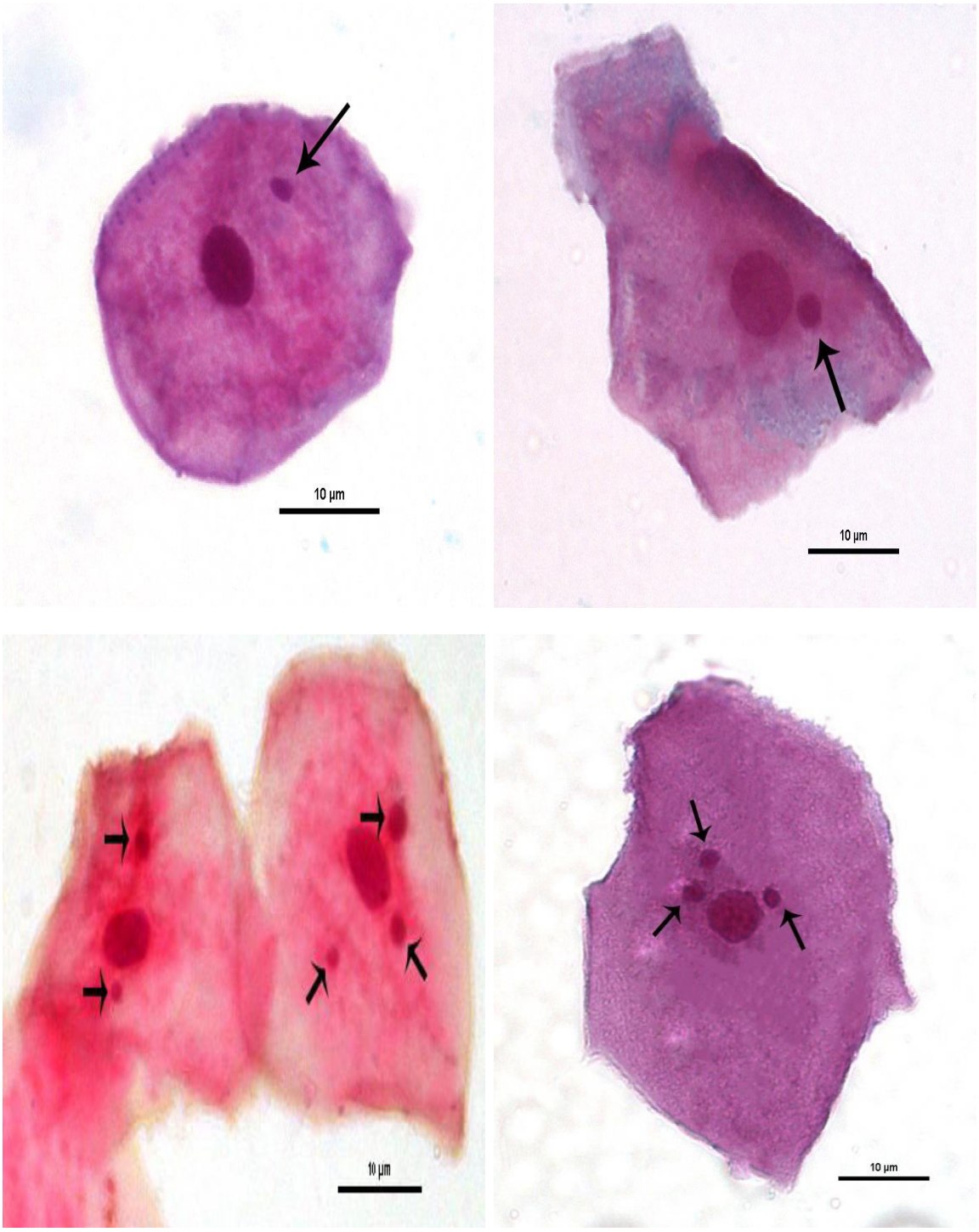


Figure 3.2: Buccal epithelial cells showing micronuclei (indicated with arrows). Bar = 10 µm.

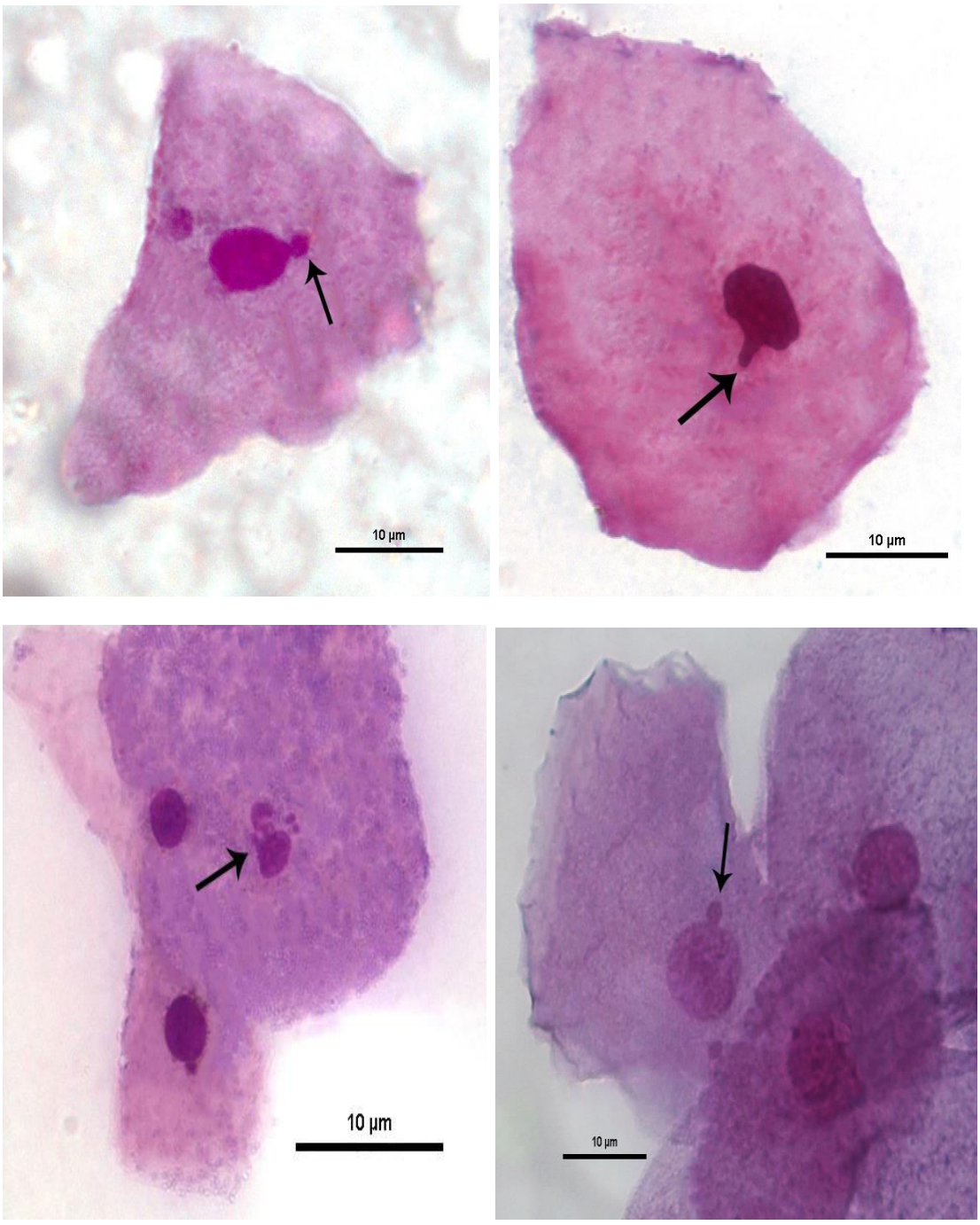


Figure 3.3: Buccal epithelial cells showing nuclear bud (indicated with arrows). Bar = 10 µm.

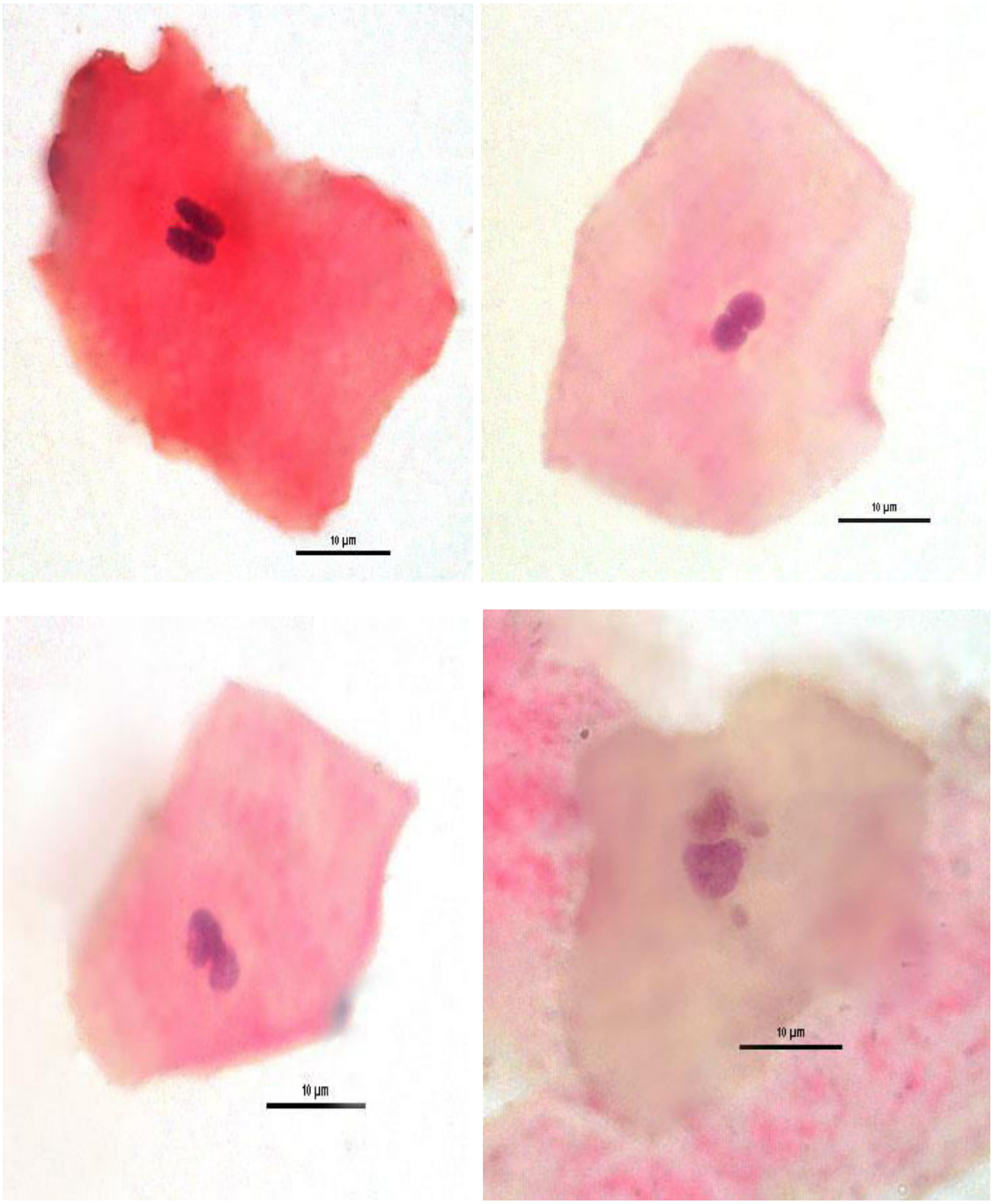


Figure 3.4: Buccal epithelial cells showing binucleated nuclei. Bar = 10 µm.

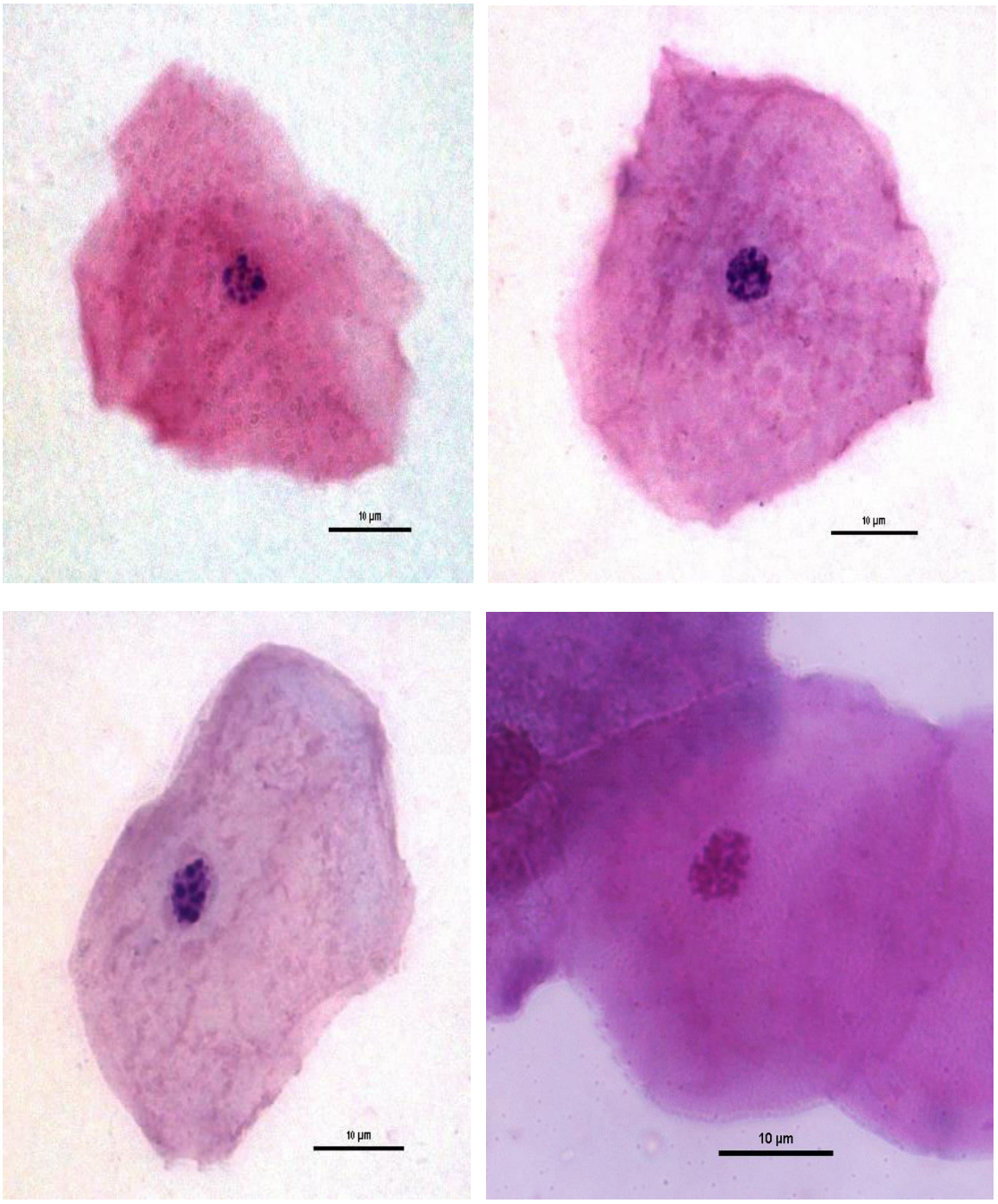


Figure 3.5: Buccal epithelial cells showing karyorrhectic nuclei. Bar = 10 µm.

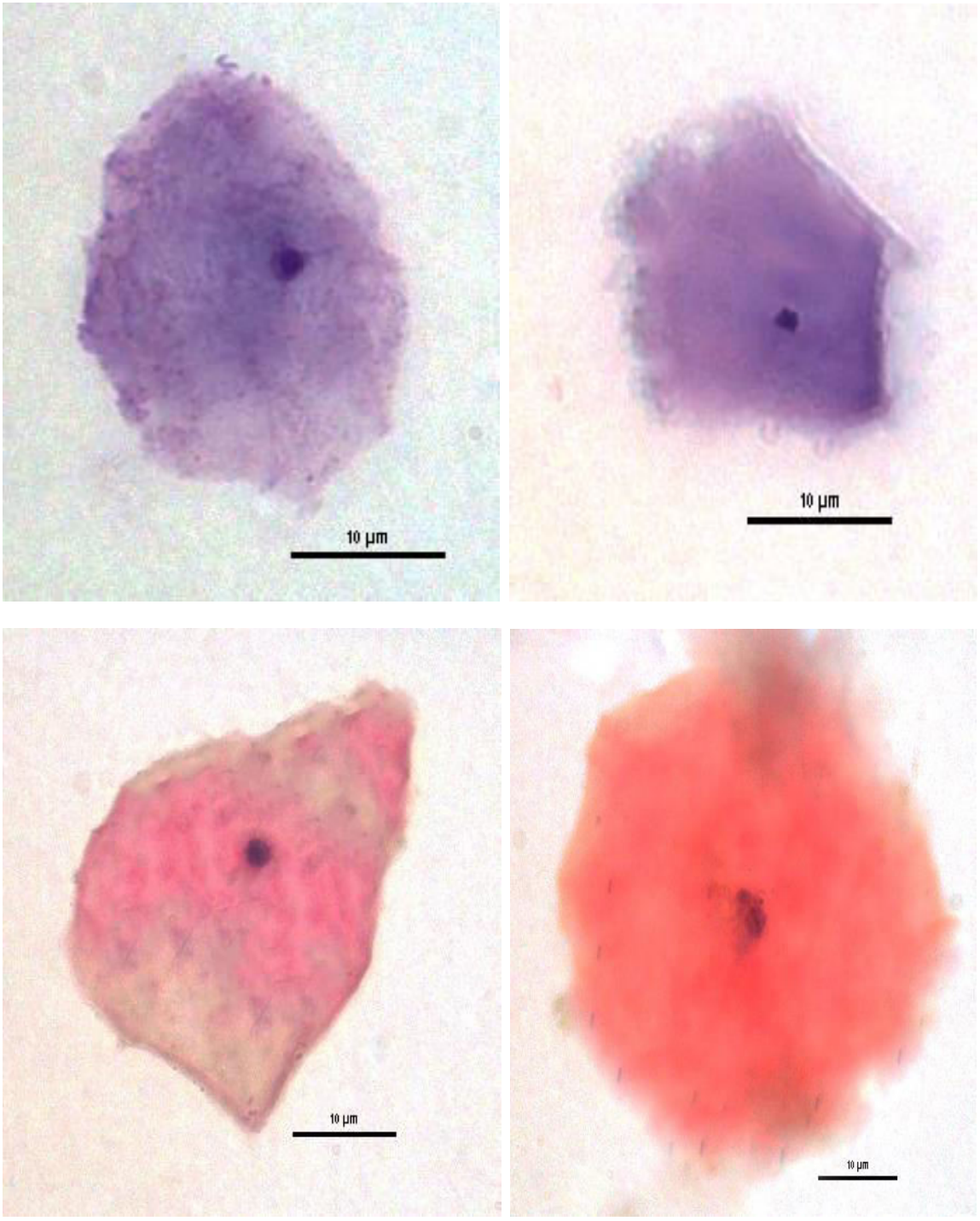


Figure 3.6: Buccal epithelial cells showing pyknotic nuclei. Bar = 10 µm.

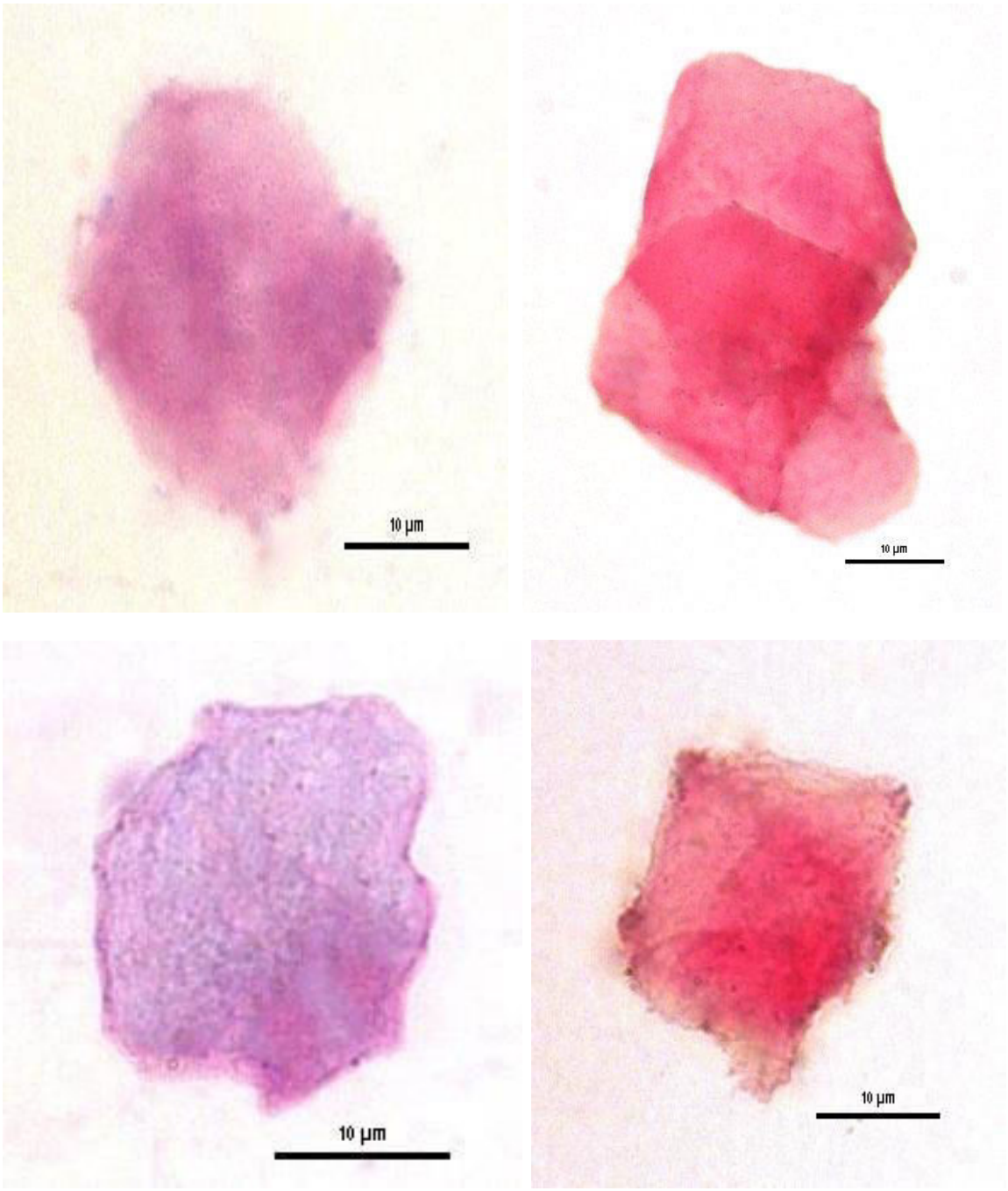


Figure 3.7: Buccal epithelial cells showing karyolytic nuclei. Bar = 10 µm.

3.4 Discussion

Chemical pesticides are increasingly used in the tea plantations in India, including Terai and the Dooars region of West Bengal to curb the menace of arthropod pests. It includes a heterogeneous mixture of compounds belonging to organophosphates, organochlorines, and pyrethroids. Therefore, the workers involved in tea leaf plucking (mainly females) and pesticide spraying (males) are at a high risk of pesticide exposure through various routes namely, inhalation, food contamination and skin contact. The study of cytogenetic damages in occupationally pesticide exposed individuals has received attention of a number of investigators from various countries, but no definite conclusion has been produced till date. Review of literatures on this subject has revealed an increase in genotoxicity biomonitoring end points in pesticide users (Bolognesi, 2003; Bull et al., 2006).

Many of these pesticides classified as “moderately hazardous” or “slightly hazardous” (WHO, 2005) are mutagens as well as “possible carcinogens to human beings” (IARC, 1991; Daniels et al., 1997; Chen et al., 2015). Different studies have indicated the genotoxic effect of pesticides on occupationally exposed human population (Scarpato et al., 1996; Pastor et al., 2001; Costa et al., 2007; Kausar et al., 2014; Gaikwad et al., 2015) and the genome alteration (Rupa et al., 1991; Shaham et al., 2001; Zhang et al., 2012). The toxic effects of pesticides on genome may be influenced by different factors, mainly extrinsic. Age, gender and smoking are the various extrinsic factors to occupational exposure that can affect the frequency of cytogenetic damage (Holland et al., 2008). The buccal epithelial cells are easily exposed to all the mutagenic components of pesticides as well as cigarettes, alcohol and the other toxic chemicals (Scarpato et al., 1996; Pastor et al., 2001; Costa et al., 2007; Kausar et al., 2014; Gaikwad et al., 2015). Pesticides of different categories generate reactive oxygen species (ROS) which can produce free

radicals, resulting in lipid peroxidation (Panemangalore et al., 1999) leading to DNA damage.

A comparison of the use of micronucleus (MN) test in detecting and quantifying the genotoxic action of carcinogens and its sensitivity has already been done with the analysis of chromatid breaks and exchanges (Adler et al., 1988). The buccal mucosal cells have been considered to be more sensitive than lymphocytes to the induction of the cytogenetic damage by the genotoxic agents (Davis, 2003). Micronucleus assay is non-invasive, neither require a cell culture nor metaphase preparation, has a low cost so is a good indicator of chromosome mutations (Majer et al., 2001; Fenech et al., 2011). As the workers get exposed to a complex mixture of chemicals, the evaluation of genotoxic effect in the pesticide exposed human population in the tea gardens of the Terai regions of Darjeeling foothills is a primary health concern. Our results showing high frequency of micronuclei, nuclear bud, binucleate, karyorrhectic, pyknotic and karyolytic cells in tea workers (both males and females) compared to the control is in agreement with the studies conducted by Bolognesi et al. (1993) and Carbonell et al. (1993). Recently, Kausar et al. (2014) have shown the percentage of micronuclei, nuclear bud, binucleate cells, karyorrhectic, karyolytic and pyknotic cells to be 1.20 ± 0.10, 0.20 ± 0.00, 0.90 ± 0.10, 2.90 ± 0.20, 1.20 ± 0.10, and 2.40 ± 0.10, respectively in the tea garden workers from Assam, India. The frequency of different damage parameters except MN in the present study is higher compared to the results of Kausar et al. (2014), which indicates that the tea garden workers in the Terai regions of the North Bengal are extensively exposed to the pesticides.

Further, MN frequency in grape garden workers reported by Gaikwad et al. (2015) is comparable to our results found in case of males engaged in pesticide mixing and

spraying. A number of studies have also shown higher frequency of MN in the pesticide exposed floriculturist (Scarpato et al., 1996; Bolognesi et al., 2011), greenhouse workers (Falck et al., 1999), agricultural workers (Ali et al., 2008), and soybean workers (Benedetti et al., 2013) compared to the control subjects. Our results showing higher frequency of MN in the pesticide exposed group corroborate the above and strongly support the genetic damages caused by pesticides. In the present study the frequency of MN (9.77 ± 2.66, $p \leq 0.001$) in the exposed group is comparable with the result of Costa et al. (2007) which is (9.03 ± 1.04, $p < 0.001$). However, Gaikwad et al. (2015), found higher frequency of micronuclei (11.4 ± 2.41, $p \leq 0.01$) in pesticide sprayers in grape garden compared to controls 6.6 ± 1.14. The probable reasons for the variability of results obtained by different investigators can best be explained by different level of exposures (Scarpato et al., 1996).

Since the male tea garden workers and few female workers in the present study smoked cigarettes/bidi and all the tea garden workers consumed alcohol, smokers and alcoholics were included in our study to see the effect of cigarettes/ bidi and alcohol on the buccal epithelial cells. Increased MN frequency in buccal epithelial cells due to smoking was reported in several studies (Sarto et al., 1987; Piyathilake et al., 1995; Kiilunen et al., 1997). Tobacco contents are identified as mutagenic in both *in vitro* and *in vivo* experiments (Riebe and Westphal, 1983; IARC, 1986; Kayal et al., 1993; Trivedi et al., 1993, 1995; Chang et al., 2001). Nersesyan et al. (2011) indicated that smoking induces MN and other nuclear anomalies (binucleate, broken egg, karyorrhexis, karyolysis and condensed chromatin) in the exfoliated buccal cells in humans and that these effects depend on the types of cigarettes consumed and the tar and nicotine contents. Our results showing a significant increase in the frequency of micronuclei (6.15 ± 2.47, $p \leq 0.001$), nuclear bud (4.13 ± 2.35, $p \leq 0.05$), binucleate (4.38 ± 2.24, $p \leq 0.01$), karyorrhectic (6.67

± 2.30, $p \leq 0.001$) and karyolytic (6.18 ± 2.19, $p \leq 0.01$) in smokers than the non-smokers (control) strongly support Nersesyan et al. (2011). The increase in the micronucleus and other cell death parameters could be due to the fact that the smoke emitted from cigarettes contains ROS and tar which cause damage to buccal epithelial cells as also suggested by Valavanidis et al. (2009). Our results are also in concurrence with the studies reporting a higher mean frequency of MN in the smokers (Sarto et al., 1987; Piyathilake et al., 1995; Kiilunen et al., 1997; Ozkul et al., 1997; Konopacka, 2003; Kamath et al., 2014). However, Falck et al. (1999) detected no increasing effect of smoking on MN.

For comparison of the effect of the number of the cigarettes on the damage parameters, the smokers were divided into two groups, group I (smoking less than 10 cigarettes/day) and group II (smoking more than 10 cigarettes/day). A significant increase of MN, nuclear bud, binucleate, karyorrhectic, pyknotic and karyolytic cells in the group II smokers having frequencies 7.72 ± 1.60, 5.61 ± 2.28, 5.83 ± 1.72, 8.00 ± 1.33, 6.28 ± 2.05 and 7.72 ± 1.32, respectively than group I with frequencies of 4.81 ± 2.29, 2.86 ± 1.56, 3.14 ± 1.88, 5.52 ± 2.36, 3.95 ± 1.99 and 4.86 ± 1.90 for above damage parameters clearly indicated a positive correlation between the number of cigarettes smoked and cell death parameters as also envisaged by Wu et al. (2004). Wu et al. (2004) reported a positive relation between micronuclei and smoking intensity and have shown that the heavy smokers have a higher frequency of MN in buccal cells. Kamath et al. (2014) also reported increased MN count in the individuals who smoked >10 cigarettes/day followed by those who smoked 5-10 cigarettes/day and <5 cigarettes/day. Though the frequency of MN, nuclear bud, pyknotic nuclei in alcoholics was not different than the control, but significantly higher binucleate, karyorrhectic and karyolytic (3.97 ± 1.99, 5.55 ± 1.84 and 6.29 ± 2.64) than control (2.75 ± 2.33, 4.48 ± 1.80 and 4.67 ± 2.01) suggests effect of alcohol on genome damage to some extent. The frequency of different cell death

parameters observed in the pesticide exposed group in the present study is not only higher than the control but also higher than the smoker and alcoholic groups (**Table 3.3**). This suggests that there may have a synergistic effect of the pesticide, and the smoking and alcohol for high cell death parameters in tea workers.

Pesticide-exposed tea garden workers were categorized into 3 groups according to age. The first group consisted of 45 individuals between 15-30 years of age, second group consisted of 30 individuals between 31-45 years of age and the third group consisted of 20 individuals between 46-62 years of age. Age was not found to have any significant effect on the frequency of MN and other cell death parameters in the present study which is in concurrence with the results shown by a number of workers (Sailaja et al., 2006; Costa et al., 2007; Moura de Bortoli et al., 2009). Moreover, the duration of pesticide exposure ranged from 1 to 36 years, so the subjects were divided into three groups, 1–7 years, 8–14 years and 15 years and above. The micronuclei and the other cell death parameters did not show any correlation with duration which is comparable to the results of Moura de Bortoli et al. (2009).

Significantly higher frequencies of micronucleus, nuclear bud and binucleate in male workers than females in the present study clearly indicate a sex bias which corroborates the study conducted by Pastor et al. (2002). The increase could be due to the sum of different factors like smoking, drinking and the intensity of pesticide exposure, which are not uniformly distributed between sexes as also suggested by Pastor et al. (2002). Among the factors that are capable of affecting cytogenetic damage are time of exposure, exposure conditions, working environment and personal protective equipment (Bolognesi, 2003). Other studies have also reported a significantly higher proportion of MN in males suggesting the effect of sex on the cell death parameters due to differential occupational

exposures (Gonsebatt et al., 1997; Martínez-Valenzuela et al., 2015). Further, male workers involved in pesticide mixing and spraying does not use any kind of protective measures against the pesticides and subsequently are at higher risk of exposure than females who are only tea leaf harvester. This may also account for the increased frequency of the cell death parameters in males. Bull et al. (2006) too emphasized on the importance of use of personal protective equipments in pesticide application.

Based on the results and discussion it can be concluded that smoking as well as alcohol intake act synergistically along with the pesticide(s), leading to the increased genome damage.

CHAPTER 4

Comet Assay

4.1 Review of Literature

The comet assay or single cell gel electrophoresis (SCGE) was first introduced by Ostling and Johanson for the detection of radiation induced DNA damages (Ostling and Johanson, 1984) and modified by Singh et al. (1988) for DNA denaturation and detection of alkali-labile sites. Collins et al. (2001) simplified the method for practical use by incorporating an additional step in which the nucleoid DNA is digested with a lesion specific endonuclease. The reasons for comet assay being so popularly accepted is that it can be performed with any type of eukaryotic cell and is less time-consuming (Hartmann et al., 2003). The genotoxicity can be tested in tissues which are in direct contact with the test substance or in tissues where absorption, distribution, metabolization or excretion takes place (Hartmann et al., 2003, 2004). The technique has the advantage of associating with fluorescence *in situ* hybridization (FISH) to analyze DNA damage (Spivak, 2015).

4.1.1 Comet assay in pesticide-exposed individuals

The comet assay has been widely used as a minimally invasive method for detecting genetic damage in pesticide exposed population (Garaj-Vrhovac and Zeljezic, 2000; Paiva et al., 2011; Benedetti et al., 2013; Carbajal-López et al., 2016). The extent of primary DNA damage and DNA repair was studied in peripheral blood lymphocytes in workers exposed to pesticides namely atrazine, alachlor, cyanazine, 2,4-dichlorophenoxyacetic acid and malathion for 6 months. Comet assay was carried out after 6 months of daily exposure to pesticides and 6 months after the end of production season. An increased tail length after 6 months of daily exposure was observed in the pesticide exposed workers than the non-exposed control (Garaj-Vrhovac and Zeljezic, 2000). After the workers were removed from pesticide exposure the DNA migration was still higher than controls. The DNA damage was repaired during the 6 months spent in

absence of pesticides but the difference between pre- and post- exposure period was still statistically significant (Garaj-Vrhovac and Zeljezic, 2000).

Comet assay in the peripheral blood lymphocytes of workers exposed to a complex mixture of pesticides in the rural Brazil showed a higher damage index and damage frequencies than control, whereas no differences were detected regarding structural and numerical chromosomal aberrations. This suggested that the damages were not enough to cause permanent mutations or damaged mitotic apparatus formation. Damages due to minimal pesticide exposure may have undergone cellular repair (Paiva et al., 2011). A study in workers employed in soybean cultivation in the state of Rio Grande do Sul (RS, Brazil) showed significantly higher damage index and damage frequency in both the male and female groups exposed to pesticides compared to the non-exposed control. Age and exposure time was not found to be correlated with the damage parameters (Benedetti et al., 2013). In a study conducted on pesticide exposed rice farmers and control subjects, Vivien et al. (2013) reported significant inhibition of blood cholinesterase which in turn was found to be associated with DNA damage in buccal mucosa cells as indicated by the comet assay. Çelik et al. (2014) estimated the genotoxic effect of fipronil (a phenyl pyrazole pesticide) using comet assay along with the sister chromatid exchange and micronuclei in the cultured human peripheral blood lymphocyte and showed a significant increase in DNA damage in a dose-dependent manner compared to negative control *in vitro*. The positive control used were mitomycin C (2 µg/ml) and hydrogen peroxide (Çelik et al., 2014). Comet assay was performed with the epithelial cells of oral mucosa to assess genotoxic effects on floriculturists in Brazil. The results showed that the frequency of cells with DNA damage was greater in the exposed group than the control subjects (Wilhelm et al., 2015). Comet assay in exfoliated buccal cells of occupationally exposed workers in Mexico revealed increased tail migration of DNA in the exposed group

(Carbajal-López et al., 2016). In a similar study, Wilhelm et al. (2015) reported higher frequency of damaged cells in floriculturists in Brazil than control subjects.

In India where pesticides are widely used in agriculture for pest control, a number of investigations have been conducted to evaluate the toxic effects of pesticides. For example, significantly large tail lengths have been reported in the peripheral blood leucocytes of workers engaged in pesticide-manufacturing and smoking was positively correlated to DNA damage as the smokers had a longer tail lengths compared to the non-smokers and ex-smokers (Grover et al., 2003; Bhalli et al., 2006). However, age was found to have a minimal effect on DNA damage (Bhalli et al., 2006). Similarly, Sailaja et al. (2006) reported significantly increased frequency of chromosomal aberration in pesticide workers compared to control but confounding factors like years of exposure, smoking, age, gender, alcohol consumption were not associated with higher levels of genetic damage. A study on oral leucocytes in immigrant agricultural workers for DNA damage using comet assay revealed significantly greater mean tail intensity and tail moment in workers than controls. No association was found between duration of exposure, age, sex and body mass with that of DNA damage (McCauley et al., 2008). However, How et al. (2015) conducted comet assay on exfoliated buccal epithelial cells among workers exposed to mixtures of organophosphate and showed that other factors such as individual (age, body mass index, smoking habit, duration of smoking, frequency of smoking), occupational and residential (distance of residence from farmland and duration of residency) together with pesticide exposure may also cause DNA damage. Recently, the comet assay in the exfoliated buccal mucosa cells of children exposed to pesticide for genotoxic risk analysis showed that the children with detectable urinary pesticide metabolite(s) had longer comet tail than control (Sutris et al., 2016).

4.1.2 Comet assay to study genotoxic effect due to occupational and environmental exposure

The comet assay is widely used for genotoxicity studies related to occupational and environmental exposures. Volatile organic compounds are of particular interest as they produce carcinogenic epoxide metabolites. Tafazoli and Kirsch-Volders (1996) evaluated the mutagenicity and cytotoxicity of 1,2-dichloroethylene, 1,1,2-trichloroethane, 1,3-dichloropropane, 1,2,3-trichloropropane and 1,1,3-trichloropropene in an *in vitro* micronucleus test along with comet assay. Their results suggested that the comet assay could be more suitable screening method for the above test chemicals than the micronucleus test. The comet assay was used to study the *in vitro* genotoxic potential of epoxides originating from various compounds namely 1,3-butadiene, isoprene, styrene, propylene and butane (Fabiani et al., 2012). Their study revealed that the epoxides could produce DNA damage at a concentration range where cell viability was not reduced (Fabiani et al., 2012).

Human peripheral blood lymphocytes were exposed to non-volatile organic agents extracted from the ground water samples from the area near textile dyeing and bleaching industries. The extracts were dissolved at different pH, without pH adjustment i.e., natural pH and acidic pH 2.0. The acidic pH caused more DNA damage. Hence, comet assay can also be used in environmental monitoring (Rajaguru et al., 2002). The confounding effect of folate and vitamin B12 in the lead-exposed workers was studied by micronucleus test and comet assay using silver nitrate staining. The lead exposed workers had significantly greater DNA damage as detected by comet assay compared to controls (Minozzo et al., 2010). Minozzo et al. indicated that protection against genotoxic damage cannot be guaranteed by high folate levels alone.

Cytogenetic damage induced by car-paint exposure (da Silva et al., 2012) and welding-fumes and solvent based paints (Sardas et al., 2010) was evaluated by comet assay in peripheral blood lymphocytes that showed higher frequency of DNA damage in the exposed subjects and suggested that welding-fumes as well as paints are capable of inducing genotoxic effects in peripheral blood lymphocytes. Eshkoor et al. (2012) reported significantly increased level of DNA damage in the mechanical workshop workers using buccal cells and the age was significantly associated with DNA damage tail length.

GC-MS/MS analyses on the serum samples of plum-tree growers to detect the presence of plant protection products (PPPs) revealed the presence of myclobutanil, propargite, cypermethrin and deltamethrin in the serum of 7 out of 19 samples (Kasiotis et al., 2012). Analysis of DNA damage by comet assay revealed increased single strand breaks in the blood samples after PPP application. Moreover, single strand DNA breaks were significantly increased in the seven samples with detectable serum PPPs compared to the subjects with no detectable PPPs (Kasiotis et al., 2012).

Comet assay has been extended to environmental monitoring too. The genotoxic potential of various compounds present in the environment was also explored using comet assay. The assay was carried out on the marine fish *Therapon jarbua* by exposing them to acute concentration of mercuric chloride. DNA damage was studied in gills, kidney and blood tissues after exposing them to various doses of mercuric chloride. DNA damage showed a dose dependent relationship. Moreover the assay also revealed that the gill cells were much more sensitive to heavy metals than the kidney and blood cells at two concentrations used for mercuric chloride (0.125 and 0.25 ppm) (Nagarani et al., 2012). A study was conducted for assessment of the pollution status of 11 sampling sites in Lagos

lagoon, Nigeria. Extracts of the organic solvent of sediment were analysed for cytotoxicity and genotoxicity in rainbow trout gill-W1 cells. The approach suggested that comet assay is very promising to identify sediments that serve as genotoxic contaminants (Amaeze et al., 2015).

4.1.3 Comet assay to detect DNA damage in carcinogenesis

The analysis of drugs, chemicals and mutagens for their carcinogenic potential is one of the primary objectives of most genotoxicity studies. A number of carcinogenic assessment protocols are in effect which require advanced laboratories and expertised personnel. However, comet assay is easy to perform and widely used for *in vitro* as well as *in vivo* carcinogenic tests. As for example, the effect of etoposide (an effective antitumor agent) and two fungicides (carbendazim and chlorothalonil) was studied by Lebailly et al. (1997) to see the induction of DNA damage, cell viability and dose dependent-DNA damage relationship using comet assay in human peripheral blood lymphocytes after 1 h treatment and 24 h post treatment. The result showed a significant dose-dependent DNA damages at concentrations in which there was less loss of cell viability after 1 h treatment. After 24 h post incubation DNA damages had disappeared. Similar results were observed with chlorothalonil alone or in association with carbendazim i.e. there was no loss of cell viability. Greater loss of cell viability was observed after 24 h with huge number of highly damaged cells. Carbendazim was not cytotoxic after 1 h treatment or after 24 h post treatment incubation (Lebailly et al., 1997). Godard et al. (1999) also found dose dependent DNA damage potentiality of etoposide and chlorothalonil *in vivo* and *in vitro* study by comet assay. Rojas et al. (1996) carried out alkaline single-cell gel electrophoresis assay in exfoliated buccal mucosal cells of the smokers and non-smokers. The total image length (including head and tail length) was

found to be greater in the smokers (89.30 ± 16.18 µm) compared to the non-smokers (52.01 ± 10.43 µm) at significant level ($p < 0.001$).

The alkaline comet assay was also performed in conjugation with the chromosomal aberration test to assess the genotoxicity of 13 drug candidates in V79 Chinese hamster cells and human lymphocytes. Result of the comet assay and the chromosomal aberration test were in agreement with each other irrespective of the type of cells used (Hartmann et al., 2003). The single cell gel electrophoresis (comet assay) has been successfully used to measure radiosensitivity in a range of tumor cell lines (Moneef et al., 2003; Niedbala et al., 2006; Smyth et al., 2007). In a study, the impact of cigarette smoke was investigated by alkaline comet assay in peripheral blood lymphocytes of white-collar active smokers and passive smokers (non- and ex-smokers) exposed to environmental tobacco smoke at workplace compared to non-smokers. Significantly high levels of basal DNA damage was observed in active smokers compared to other groups. A significant increase in DNA damage associated with decreased DNA repair capacity was observed not only in active smokers but also in non- and ex- smokers who were exposed to second-hand tobacco smoke at workplace (Fracasso et al., 2006). Cigarette smokers being at higher risk of cervical cancers (Castle et al., 2002; McIntyre-Seltman et al., 2005) the DNA-damage induced by cigarette smoke was studied in HPV 16-transformed human ectocervical cells with or without cigarette smoke condensate for varying times to detect DNA damage by comet assay (Moktar et al., 2009). The mean tail length of cells with single and double strand breaks increased in dose-dependent manner. It was found that the single strand and double strand breaks were repaired during the initial 24 h, but after this period, repair of DNA damage did not take place (Moktar et al., 2009). Effect of exposure of gingival tissue in case of oral submucous fibrosis (OSF) compared to control was studied using

comet assay as a reliable tool. All the patients showed large number of comet cells corresponding to control (Dodani et al., 2012).

Increased DNA damage (higher proportion of DNA single and double strand breaks) in patients with breast cancer (Rajeswari et al., 2000), ovarian cancer (Baltaci et al., 2002) and cervical neoplasia (Cortés-Gutiérrez et al., 2012) compared to control subjects was observed using comet assay. Alkaline single cell gel electrophoresis was also carried out in peripheral blood leucocytes of obese and normal weight subjects which revealed significantly elevated DNA damage in obese subjects than non-obese people. This could be due to greater production of free radicals in the obese people. This implies that obese subjects are susceptible to cancer and precocious aging (Gandhi and Kaur, 2012). Comet assay has also been used to assess the effects of chemotherapeutic drugs such as melphalan, cisplatin, mechlorethamine and doxorubicin that are used in breast and colon cancer (Apostolou et al., 2014). Drug activity was found to differ even in same cancer types. Thus the comet assay can be used for a range of drugs and is a reliable and quick technique (Apostolou et al., 2014).

A Brazilian plant *Cordia ecalyculata*, with anorectic properties and *Spirulina maxima*, a cyanobacterium with antioxidant and anti-genotoxic activity are two weight reduction drugs (Araldi et al., 2014). The mutagenic potential of *C. ecalyculata* in association with *S. maxima* at various concentrations of doses was tested *in vitro* by comet assay using human peripheral blood. Cyclophosphamide was used as a positive control. Anorectic effect resulted from treatment of 300 mg/kg of *C. ecalyculata* and a combined treatment of 500 mg/kg *C. ecalyculata* together with 250 mg/kg *S. maxima*. No clastogenic or genotoxic activity was revealed by mutagenic tests for any kind of treatment which indicates that these weight reducing drugs can be used safely (Araldi et al., 2014). Comet

assay has also been used to assess the genotoxicity of benzo[a]pyrene (an environmental pollutant) on *Caenorhabditis elegans* exposed for 48 h. Formation of comet was concentration-dependent up to a concentration of 20 µM (Imanikia et al., 2016).

4.1.4 Comet assay in reproductive biology

Comet assay is also widely used on the germ cells to test genetic toxicity. Alkaline comet assay was performed on human spermatozoa after treating them with X-rays or H_2O_2. They were found to be resistant to damage compared to the somatic cells. The authors suggested that this insensitivity might be due to marked condensation of DNA of mature spermatozoa (Hughes et al., 1996). Haines et al. (1998) observed that 100 Gy gamma irradiation caused an increase in % tail DNA from 33.3 to 56.6 in neutral comet assay. Alkaline conditions led to occurrence of 97.4% of DNA in the tail and no further increase took place after irradiation with doses up to 100 Gy. Similar results were obtained with mouse sperms also (Haines et al., 1998). Baumgartner et al. (2004) showed that doxorubicin (an anticancer drug) induced DNA damage in human sperm and lymphocytes *in vitro*. Though the drug reduced the percentage of head DNA in human lymphocytes and sperm, the effect on lymphocytes was not found to be related to the drug concentration. The sperm was treated for 1 h and the analysis was done directly and lymphocytes were treated for 20 h and then set for chromosome preparations (Baumgartner et al., 2004). Alkaline and neutral comet assay has been performed from time to time to detect single and double strand DNA breaks which is of typical interest in reproductive biology be it on the sperms of different clinical groups or to detect the level of DNA damage on cryopreserved sperms (Ribas-Maynou et al., 2012, 2014).

4.1.5 Comet assay in drug response

Demir et al. (2010) used comet assay to test the genotoxic effects of the flavor ingredients of benzyl derivatives such as benzyl alcohol, benzyl acetate, benzoic acid and benzaldehyde and showed increased % tail DNA and tail moment at different concentrations for different chemicals. In order to assess the genotoxic properties of halothane and isoflurane- two inhalation anesthetics, alkaline single cell gel electrophoresis was performed in peripheral blood lymphocytes of humans. The cells were exposed to the anesthetics in a dose-dependent way. It was found that both the drugs were capable of inducing DNA strand breaks and alkali-labile sites as indicated by the comet assay (Jaloszyński et al., 1999). When the cells were incubated in a drug-free medium the cells were capable of repair up to certain limit. This indicated the genotoxic action of the drugs. The quantum of damage detected after the specified dose was the result of DNA fragmentation, i.e. cell death (Jaloszyński et al., 1999).

4.1.6 Comet assay in other studies

In an early study, the alkaline comet assay was used to detect alkylation damage in human lymphocyte DNA using bacterial repair enzyme 3-methyladenine DNA glycosylase II. It was found that 3-methyladenine DNA glycosylase II was capable of detecting DNA damage when treated with low concentration of methyl methanesulfonate (Collins et al., 2001). Calinisan et al. (2002) designed a study to see the effect of human papilloma virus (HPV) DNA of the E6-E7 region on the DNA of blastocyst stage embryonic cells by a modified version of comet assay. The data showed that after 24 h of exposure, of HPV DNA type 16 caused DNA fragmentation in blastocysts by initiating apoptosis but types 18, 31 and 33 did not affect blastocyst DNA (Calinisan et al., 2002).

Comet assay has been combined with fluorescence *in situ* hybridisation to study mitomycin-C induced formation and repair of inter-strand DNA crosslinks and also for the analysis of region-specific hypomethylation (McKenna et al., 2003; Wasson et al., 2006). The induction and repair of DNA damage in individual cell could be measured by single cell gel electrophoresis. For this purpose homogenous cell population of blood mononuclear $CD34^+$ was used. It was noticed that the total comet intensity corresponded to the position of cells in cell cycle. The results were comparable to that obtained from flow cytometry (Kruszewski et al., 2012). Healthy individuals were examined in order to determine background level of DNA strand breaks considering age, sex and smoking as confounding factors by comet assay. Age was found to have a significant effect on DNA damage. The authors graded the lymphocytes by eye and classified them into 5 groups. The procedure can be used for a sensitive and fast quantification of DNA damage (Diem et al., 2002).

The alkaline version of the comet assay has also been used to study the difference in the level of DNA damage between fresh and frozen blood samples of smoker and non-smoker population. The whole blood was frozen using dimethyl sulfoxide and stored at -80^{o}C. The study did not show significant difference between the fresh and frozen blood samples suggesting that the frozen whole blood can be used to perform comet assay for the assessment of genetic damage in populations (Hininger et al., 2004). Aphidicolin (APC)-block nucleotide excision repair (NER) comet assay was performed to evaluate the intra and inter-assay variation as well as inter-individual variation in cryopreserved peripheral blood mononuclear cells. The variation was also measured for internal standard (K562 erythroleukemia cell line). Other cell lines like lymphoblastoid cell line (LCLs) may also be tried as surrogate of peripheral blood mononuclear cells. The assay showed that it could be used on cryopreserved peripheral blood mononuclear cells to measure

DNA repair capacity variation in epidemiological studies (Allione et al., 2013). During a study to compare DNA damage in fresh and cryopreserved peripheral blood mononuclear cells (PBMCs) by detecting the formamidopyrimidine-DNA glycosylase (FPG) sites using the intervention with blueberry or the placebo drink. Bo et al. (2015) showed that the long-term storage of samples participating in dietary intervention can significantly modify the DNA damage at FPG-sensitive sites and H_2O_2 induced damage. It indicates that storage samples should be used with appropriate care and control.

4.2 Materials and methods

4.2.1 Sampling area

Sampling area was same as mentioned under **section 2.2.1**

4.2.2 Characteristics of the participants

Since the individuals used for enzyme assay and micronucleus assay were also used for comet assay the characteristics of the participants were same as mentioned under **section 2.2.2 Characteristics of the participants.**

4.2.3 Sampling procedure

After obtaining consent from individuals, venous blood was collected by venepuncture using 5 ml sterile disposable syringe, transferred immediately in tubes containing EDTA as anticoagulant and brought to the laboratory and kept at 4^oC. Comet assay was performed under alkaline conditions within 24 hours of blood collection.

4.2.4 DNA damage analysis using single cell alkaline gel electrophoresis

The comet assay was performed following the procedure of Singh et al. (1988) with slight modification. Duplicate slides were prepared per subject. Fully frosted microscopic slides were coated with 140 µl of 0.75% agarose with normal melting point and allowed to gel at 4^{o}C for 10 minutes. To 0.5% of 110 µl of low melting point agarose (melting temperature 65^{o}C, gelling temperature 35^{o}C) an aliquot of 20 µl of whole blood was added. This was then layered onto the slides coated with 0.75% normal melting point agarose and spread with a cover slip. After solidification and removal of the cover slip, third layer of 110 µl of 0.5% low melting agarose was layered onto the slides and allowed to gel at 4^{o}C for 10 minutes. The cover slips were removed and immersed in cold, fresh lysis buffer (2.5M NaCl, 100mM EDTA, 10mM Tris-HCl, 1% Triton X-100, 10% DMSO, pH 10, DMSO was added just before use) and refrigerated at 4^{o}C for 1 hour. Slides were then placed in alkaline buffer (300mM NaOH, 1 mM EDTA, pH $\geq$ 13) for 20 minutes to allow DNA unwinding. Electrophoresis was performed for 25 minutes at 300mA in the same buffer. After electrophoresis slides were washed gently thrice for 5 minutes each in fresh neutralization buffer (0.4M Tris-HCl, pH 7.5), dehydrated with absolute methanol for 10 minutes and dried at room temperature. The entire process was carried out in dark to minimize artefactual DNA damage. Prior to analysis slides were stained with 50 µl of ethidium bromide (EtBr) (20 µg/ml). Slides were examined under a fluorescent microscope (Nikon Eclipse E200, Nikon) with barrier filter of 590 and excitation filter of 510-560 connected to a CCD camera at 40x magnification.

4.2.5 Scoring criteria

Slides were blindly coded. 50 cells were analyzed per subject (25 cells from each slide). Damaged cells have the appearance of a comet and undamaged cells have intact nucleus.

Tail length (Ltail), tail moment (TM) and olive tail moment (OTM) was calculated to estimate DNA damage using CASPlab image analysis software.

LTail: It is the comet tail length measured from right border of head area to end of tail.

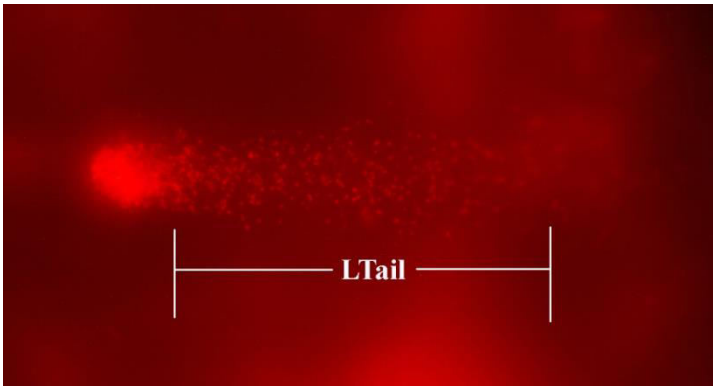


Figure 4.1: Photograph showing length of tail (LTail).

TM: Percent of DNA in the tail X Tail length. This gives the extent of DNA damage.

OTM: Percent of DNA in the tail X Distance between the center of gravity of DNA in the tail and the center of gravity of DNA in the head in x- direction.

4.2.6 Ethical consideration

Ethical consideration was the same as mentioned under **section 2.2.8**

4.2.7 Statistical analysis

Dependent variables Ltail, TM, OTM along with their log transformations and square root transformations have been tested for normality using Kolmogrov-Smirnov test and Shapiro-Wilks test. Both tests showed that all the dependent variables were non-normal ($p \leq 0.001$). Therefore, nonparametric tests Kruskal-Wallis test and Mann-Whitney U test were applied for testing equality of means of multiple independent populations and 2

independent populations (pair-wise), respectively. The mean and standard error of mean (SEM) were calculated for the dependent variables.

4.3 Results

For analysis, 50 cells were scored per subject (25 cells from each slide). Tail length (LTail), tail moment (TM) and olive tail moment (OTM) were measured in tea garden workers (exposed), controls, smokers and the alcohol consumers using CASPlab image analysis software. The results have been presented in the **Tables 4.1** and **4.2**. The results showed a mean tail length (LTail) of 45.98 ± 4.25 µm (0.99-187.64 µm) and 15.14 ± 2.99 µm (0.36-36.72 µm) in the tea garden workers and the control individuals, respectively. The LTail was about 3 times longer than cells from the control, which was highly significant at $p \leq 0.001$. In contrast to the tea garden workers, the smokers and alcohol consumers had tail length of 16.83 ± 2.60 µm (0.36-66.64 µm) and 14.24 ± 1.32 µm (2.52-29.88 µm) which were almost similar to that of control subjects (**Table 4.1**). The mean TM and OTM were found to be 8.86 ± 1.18 (0.001-50.62) and 6.41 ± 0.78 (0.0002-33.58) in tea garden workers and 2.89 ± 0.48 (0.000068-16.51) and 2.32 ± 0.36 (0.000087061-10.40) in controls, respectively. Analysis showed that the TM and OTM were significantly higher in the workers ($p \leq 0.001$). The smokers and the alcoholics were found to have a mean TM and OTM of 3.96 ± 0.89 (0.00003-19.21) and 2.69 ± 0.48 (0.0002-12.48) and 3.19 ± 0.55 (0.00162-11.79) and 2.60 ± 0.42 (0.0002-10.11), respectively. The differences of TM and OTM observed in smokers and alcohol consumers were statistically non-significant than control (**Table 4.1**). Comet assay revealed differential degree of DNA damage in pesticide exposed workers (**Figure 4.2**) compared to control (**Figure 4.3**), smokers (**Figure 4.4**) and alcohol consumers (**Figure 4.5**). The damage varied from very low to very high.

Analyses were also done among the tea garden workers, smokers and alcohol consumers to see the effect of cigarette smoking and alcohol consumption. A mean tail length of 45.98 ± 4.25 µm, 16.83 ± 2.60 µm and 14.24 ± 1.32 µm were recorded in tea garden workers, smokers and alcohol consumers, respectively. The tail length in worker group was 3 times longer than the smokers and alcohol consumers which were highly significant at $p \leq 0.001$. The mean TM and OTM in tea garden workers, smokers and alcohol consumers were observed to be 8.86 ± 1.18 and 6.41 ± 0.78, 3.96 ± 0.89 and 2.69 ± 0.48, 3.19 ± 0.55 and 2.60 ± 0.42, respectively. The damage revealed by TM and OTM were about 2.5 times ($p \leq 0.05$) and 3 times ($p \leq 0.01$) greater in the exposed group than the other two groups (**Table 4.2**).

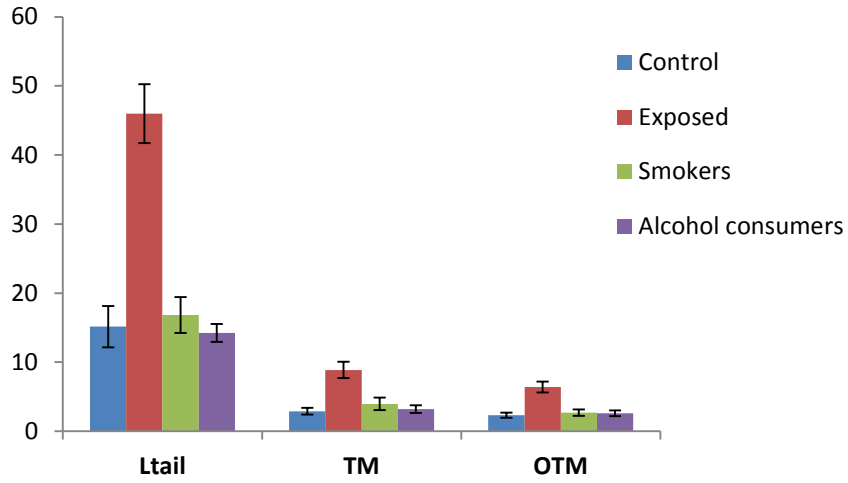
In order to see the effect of gender (sex) on damage parameters (LTail, TM, OTM), both control and the tea garden workers (exposed) were categorized into male and female groups. The results showed a mean LTail, TM and OTM of 17.20 ± 4.07 µm, 3.25 ± 0.62 and 2.59 ± 0.47 in males and 9.93 ± 2.11 µm, 1.99 ± 0.62 and 1.65 ± 0.47 in females of control group, while LTail, TM and OTM were 51.92 ± 6.17 µm, 9.97 ± 1.65 and 6.64 ± 1.08 in males and 35.78 ± 4.18 µm, 6.95 ± 1.44 and 6.02 ± 1.02 in females of exposed group, respectively. Pesticide exposed males and females had almost 3 times higher LTail, TM and OTM compared to the control males and females. The significance level for LTail, TM and OTM were $p \leq 0.001$, 0.01, 0.01 between exposed and control males and $p \leq 0.001$, 0.01, 0.001 between exposed and control females. When the males and females under the exposed and control category were tested for significance, in spite of the apparently high difference, it was statistically non-significant. This may be due to the fact that for testing equality of means of two independent variables, the test statistics is related to their mean and standard error of means, a very high standard error of means may lead to acceptance of equality of means. Again, if standard error of means is very

low the test statistics may lead to rejection of equality of means. A similar condition may account for the non-significant differences observed between males and females of control and exposed group (**Table 4.3**).

To see the effect of age on the LTail, TM and OTM, the pesticide exposed group (workers) was classified into 3 age groups 15-30, 31-45, 46-62 years. The analysis revealed a mean LTail, TM and OTM of 46.09 ± 6.08 µm, 9.13 ± 1.64 and 7.02 ± 1.15 in the age group 15-30 years, 53.49 ± 8.76 µm, 8.90 ± 2.03 and 6.58 ± 1.56 in the age group 31-45 years and 34.46 ± 6.69 µm, 8.19 ± 3.00 and 4.80 ± 1.22 in the age group 46-62 years, respectively. Though none of the groups differed significantly for any of the parameters (LTail, TM and OTM), the LTail was slightly high for age group 31-45 years, whereas TM and OTM were slightly high for age group 15-30 years (**Table 4.4**).

Table 4.1: Comparison of LTail, TM and OTM between controls, exposed, smokers and alcohol consumers. For each participant 50 cells were scored.

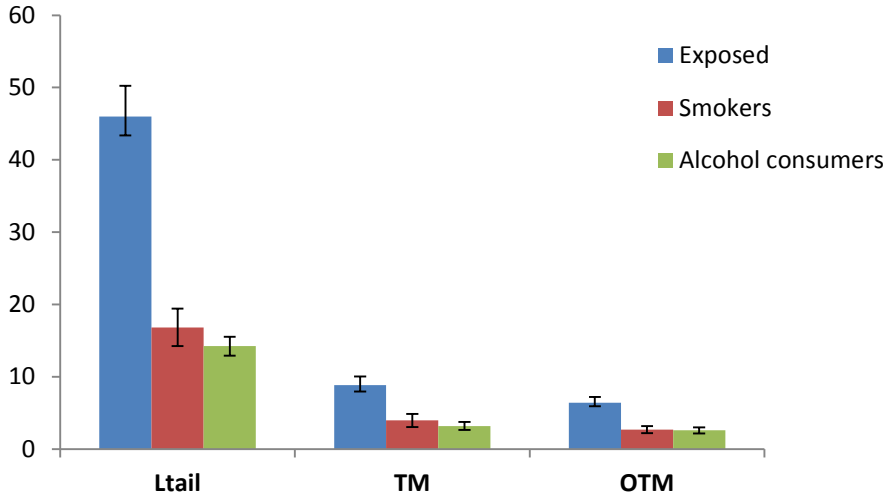
	LTail	TM	OTM
Control(N=60)	15.14 ± 2.99	2.89 ± 0.48	2.32 ± 0.36
Exposed(N=95)	45.98 ± 4.25***	8.86 ± 1.18***	6.41 ± 0.78***
Smokers(N=39)	16.83 ± 2.60	3.96 ± 0.89	2.69 ± 0.48
Alcohol consumers(N=31)	14.24 ± 1.32	3.19 ± 0.55	2.60 ± 0.42



Results are expressed as mean ± SEM
LTail is measured in µm
***$p \leq 0.001$ compared with control using Mann-Whitney U test

Table 4.2: Comparison of LTail, TM and OTM between exposed, smokers and alcohol consumers. For each participant 50 cells were scored.

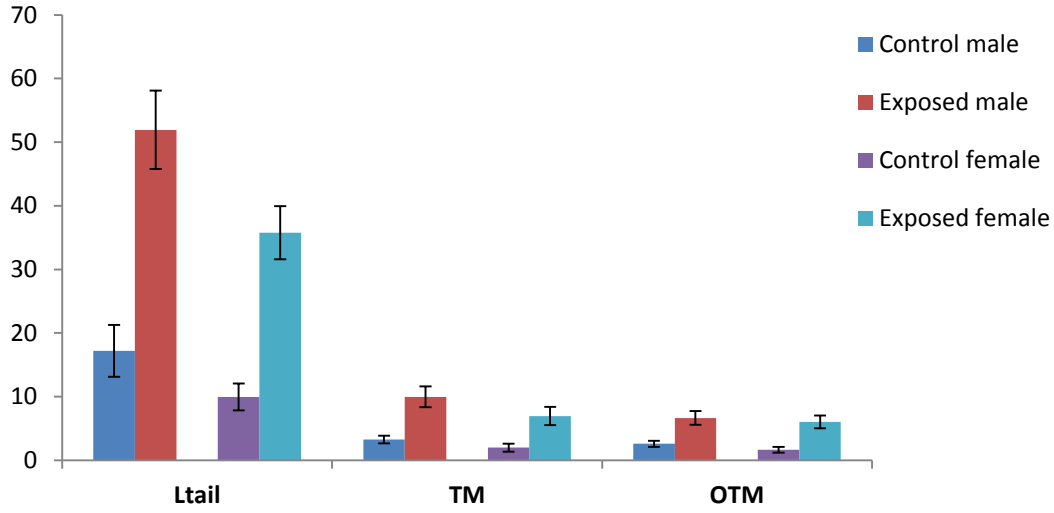
	LTail	TM	OTM
Exposed(N=95)	45.98 ± 4.25	8.86 ± 1.18	6.41 ± 0.78
Smokers(N=39)	16.83 ± 2.60***	3.96 ± 0.89*	2.69 ± 0.48**
Alcohol consumers(N=31)	14.24 ± 1.32***	3.19 ± 0.55*	2.60 ± 0.42**



Results are expressed as mean ± SEM
LTail is measured in µm
***$p \leq 0.001$ compared with exposed using Mann-Whitney U test
** $p \leq 0.01$ compared with exposed using Mann-Whitney U test
* $p \leq 0.05$ compared with exposed using Mann-Whitney U test

Table 4.3: Comparison of LTail, TM and OTM between males and females of controls and exposed. For each participant 50 cells were scored.

	LTail	TM	OTM
Control			
Male(N=43)	17.20 ± 4.07	3.25 ± 0.62	2.59 ± 0.47
Female(N=17)	9.93 ± 2.11	1.99 ± 0.62	1.65 ± 0.47
Exposed			
Male(N=60)	51.92 ± 6.17***	9.97 ± 1.65**	6.64 ± 1.08**
Female(N=35)	35.78 ± 4.18***	6.95 ± 1.44**	6.02 ± 1.02***



Results are expressed as mean ± SEM
LTail is measured in µm
***$p \leq 0.001$ compared with control using Mann-Whitney U test
** $p \leq 0.01$ compared with control using Mann-Whitney U test

Table 4.4: LTail, TM and OTM of the exposed group classified according to age.

Age Groups (years)	LTail	TM	OTM
15-30 (N=45)	46.09 ± 6.08	9.13 ± 1.64	7.02 ± 1.15
31-45 (N=30)	53.49 ± 8.76	8.90 ± 2.03	6.58 ± 1.56
46-62 (N=20)	34.46 ± 6.69	8.19 ± 3.00	4.80 ± 1.22

Results are expressed as mean ± SEM
LTail is measured in µm

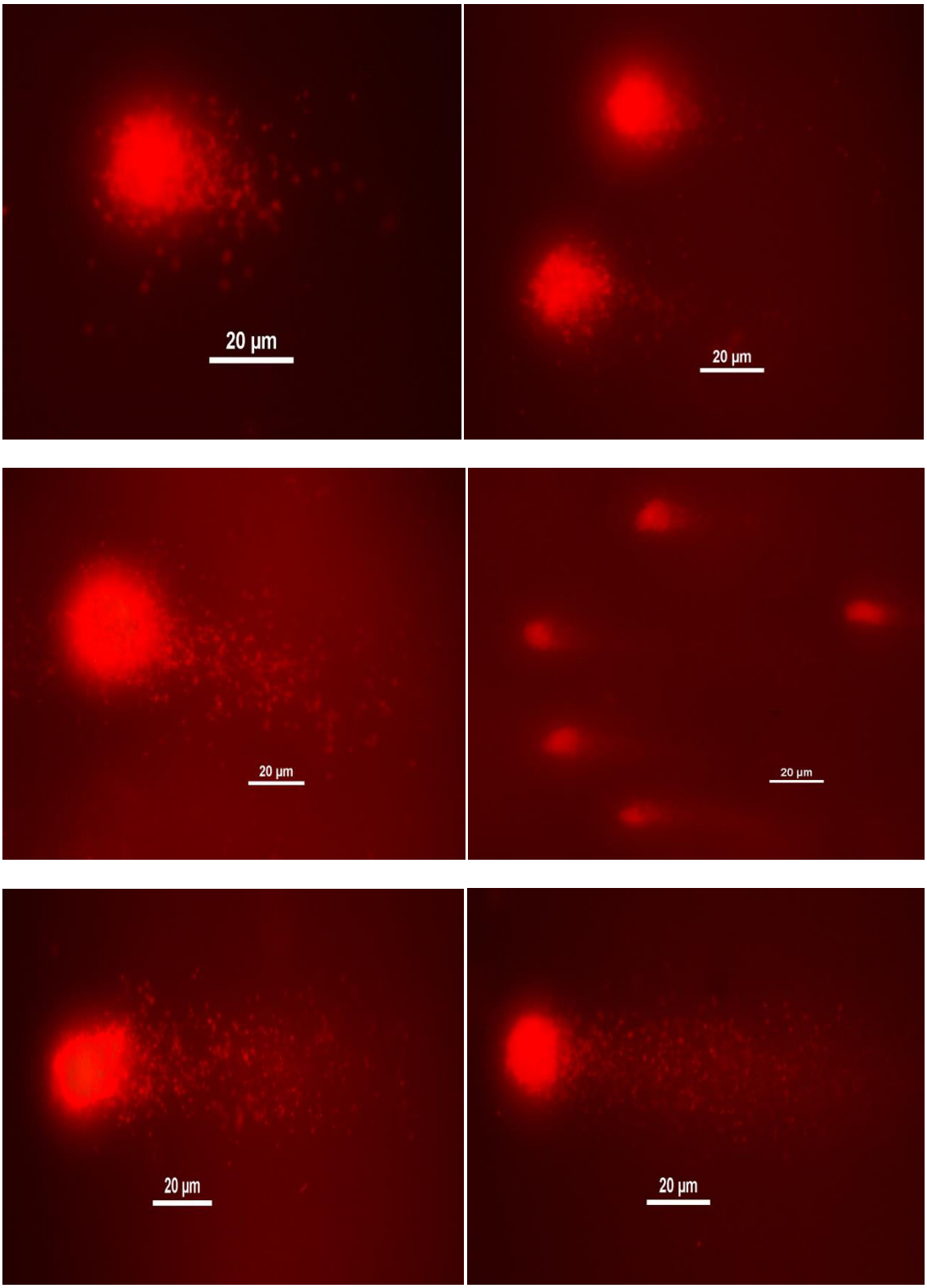


Figure 4.2: Levels of DNA damage detected in tea garden workers by comet assay. Bar = 20 µm.

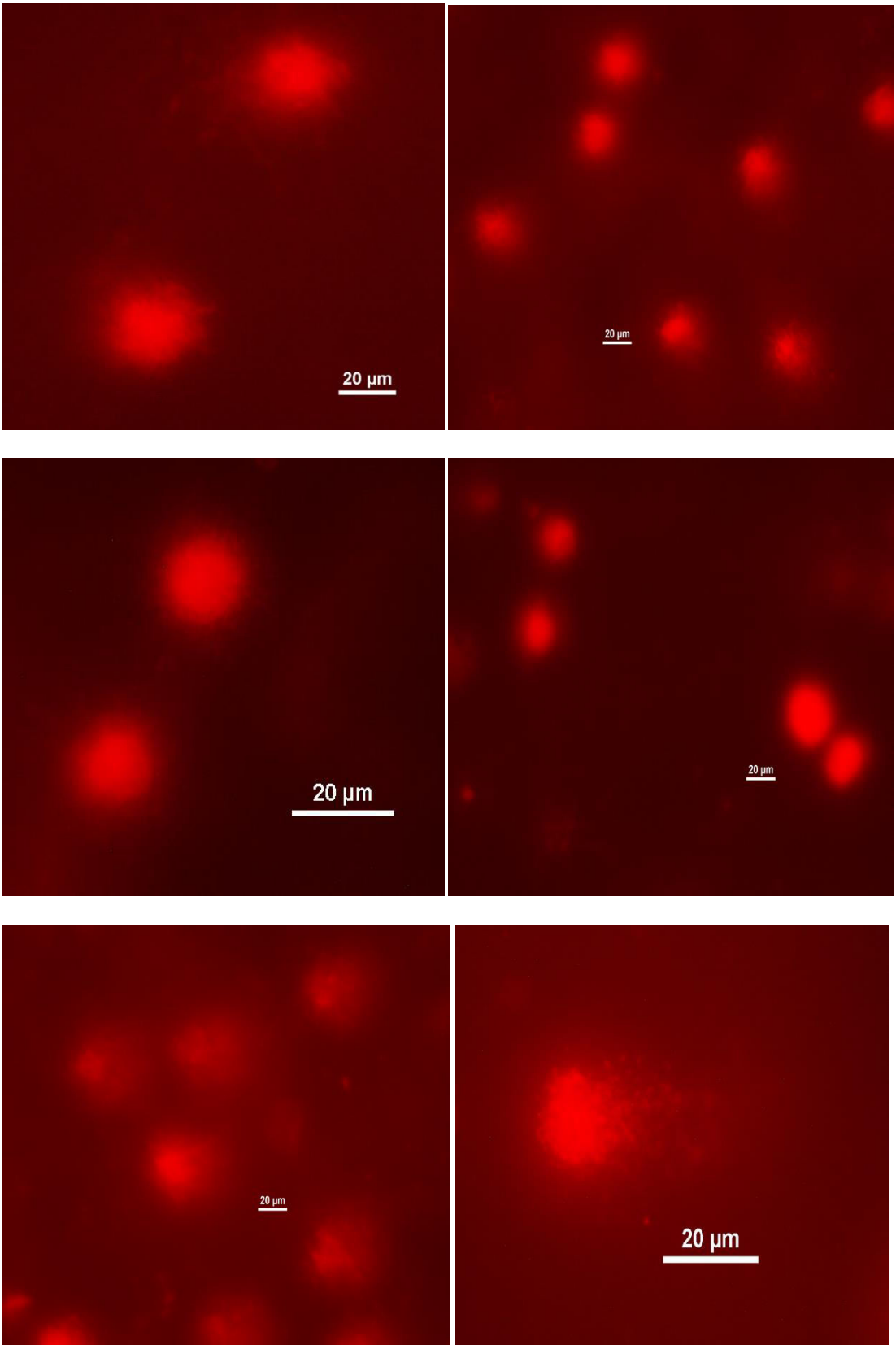


Figure 4.3: Levels of DNA damage detected in controls by comet assay. Bar = 20 µm.

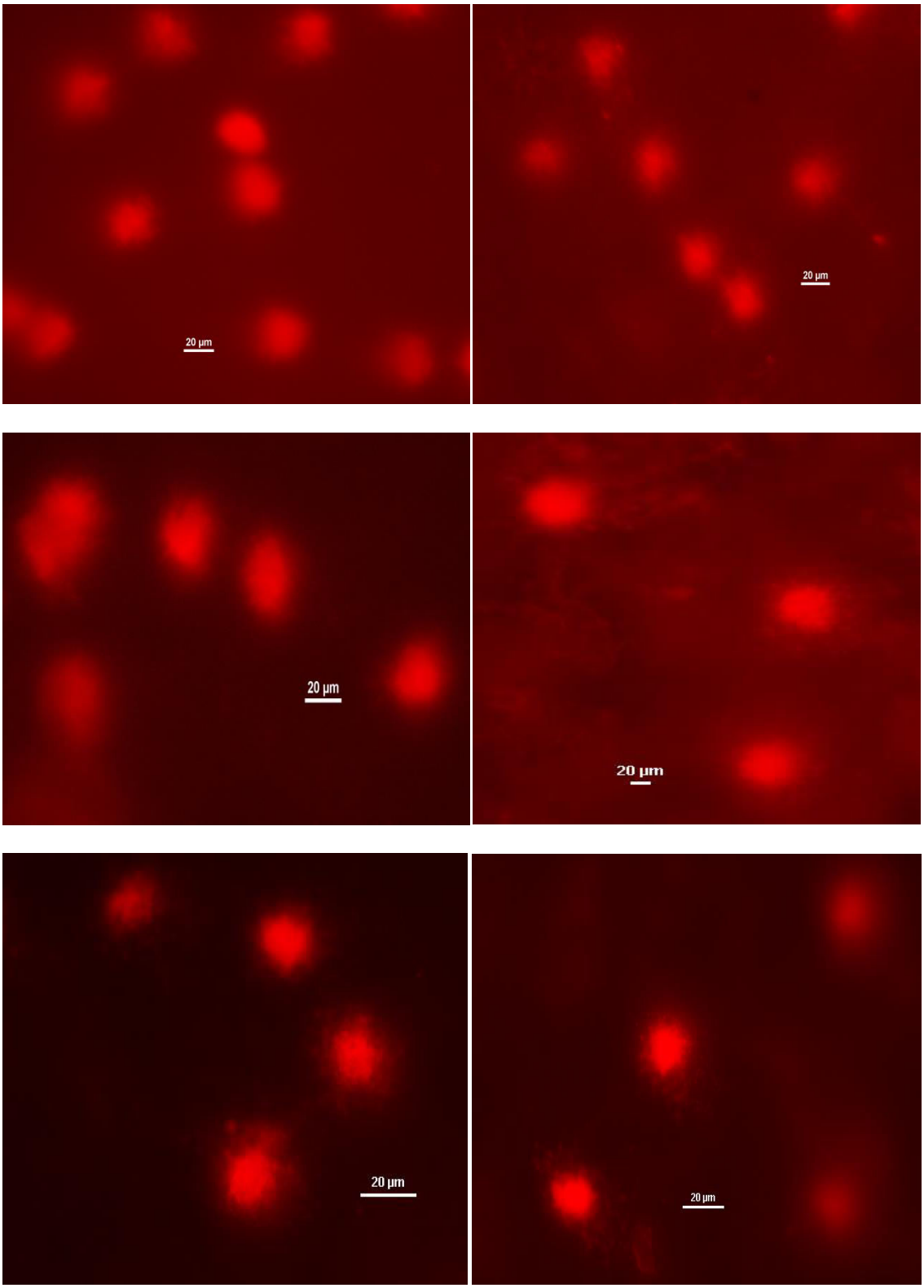


Figure 4.4: Levels of DNA damage detected in smokers by comet assay. Bar = 20 µm.

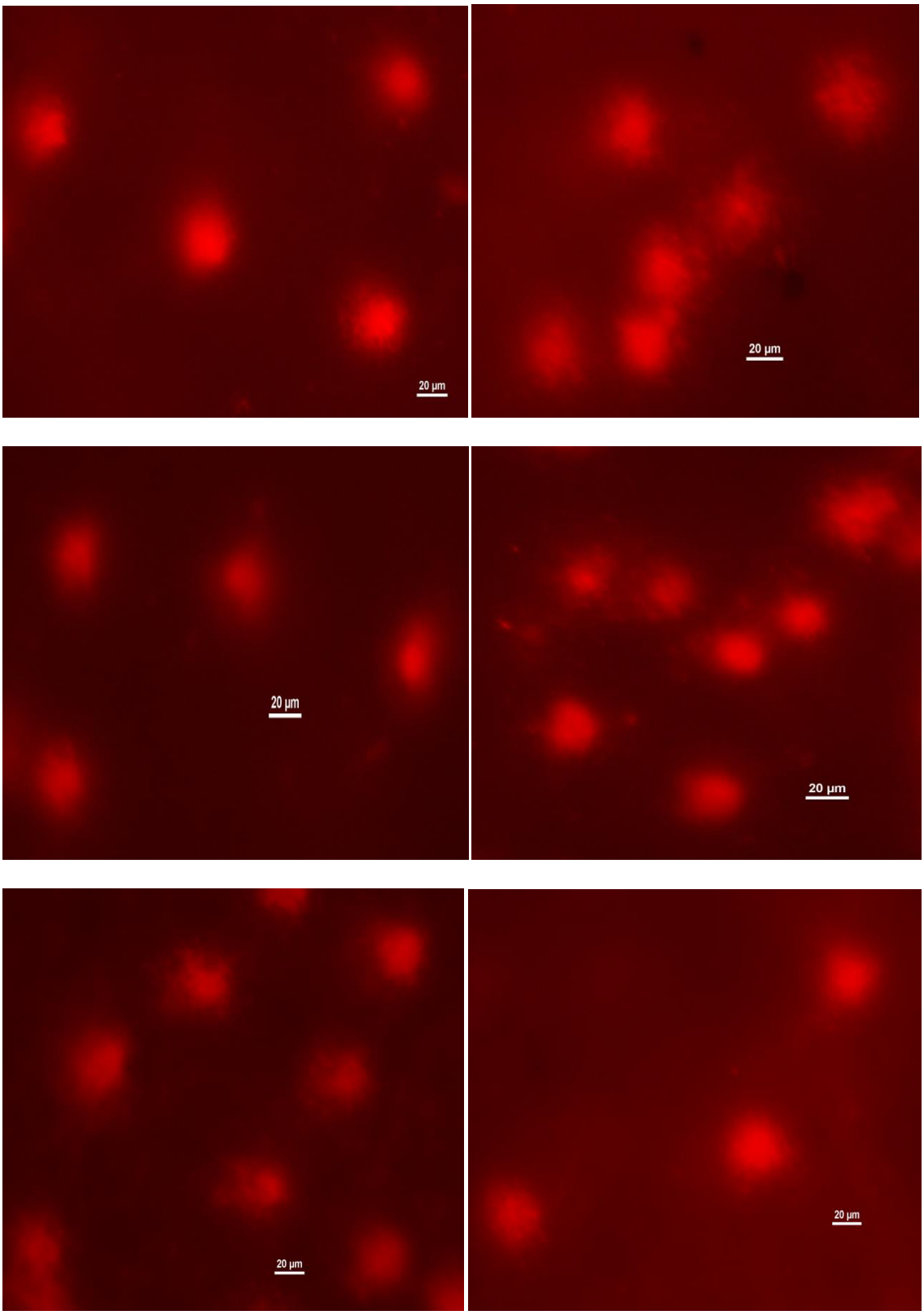


Figure 4.5: Levels of DNA damage detected in alcohol consumers by comet assay. Bar = 20 µm.

4.4 Discussion

The northern part of West Bengal is popular for its tea industry, which contributes a considerable share to the Indian economy and provides employment to a large sector of population (Gurusubramanian et al., 2008). A number of arthropod pests attack the tea plantation (Gurusubramanian et al., 2008) causing severe loss (Das, 1965). Planters use various formulations of the chemical pesticides to keep the pest populations under control and increase the yield. In the tea industry a large number of people (both males and females) are involved in tea leaf plucking and pesticide spraying or mixing and thus these worker populations are directly or indirectly at high risk of exposure to the toxic pesticides. From the agricultural point of view, the importance of pesticides cannot be denied; however, these biologically active compounds have adverse effects on the environment and non-target organisms including humans (Murphy, 1986; Bianchi et al., 1988).

Pesticides have been shown to cause mild to severe genome damage leading to different health hazards which includes a wide range of subclinical and clinical effects (cancer, adverse reproductive outcomes and other chronic illness) (IARC 1991; Arbucle and Sever, 1998; Lander et al., 2000; Meinert et al., 2000; Priyadarshi et al., 2000; Hagmar et al., 2001; Jenner, 2001; Ji et al., 2001; Alavanja et al., 2004; Hanke and Jurewicz, 2004; Kamel and Hoppin, 2004). Occupational exposure to xenobiotics creates covalent bond with DNA, leading to chromosome alterations which could result in chemical carcinogenesis (Fairbairn et al., 1995; Shah et al., 1997). In the present study the effect of pesticides on DNA damage in the worker population involved in tea leaf plucking, pesticide mixing and spraying was determined by comet assay. Results of the comet assay performed on peripheral blood lymphocytes of tea workers, controls, smokers and alcohol

consumers indicated occupational exposure to pesticide mixture leading to significant increase in DNA damage. As early as 1973, Yoder et al. (1973) had reported a marked increase in chromatid lesions in the peripheral lymphocytes of pesticide exposed individuals. About 3 times longer comet tail length (LTail), tail moment (TM) and olive tail moment (OTM) in the exposed workers than control subjects indicated high genome damage ($p \leq 0.001$) (**Table 4.1**). Bhalli et al. (2006) had reported almost three times longer comet tail length in the Pakistani pesticide manufacturing workers compared to the controls (20.0 ± 2.87 µm vs. 7.4 ± 1.48 µm, $p < 0.001$). Results of the present study showing 3-fold higher LTail in pesticide exposed workers are comparable to the findings of Bhalli et al. (2006). A number of studies have also revealed DNA fragmentation in the lymphocytes of the populations occupationally exposed to pesticides (Garaj-Vrhovac and Zeljezic 2000, 2001; Undeğer and Başaran, 2002; Grover et al., 2003; Castillo-Cadena et al., 2006; Remor et al., 2009; Paiva et al., 2011; Wilhelm et al., 2015). Comet assay study on the exfoliated buccal cells of pesticide exposed agricultural workers revealed increased DNA damage in the individuals exposed to a complex mixture of pesticides compared to the non-exposed individuals (Carbajal-López et al., 2016). Garaj-Vrhovac and Zeljezic (2000) used the alkaline version of comet assay on the peripheral blood lymphocytes of workers employed in pesticide production and observed that the tail length and tail moment were higher in pesticide exposed workers during high pesticide exposure period than controls. When the pesticide exposed workers spent 6 months out of pesticide exposure, inspite of the significant recovery of the damage, the tail length and tail moment were still high compared to the first result. It has been reasoned that the DNA damage revealed by comet assay might be the result of single-strand DNA breaks, repair of double-strand DNA-breaks, DNA adduct formations, DNA-DNA or DNA-protein

crosslinks and single strand breaks due to incomplete excision repair sites (King et al., 1993; Tice et al., 2000).

Since all male workers smoked cigarettes or bidis and consumed alcohol and few female workers in the present study smoked cigarettes or bidis and all consumed alcohol, smokers and alcoholics were included for comparison. Smoking has been reported to increase DNA damage as measured by comet assay (Frenzilli et al., 1997; Piperakis et al., 1998; Palus et al., 1999). The pesticide exposed workers had almost 3 fold higher LTail, TM and OTM than smokers and alcohol consumers (**Table 4.2**); however a comparison between control and smokers did not show any significant difference in damage parameters (**Table 4.1**) indicating that smoking had very little or no effect on the damage. This is in good agreement with the results obtained by other workers who also did not find any association between DNA damage and smoking (Undeğer and Başaran, 2002; Hoffmann et al., 2005; Castillo-Cadena et al., 2006; Kaur et al., 2011). Kaur et al. (2011) observed that the mean tail length among the smoker and non-smoker agricultural workers exposed to pesticides were 66.48 ± 19.36 µm and 75.08 ± 21.03 µm, which was statistically non-significant. Carbajal-Lopez et al. (2016) too found that smoking had no effect on genetic damage. Garaj-Vrhovac and Zeljezic (2000) found that within the pesticide exposed group no significant difference was observed in mean tail length and tail moment between smokers and non-smokers, whereas both the parameters were increased in smokers compared to the non-smokers of the control group. This may be due to the fact that cigarette smoke is not a very potent confounding factor of DNA damage among pesticide exposed subjects when peripheral lymphocytes are used (Garaj-Vrhovac and Zeljezic, 2000). Likewise, in the present study the LTail, TM and OTM in alcoholics were not significantly different than control. Though non-significant, the LTail, TM and OTM in smokers are slightly higher than the control.

In order to test the confounding effect of gender (sex), the control and exposed individuals were divided into males and females. On comparing the control males with exposed males and the control females with exposed females, the comet parameters (LTail, TM, OTM) were found to be significantly greater in the exposed males and females compared to the control males and females (**Table 4.3**). Though the comet parameters, LTail, TM and OTM between control and exposed groups were higher in the males compared to females of the respective category (group), the difference was statistically non-significant. This is because the rise did not reach significance level inspite of the greater difference. This may be due to the fact that for testing equality of means of two independent variables, the test statistics is related to their mean and standard error of means, a very high standard error of means may lead to acceptance of equality of means. Again, if standard error of means is very low the test statistics may lead to rejection of equality of means. A similar condition may account for the non-significant differences observed between males and females of control and exposed group. Other researchers too did not find any effect of gender (sex) on the DNA damage (Grover et al., 2003; Piperakis et al., 2003; Kaur et al., 2011).

To check if age acts as a confounding factor, the pesticide exposed individuals were categorized into 15-30, 31-45 and 46-62 age groups. LTail was slightly higher in the age group 31-45 years, whereas TM and OTM were slightly higher in the age group 15-30 years but the increase was non-significant (**Table 4.4**). No significant difference was observed in any of the age groups suggesting that age was not associated with DNA damage. The non-significant association of age and DNA damage was also reported by other workers (Grover et al., 2003; Kaur et al., 2011). In order to check if duration of exposure had any effect on LTail, TM and OTM, the pesticide exposed workers were classified into 3 groups (1-7 years, 8-15 years and >15 years) based on their employment

duration. Duration of exposure was found to have no effect on DNA damage, which corroborates the findings of Undeğer and Başaran (2002) and Kaur et al. (2011).

Therefore, a 3-fold greater comet LTail, TM and OTM in the tea garden workers engaged in tea leaf plucking or pesticide spraying strongly indicated higher extent of DNA damage than the non-exposed control, smokers and the alcohol consuming individuals and was independent of gender, age and duration of exposure.

CHAPTER 5

CYP2C9 polymorphism

5.1 Review of Literature

Despite the health concern, there is an increasing trend of application of new formulations of pesticides in agriculture, which are more toxic than the earlier ones. These pesticides exert the toxic effect on target and non-target organisms including humans. The pesticides, entering the human system, are detoxified through enzymatic action to hydrophilic metabolites and are eliminated, a process called biotransformation. These reactions are divided into phase I and phase II reactions (Abass et al., 2009). Cytochrome P450 (CYP) mixed function oxygenases are mainly responsible for phase I conversion. The CYP genes constitute a multigene family of CYP super family. Based on structural homology the CYP superfamily is divided into families and subfamilies. At present there are 18 families and 44 subfamilies in humans covering 57 functional CYP genes (Abass et al., 2009). A single nucleotide exchange causes allelic variants leading to non-synonymous amino acid substitution, differing only at few residues in the coding region (Schwarz, 2003). Rosemary and Adithan (2007) reviewed that oxidative biotransformation of exogenous compounds (drugs, procarcinogens, alcohols) are catalyzed by enzymes belonging to the families CYP1, CYP2 and CYP3, while others are involved in the metabolism of endogenous compounds (fatty acids, prostaglandins, steroids). CYP3A4 is the most abundant of CYP450 content in human liver (~28%) followed by CYP2C family (18%), CYP1A2 (~12%), CYP2E1 (7%), CYP2A6 (4%), CYP2D6 (1.5%) and CYP2B6 (0.2%). More than half of the drugs currently prescribed are metabolized by CYP3A (51%) followed by CYP2D6 (24%) and CYP2C (~20%) (Rosemary and Adithan, 2007).

Human cytochrome CYP2C9 gene is located on chromosome 10q24 and has an approximate length of 55 kb (Lee et al., 2002). In humans, this major enzyme of the

CYP2C subfamily constitutes approximately 20% of the hepatic cytochrome P450 enzyme (Takahashi and Echizen, 2001). The biotransformation of a huge variety of xenobiotics including the organophosphates, chlorpyrifos and parathion are found to be associated with CYP2C9 (Daly, 2003; Foxenberg et al., 2007). Mitochondrial membrane transportation is affected by organophosphate pesticides (Nakagawa and Moore, 1999). It disturbs cytochrome P450 system in human liver (Kappers et al., 2001; Sams et al., 2003) which plays a crucial role in pesticide metabolism. CYP2C9 also metabolizes approximately 10% of important therapeutic drugs (Goldstein and de Morais, 1994; Miners and Birkett, 1998; Streetman et al., 2000). There are 67 variant alleles of CYP2C9 located in the coding region (Du et al., 2016; http://www.cypalleles.ki.se/cyp2c9.htm). CYP2C9*2 and CYP2C9*3 allelic variants are most common and are associated with decreased metabolism of respective substrates (Sullivan-Klose et al., 1996; Crespi and Miller, 1997; Niemi et al., 2002).

In all ethnic population, the allelic variants of CYP2C9 namely CYP2C9*1, CYP2C9*2 and CYP2C9*3 are frequently found (Lee et al., 2002). The most characterized alleles of CYP2C9 gene are the CYP2C9*2 situated in exon 3 and CYP2C9*3 situated in exon 7. The CYP2C9*2 and CYP2C9*3 allelic variants results from CYP2C9*1 by a nucleotide substitution of $C_{430}\rightarrow T$ located in exon 3, leading to amino acid substitution of arginine by cysteine at position 144 and $A_{1075}\rightarrow C$ located in exon 7 leading to amino acid substitution of isoleucine by leucine at position 359 (Yoon et al., 2001). Studies have shown that the frequency of CYP2C9*2 allele varies significantly among the populations of north Indians and south Indians (Jose et al., 2005; Sistonen et al., 2009; Rathore et al., 2010; Yadav et al., 2010; Singh et al., 2011), while CYP2C9*3 allele was found to be equally distributed among the Indian populations (Paul et al., 2011). Enzyme activity is significantly reduced in the individuals with the allelic variants CYP2C9*2 and

CYP2C9*3 and these individuals may not be able to metabolize the substrate efficiently which in turn may lead to toxicity (Miners, 2002). Substantial reduction in metabolic activity has been found to be associated with the allele CYP2C9*3, while intermediate reduction in enzyme activity has been found to be associated with CYP2C9*2 allele in comparison to CYP2C9*1 allele (Lee et al., 2002). Both the CYP2C9 and CYP2C19 participate in the oxidative metabolism of warfarin, an anticoagulant (Kaminsky et al., 1993), diclofenac and ibuprofen, naproxen, piroxicam, aceclofenac (non steroidal anti-inflammatory agents), sulfamethoxazole and dapsone (antibacterials) (Leemann et al., 1993; Barry et al., 1997; Winter et al., 2000), H2 antagonist (Omeprazole) (Karam et al., 1996; Yamazaki et al., 1997), diazepam (benzodiazepine) (Jung et al., 1997; McGinnity et al., 1999) phenytoin (antiepileptics), rosiglitazone, tolbutamide, glyburide, glibenclamide, glimepiride, glipizide, losartan, irbesartan, torsemide, tamoxifen, fluvastatin, fluoxetine, amitriptyline (Lee et al., 2002; García-Martín et al., 2006). In a population study, Sullivan-Klose et al. (1996) have also reported the association of CYP2C9 with the metabolism of tolbutamide and the homozygotes of Leu^{359} allelic variant of CYP2C9 were identified as poor metabolizers of tolbutamide. A constant equilibrium exists between the metabolic activation and detoxification of xenobiotics in human body. Inter-individual variation is responsible for the expression and characterization of the enzymes. Oxidative desulphuration of the organophosphate parathion, chlorpyrifos and diazinon are mediated by the cytochrome P450 (Neal, 1967; Butler and Murray, 1997; Mutch et al., 1999; Sams et al., 2000).

5.1.1 CYP2C9 gene polymorphism in pesticide metabolism

Human hepatocytes when cultured for 72 h in a serum free medium on collagen with inducers namely rifampicin, dexamethasone and phenobarbital, CYP2C9 was found to be

expressed along with CYP2C8 and CYP2C19 (Raucy et al., 2002), while only CYP2C9 was expressed at an appreciable level in untreated hepatocytes. Biotransformation of organophosphorothioate insecticides (chlorpyrifos and diazinon) were investigated in human liver microsomes as well as recombinant human cytochrome P450s (Sams et al., 2004). At low substrate concentration some of the cytochromes (eg cytochromes 2C9, 2D6 and 3A4) made a minor contribution to the biotransformation of chlorpyrifos and diazinon (Sams et al., 2004). Chlorpyrifos is toxic *in vivo* due to its bioactivation to chlorpyrifos-oxon to a more potent cholinesterase inhibitor. The oxidation reaction possibly proceeds via a phospho oxythiiran intermediate in which the oxon is generated by a desulfuration reaction or the parent compound is degraded by a dearylation reaction (Chambers, 1992). Abass et al. (2009) studied the inhibition ability of 18 pesticides on CYP1A1/2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 2A4 and showed that CYP1A1/2 was most inhibited by organophosphorus insecticides followed by CYP2C8, CYP2C9, CYP2D6, CYP2A4. Organophosphates differentially inhibited different cytochromes. Deltamethrin and fenvalerate was found to potentially inhibit CYP2D6. At least one cytochrome was found to be inhibited by most of the pesticides (Abass et al., 2009).

A study was conducted to explore the DNA damage in workers occupationally exposed to organophosphate pesticides. A significantly higher DNA damage was observed in workers with CYP2D6*3 PM (poor metabolizers) and PON1 (QQ and MM) genotypes indicating that these genotypes can modulate DNA damage by gene-environment interactions (Singh et al., 2011). Enzymes involved in the metabolism of pyrethroid pesticides were studied in rat and human hepatic microsomes. In presence or absence of NADPH, rat and human hepatic microsomes were incubated with pyrethroids bifenthrin, *S*-bioallethrin, bioresmethrin, β-cyfluthrin, cypermethrin, *cis*-permethrin, and *trans*-permethrin. Rat cytochrome P450 (P450) isoforms CYP1A1, CYP1A2, CYP2C6,

CYP2C11, CYP3A1 and CYP3A2 and the human p450 isoforms CYP2C8, CYP2C9, CYP2C19 and CYP3A4 showed activity towards several pyrethroids (Scollon et al., 2009). Scollon et al. also commented that variable detoxification of pyrethroids results from species-specific difference in metabolism and these must be considered when assessing the adverse health effect of pyrethroids.

Studies have also been conducted to see the effect of CYP2C9 gene polymorphism on colorectal cancer (Zhao et al., 2013). Recently, Zhao et al. (2013) showed that CYP2C9*2, CYP2C9*3 alleles were not found to be associated with colorectal cancer susceptibility. Studies on human lymphoblastoma cells exposed to chlorpyrifos have revealed that CYP1A2, 2B6, 2C9*1, 2C19 and 3A4 are responsible for dearylation and desulfuration of chlorpyrifos and the highest dearylation and desulfuration activities were displayed by CYP2C19 and 2B6 (Tang et al., 2001).

Glimepiride is a drug used to treat Type 2 diabetic patients and is metabolized by CYP2C9 (Langtry and Balfour, 1998; Elsby et al., 2012). Dutta et al. (2015) studied the association between CYP2C9 genetic polymorphism and advanced drug reaction of glimepiride. The result indicated that CYP2C9*3 polymorphism was statistically significant in individuals with adverse drug reaction. The CYP2C9*1 allele is predominant in the individuals with no adverse drug reaction, while none of the individuals showing either drug reaction or no drug reaction had CYP2C9*2 allele (Dutta et al., 2015).

For the analysis of mutations in CYP2C9 gene, several protocols are available (Wang et al., 1995; Sullivan-Klose et al., 1996; Nasu et al., 1997). The validity of those methods for the genotyping was determined in Swedish population by Yasar et al. (1999). Using the protocol described by Sullivan-Klose et al. (1996) and Nasu et al. (1997) the

frequency of CYP2C9*2 and CYP2C9*3 alleles was determined to be 0.107 and 0.074 for 430 Swedish population (Yasar et al., 1999). It has been reported that CYP2C9*2 and CYP2C9*3 polymorphisms cause a decrease in enzymatic activity and the individuals carrying these alleles have clinical implications (Crespi and Miller, 1997; Takahashi and Echizen, 2001; Schwarz, 2003). Impaired metabolic activity of the CYP2C9 is observed in affected individuals when CYP2C9*3 allele appears in homozygous condition (Schwarz, 2003). CYP2C9*3 allele has been shown to cause the largest reduction in the metabolic capacity for many substrates of CYP2C9 followed by intermediate reduction caused by CYP2C9*2 allele, while CYP2C9*1 is least reduced (Lee et al., 2002). Studies have shown that severe toxicity may be experienced by CYP2C9*3/CYP2C9*3 homozygotes while metabolizing warfarin and phenytoin. These individuals may not respond adequately to drugs, such as losartan and cyclophosphamide that may result in therapeutic failure (Yasar et al., 2002). Review of literatures reveals that CYP2C9 and other cytochrome P450 alleles can be used as a good DNA marker for genetic association studies with respect to the xenobiotic exposure.

The present study contemplates a population study of CYP2C9 polymorphism in the pesticide-exposed tea garden workers to see association between CYP2C9 polymorphisms and pesticide exposure, if any.

5.2 Materials and methods

5.2.1 Sampling area

Sampling area was same as mentioned under **section 2.2.1**

5.2.2 Characteristics of the participants

Characteristics of the participants were same as mentioned under **section 2.2.2 Characteristics of the participants.**

5.2.3 Sampling procedure

As mentioned earlier venous blood was drawn by venepuncture using 5 ml sterile, disposable syringe, transferred to EDTA containing tubes and brought to the laboratory. The blood samples were kept in -20^{o}C until further use.

5.2.4 Ethical consideration

Ethical consideration was the same as mentioned under **section 2.2.8**

5.2.5 Genomic DNA extraction

For DNA extraction, 1 ml of R.B.C. lysis buffer (155mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) was added to 1 ml of peripheral blood in a 2 ml microcentrifuge tube and mixed thoroughly, centrifuged at 5000 rpm for 6 minutes at 4^{o}C. Supernatant was discarded. Approximately 1.5 ml chilled SET buffer (0.3M Sucrose, 0.002M EDTA pH 8.0, 0.025M Tris pH 8.0) was added to it, mixed, kept for 10 minutes at -20^{o}C and centrifuged at 5000 rpm for 15 minutes at 4^{o}C. Supernatant was discarded, chilled TEN buffer (0.01M Tris pH 8.0, 0.1 mM EDTA pH 8.0, 0.1M NaCl) was added to the pellet upto 1 ml volume and SDS was added to a final concentration of 1% and kept at 50^{o}C for 10 minutes. The mixture was then kept at room temperature for 10 minutes, 5M NaCl was added to the mixture to a final concentration of 1M, mixed slowly and kept in ice for 60 minutes. The mixture was centrifuged at 11,000 rpm for 15 minutes at 4^{o}C, supernatant was collected and equal volume of Tris-saturated phenol was added to it and mixed,

centrifuged at 8000 rpm for 10 minutes at 4°C. Upper aqueous phase was collected and equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to it, mixed and centrifuged at 8000 rpm for 10 minutes at 4°C. The aqueous phase was collected and equal volume of chloroform-isoamyl alcohol (24:1) was added, centrifuged at 8000 rpm for 10 minutes at 4°C. Finally, to the aqueous phase 1/10th volume of Na-acetate (3M pH 5.2) and twice the volume of chilled ethanol was added, kept at -20°C for 1 hour. DNA was peletted by centrifugation at 15,000 rpm for 15 minutes at 4°C. Pellet was washed with chilled 70% ethanol by centrifuging at 15,000 rpm for 15 minutes at 4°C. DNA was dried and dissolved in 25 µl Tris-EDTA pH 8.0 (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). DNA samples were kept at -20°C until further use.

5.2.6 PCR amplification

CYP2C9 region of DNA was amplified using the polymerase chain reaction (PCR). Amplification was carried out in a thermalcycler (primus ^{advanced} 96 of PEQLAB) using the appropriate primers (**Table 5.1**) and PCR cycling conditions (**Table 5.2**).

Table 5.1: CYP2C9 specific primer sequences.

Primer Name	**Sequence 5' → 3'**	**Detection**	**Amplicon size (bp)**	**Reference**
1F	cac tgg ctg aaa gag cta aca gag	CYP2C9*2 *Ava*II, 37°C Wt: 296, 79; variant: uncut	375	Aynacioglu et al. (1999); Burian et al. (2002)
1R	gtg ata tgg agt agg gtc acc cac			
3F	aat aat aat atg cac gag gtc cag aga tGc	CYP2C9*3 *Nsi*I, 37°C Wt: 112,29; variant: uncut	141	Sullivan-Klose et al. (1996)
3R	gat act atg aat ttg gga ctt c			
4F	aat aat aat atg cac gag gtc cag agG tac	CYP2C9*3 *Kpn*I, 37°C Wt: uncut; variant: 111,30	141	Sullivan-Klose et al. (1996)
4R	gat act atg aat ttg gga ctt c			

Table 5.2: PCR cycling condition for detection of CYP2C9*2 and CYP2C9*3 allele.

Cycling Condition	**CYP2C9*1/CYP2C9*2 using *Ava*II restriction enzyme**	**CYP2C9*1/CYP2C9*3 using *Nsi*I restriction enzyme**	**CYP2C9*1/CYP2C9*3 using *Kpn*I restriction enzyme**
Lid temperature	105^{o}C	105^{o}C	105^{o}C
Initial denaturation	95^{o}C, 10 min	95^{o}C, 5 min	95^{o}C, 5 min
Number of cycle(s)	45	35	35
Denaturation	95^{o}C, 5 secs	95^{o}C, 45 secs	95^{o}C, 45 secs
Annealing	67^{o}C, 10 secs	58^{o}C, 20 secs	58^{o}C, 20 secs
Extension	72^{o}C, 15 secs	72^{o}C, 2 minutes	72^{o}C, 2 minutes
Final extension step	72^{o}C, 5 min	72^{o}C, 5 min	72^{o}C, 5 min
Store forever	4^{o}C	4^{o}C	4^{o}C

5.2.7 Preparation of reaction mixture

The PCR reaction was set in 0.2 ml PCR tubes. For several parallel reactions, a master mix was prepared by multiplying the quantity of each reagent for 25 µl reaction mixture as shown in **Table 5.3**. Master mix containing water, buffer, dNTPs, primers and Taq DNA polymerase was dispensed in individual tubes and the template DNA was added to each tube. The reaction mixture was gently vortexed followed by brief centrifugation and placed in a thermalcycler.

Table 5.3: Composition of reaction mixture for detection of CYP2C9*2 and CYP2C9*3 allele.

Reagent	Final Concentration		Quantity for 25 µl reaction mixture	
	CYP2C9*2	CYP2C9*3	CYP2C9*2	CYP2C9*3
Sterile deionized water			13.6 µl	14.3 µl
5X Taq buffer	1X	1X	5 µl	5 µl
dNTP mix	200 µM	200 µM	0.5 µl	0.5 µl
25mM $MgCl_2$	2.5 mM	3 mM	2.5 µl	3 µl
1F Primer	1 µM	0.4 µM	1 µl	0.4 µl
1R Primer	1 µM	0.4 µM	1 µl	0.4 µl
Taq DNA polymerase	5 U/ λ	5 U/ λ	0.4 µl	0.4 µl
Template DNA	90 ng	90 ng	1 µl	1 µl

5.2.8 Amplification check by Agarose gel electrophoresis

The amplified PCR products were checked by separating on 2.5% (for 1F 1R primers) and 3% (for 3F 3R and 4F 4R primers) agarose gel containing 0.5 µg/ml ethidium bromide. The electrophoresis was carried out in TAE (40 mM Tris, 40 mM acetic acid, 1 mM EDTA pH 8.0) containing buffer with 100 bp DNA ladder as size marker. Gels were viewed on Gel Documentation system (Spectroline of Bio-vision) and photographed with transmitted UV light (**Figure 5.1**).

DNA sequencing of representative samples were done to validate the PCR-RFLP protocol (**Figures 5.2, 5.3, 5.4**).

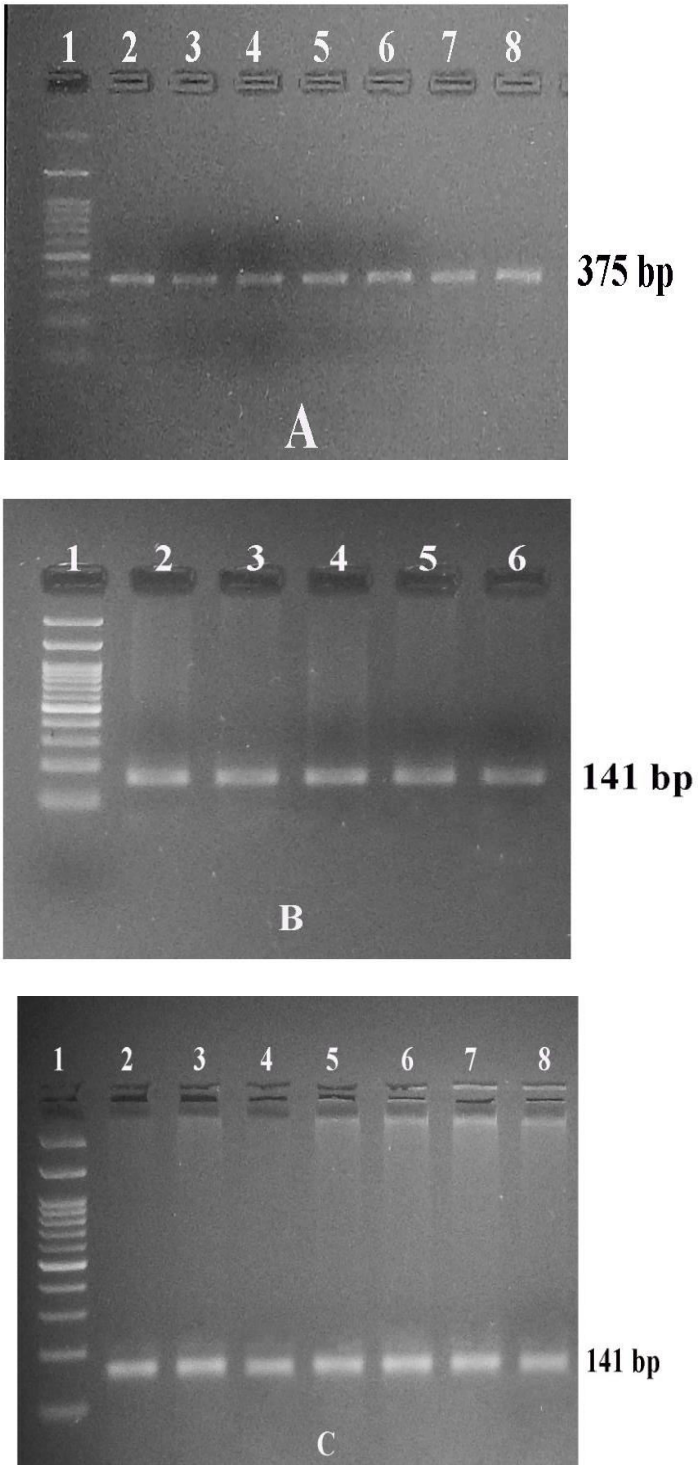
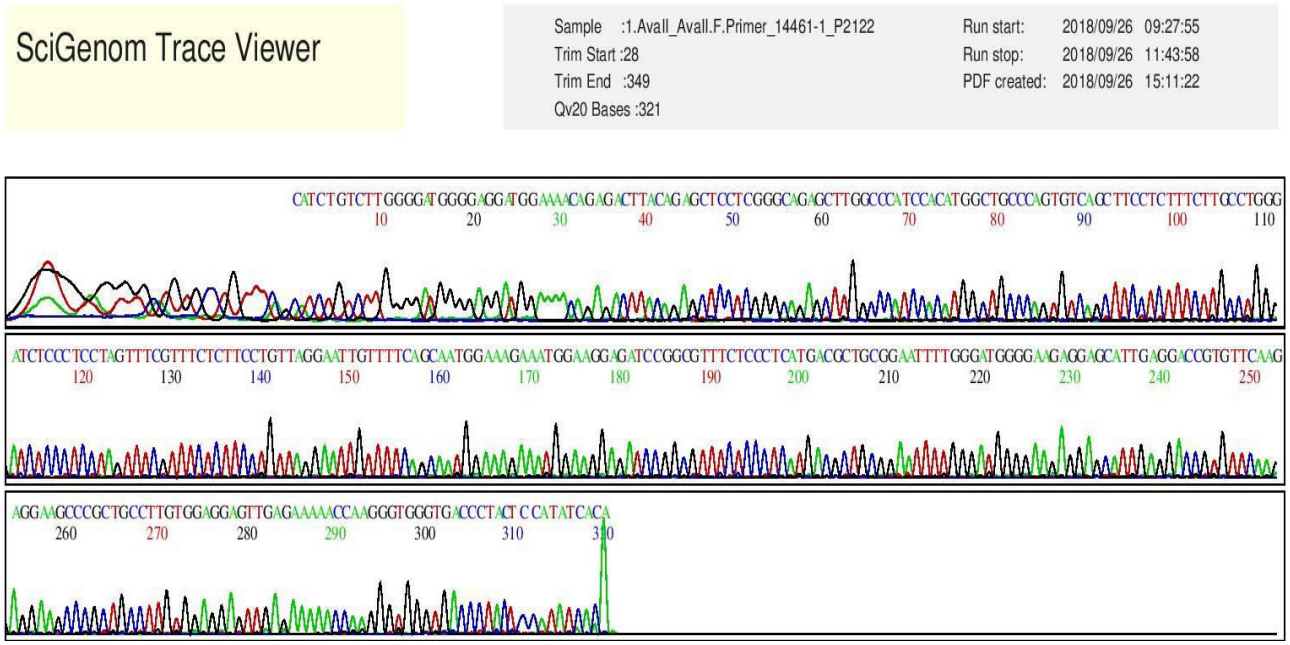


Figure 5.1: Gel showing PCR amplicons using Primer sets, **(A)** 1F and 1R **(B)** 3F and 3R **(C)** 4F and 4R (as shown in **Table 5.1**). Lane 1 in each gel indicates 100 bp DNA marker.



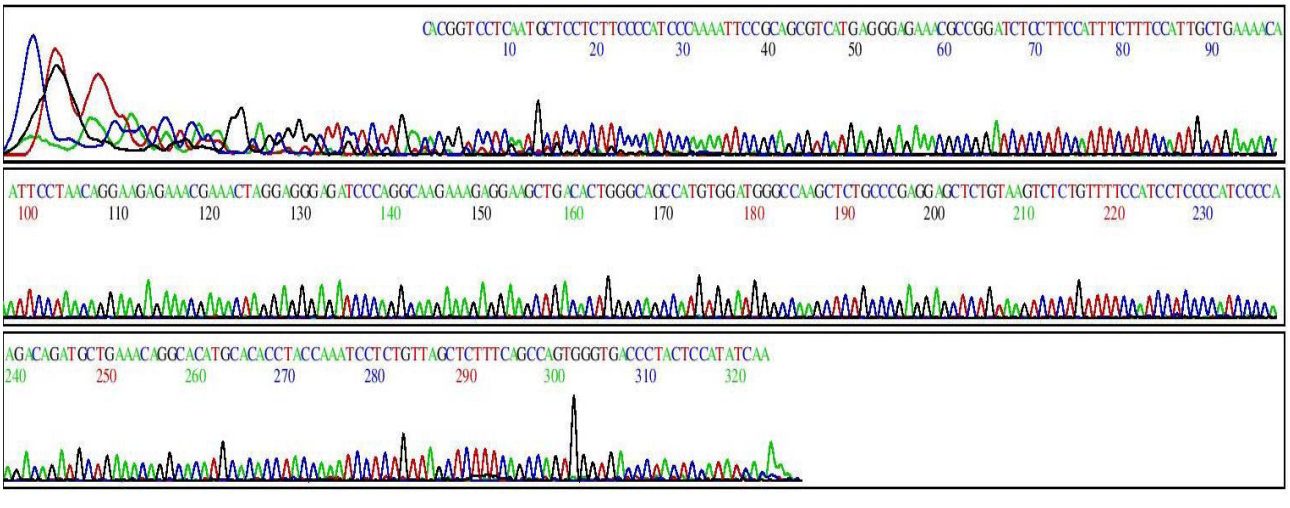


Figure 5.2: Chromatogram of PCR product sequence (exon 3, *Ava*II recognition site) generated by automated sequence analyzer showing 4 peaks of four nucleotides.

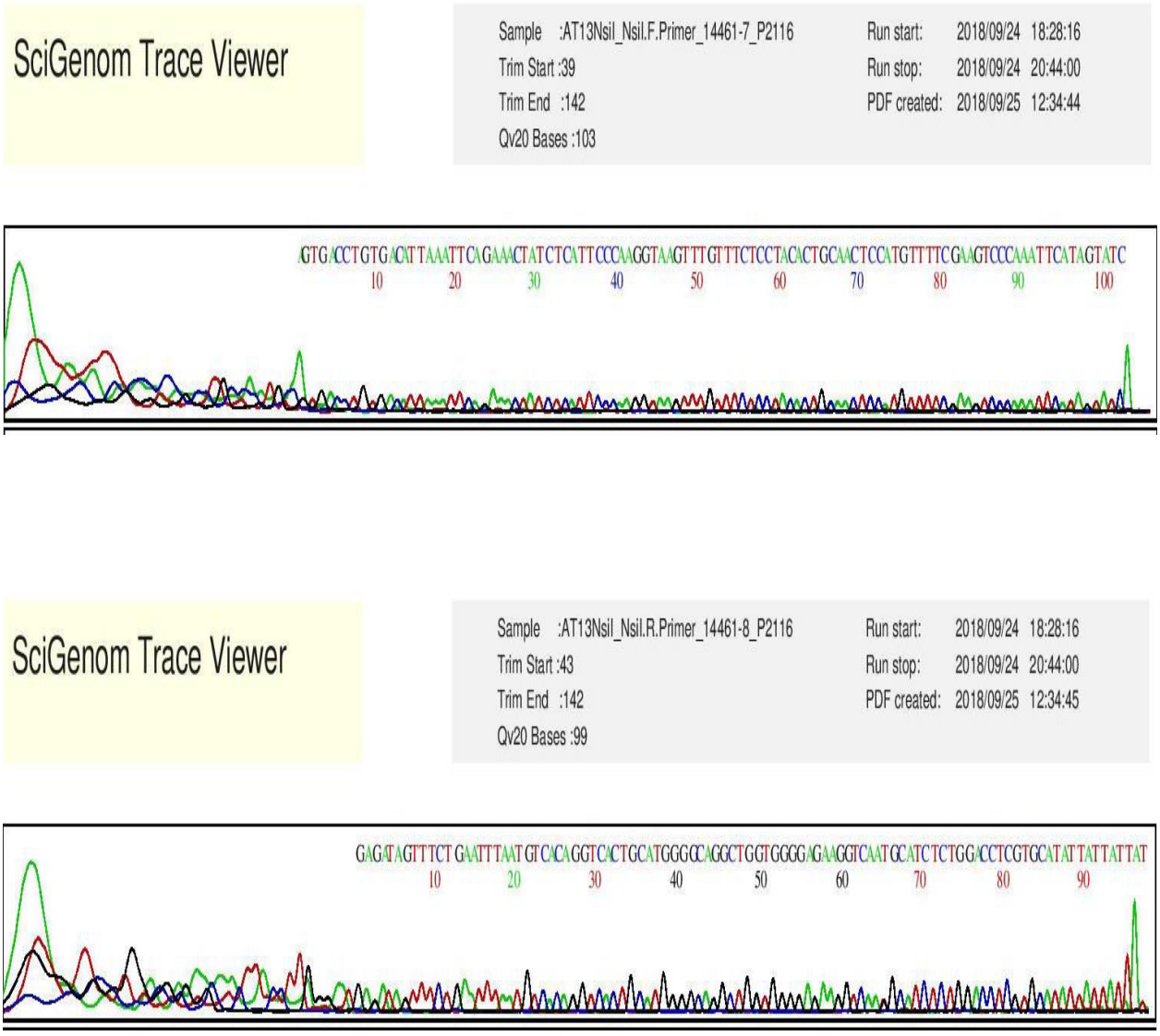


Figure 5.3: Chromatogram of PCR product sequence (exon 7, *Nsi*I recognition site) generated by automated sequence analyzer showing 4 peaks of four nucleotides.

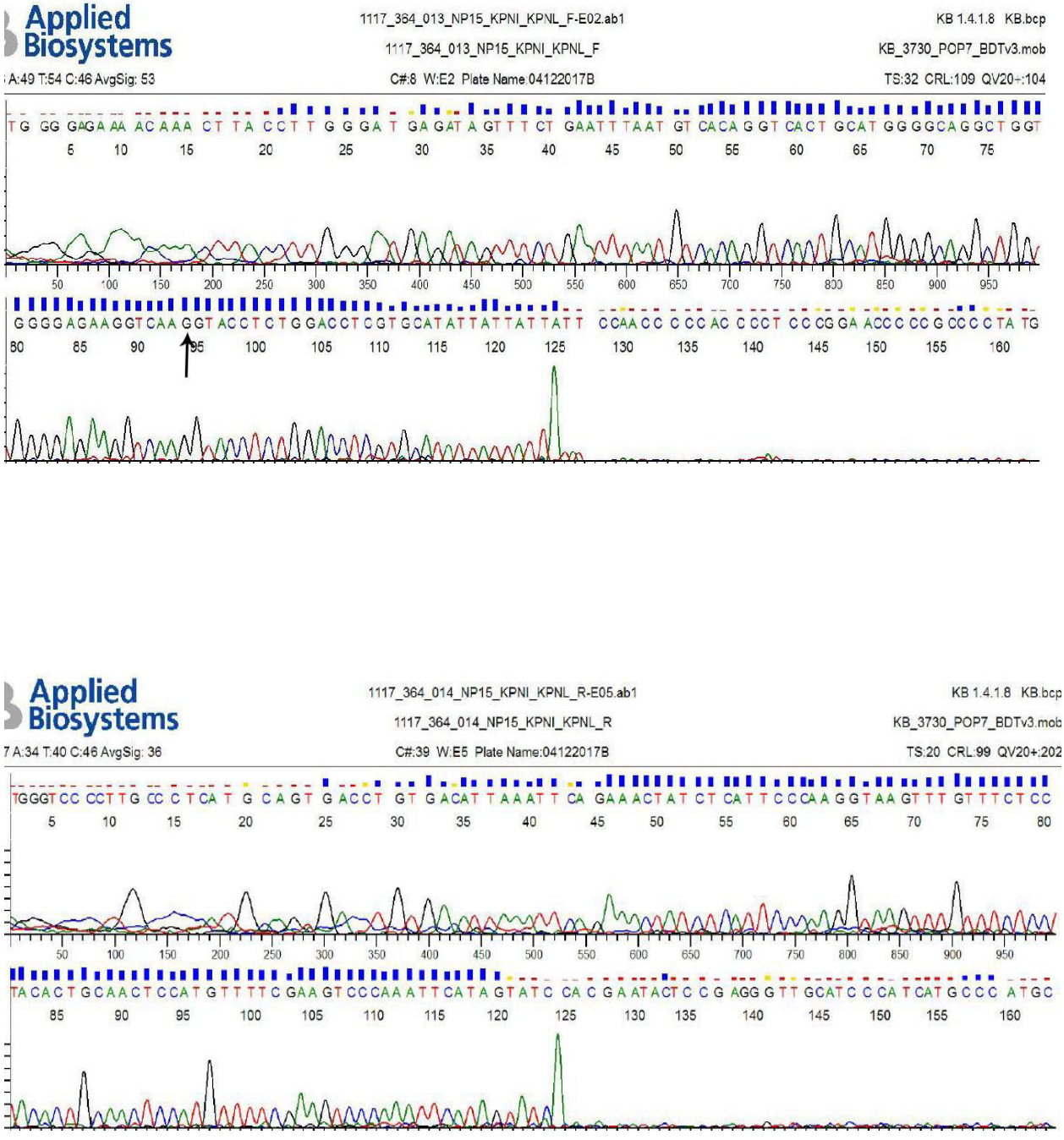


Figure 5.4: Chromatogram of PCR product sequence (exon 7, *Kpn*I recognition site) generated by automated sequence analyzer showing 4 peaks of four nucleotides (arrow indicates a variant for CYP2C9*3 allele).

5.2.9 Restriction digestion of PCR products

Aliquotes of each PCR product (10 µl) were digested with restriction endonucleases (*Ava*II for CYP2C9*2 and *Nsi*I and *Kpn*I for CYP2C9*3) at 37^{o}C overnight for complete digestion. The DNA fragments were electrophoresed either on 2.5% agarose gel to detect CYP2C9*2 allele or 3% agarose gel to detect CYP2C9*3 allele. Bands were detected by viewing on Gel Documentation system (Spectroline of Bio-vision) and photographed with transmitted UV light (**Figures 5.5, 5.6 and 5.7**).

Table 5.4: Composition of reaction mixture for restriction digestion.

Reagent	Final Concentration	Quantity for 20 µl reaction mixture
Sterile deionized water		4.6 µl
Buffer	2X	4 µl
BSA	0.2 mg/ml	0.4 µl
Restriction enzyme	1U/ µl	1 µl

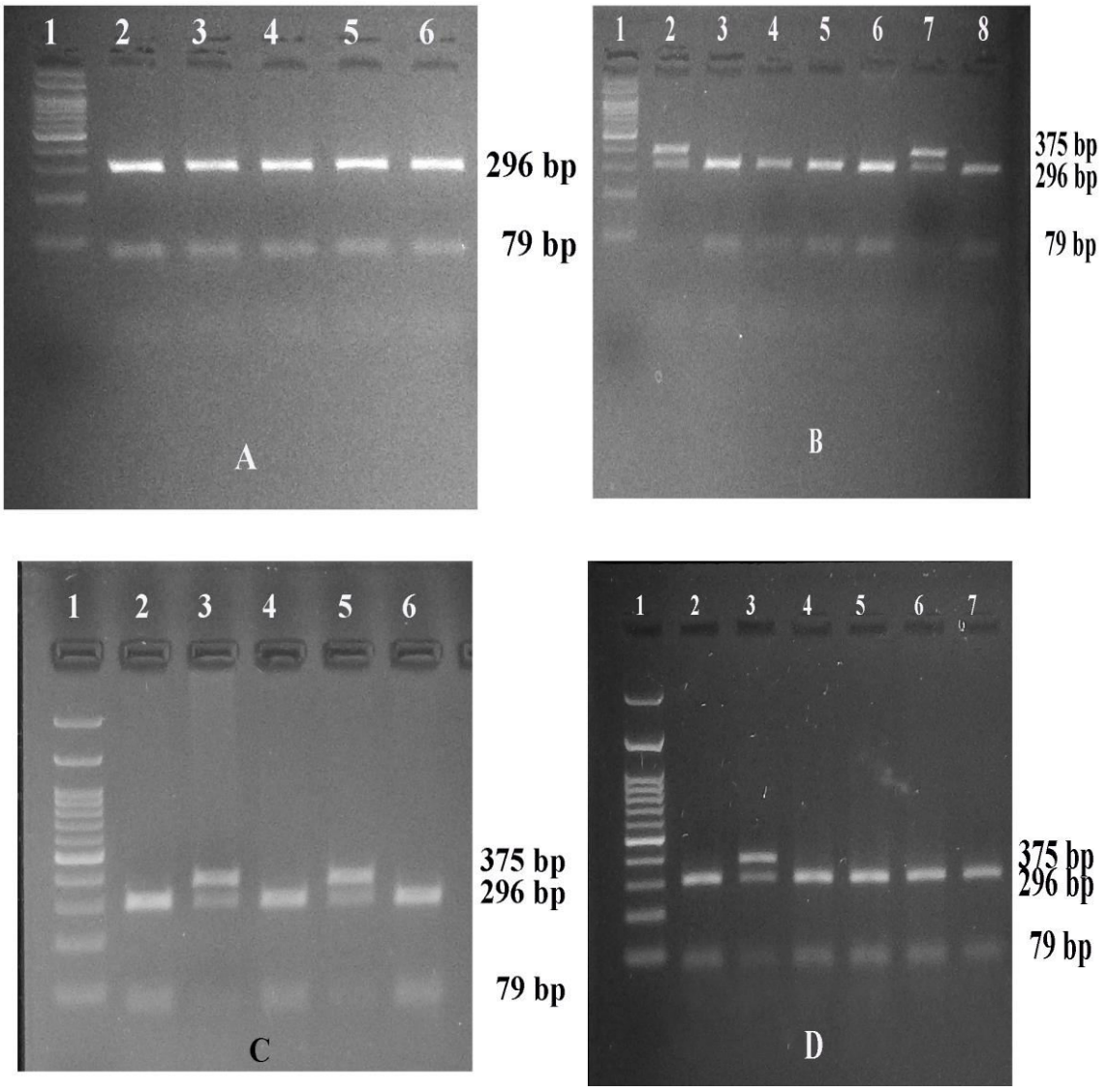


Figure 5.5: PCR-RFLP analysis of CYP2C9 gene using *Ava*II
(**A)** Lanes 2-6 indicate CYP2C9*1/*1 (296 bp and 79 bp).
(B) Lanes 2,7 indicate CYP2C9*1/*2 (375 bp, 296 bp and 79 bp), Lanes 3,4,5,6,8 indicate CYP2C9*1/*1.
(C) Lanes 2,4,6 indicate CYP2C9*1/*1, Lanes 3,5 indicate CYP2C9*1/*2.
(D) Lanes 2,4,5,6,7 indicate CYP2C9*1/*1, Lane 3 indicates CYP2C9*1/*2.
Lane 1 denotes 100 bp DNA marker (A, B, C and D).

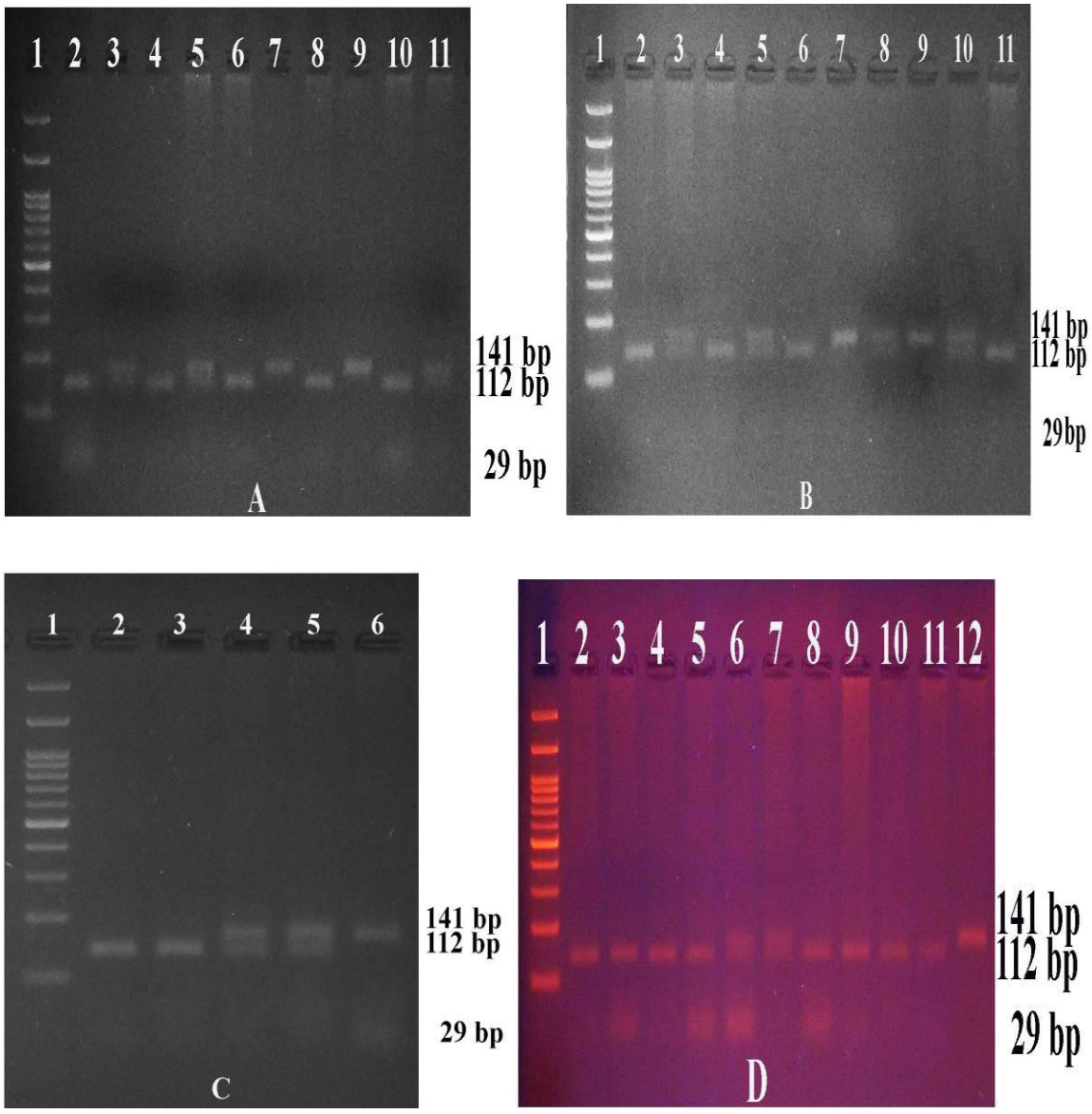


Figure 5.6: PCR-RFLP analysis of CYP2C9 gene using *Nsi*I.
(A) Lanes 2,4,6,8,10 indicate CYP2C9*1/*1 (112 bp and 29 bp), Lanes 3,5,11 indicate CYP2C9*1/*3 (141 bp, 112 bp and 29 bp), Lanes 7,9 indicate CYP2C9*3/*3 (141 bp).
(B) Lanes 2,4,6,11 indicate CYP2C9*1/*1. Lanes 3,5,8,10 indicate CYP2C9*1/*3. Lanes 7,9 indicates CYP2C9*3/*3.
(C) Lanes 2,3 indicate CYP2C9*1/*1. Lanes 4,5 indicate CYP2C9*1/*3, Lane 6 indicates CYP2C9*3/*3.
(D) Lanes 2,3,4,5,8,9,10,11 indicate CYP2C9*1/*1, Lanes 6,7 indicate CYP2C9*1/*3, Lane 12 indicates CYP2C9*3/*3.
Lane 1 denotes 100 bp DNA marker (A, B, C and D).

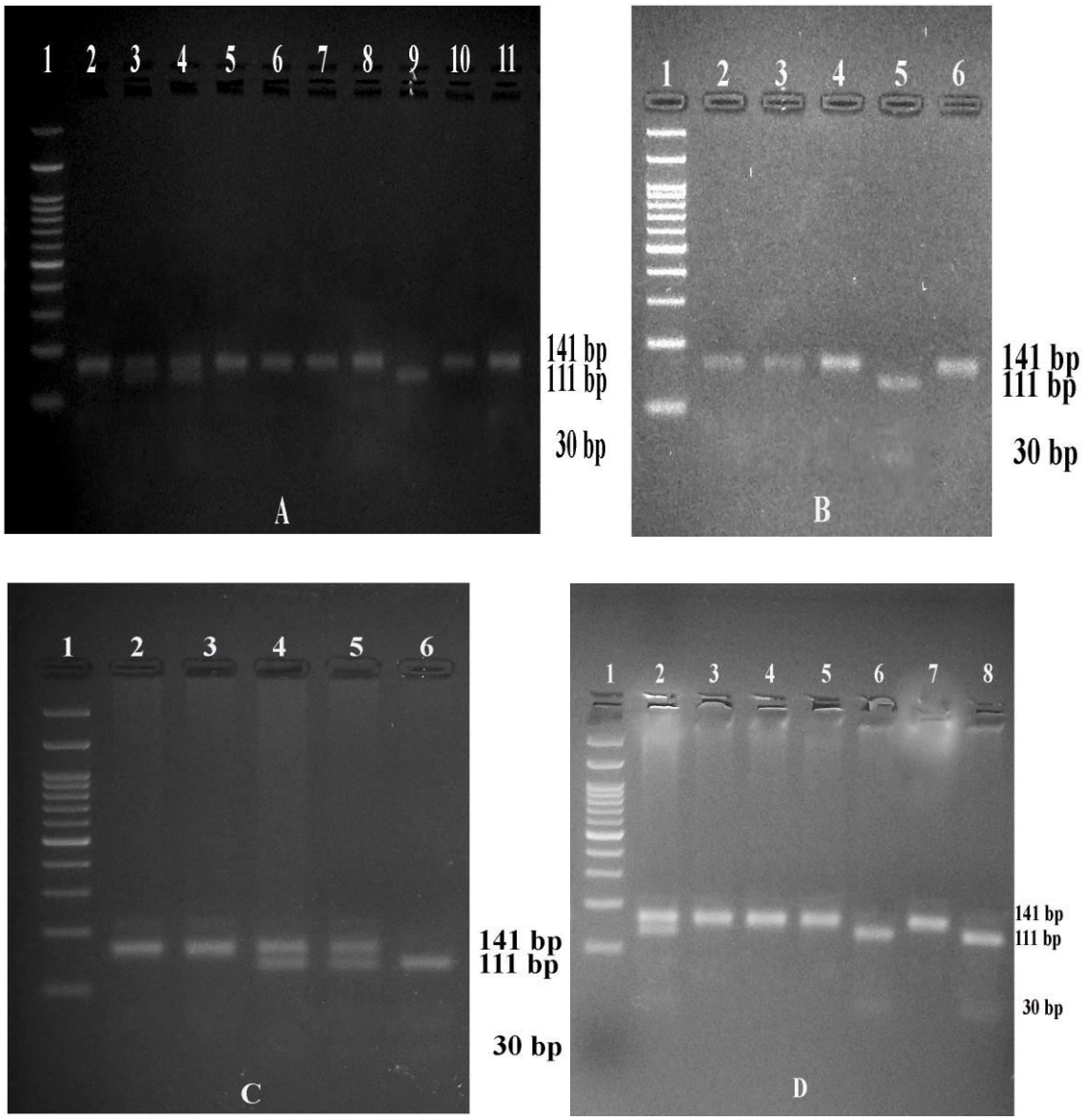


Figure 5.7: PCR-RFLP analysis of CYP2C9 gene using *Kpn*I.
(A) Lanes 2,5,6,7,8,10,11 indicate CYP2C9*1/*1 (141 bp), Lanes 3,4 indicate CYP2C9*1/*3 (141 bp, 111 bp and 30 bp), Lane 9 indicates CYP2C9*3/*3 (111 bp and 30 bp).
(B) Lanes 2,3,4,6 indicate CYP2C9*1/*1, Lane 5 indicates CYP2C9*3/*3.
(C) Lanes 2,3 indicate CYP2C9*1/*1, Lanes 4,5 indicate CYP2C9*1/*3, Lane 6 indicates CYP2C9*3/*3.
(D) Lane 2 indicates CYP2C9*1/*3, Lanes 3,4,5,7 indicate CYP2C9*1/*1, Lanes 6,8 indicate CYP2C9*3/*3.
Lane 1 denotes 100 bp marker (A, B, C and D).

5.2.10 Detection of restriction digestion product

Interpretation of CYP2C9*2 allele was done according to Burian et al. (2002). PCR with 1F and 1R primer produced a 375 bp amplicon. Samples containing CYP2C9*1 allele produced 296 bp and 79 bp after digestion with *Ava*II, CYP2C9*2 allele was not cleaved and remained as 375 bp. Samples heterozygous for CYP2C9*1/*2 allele produced bands of 375 bp, 296 bp and 79 bp (**Figure 5.8**).

Interpretation of CYP2C9*3 allele was done according to Sullivan-Klose et al. (1996). Both the primer sets 3F 3R and 4F 4R produced 141 bp amplicon. When digested with *Nsi*I and *Kpn*I samples containing CYP2C9*1 allele produced 112 bp and 29 bp after digestion with *Nsi*I, CYP2C9*3 allele was not cleaved. Samples heterozygous for CYP2C9*1/*3 allele produced bands of 141 bp, 112 bp and 29 bp (**Figure 5.9**). Samples containing CYP2C9*3 allele produced 111 bp and 30 bp after digestion with *Kpn*I, CYP2C9*1 allele was not cleaved by *Kpn*I. Samples heterozygous for CYP2C9*1/*3 alleles produced bands of 141 bp, 111 bp and 30 bp (**Figure 5.10**).

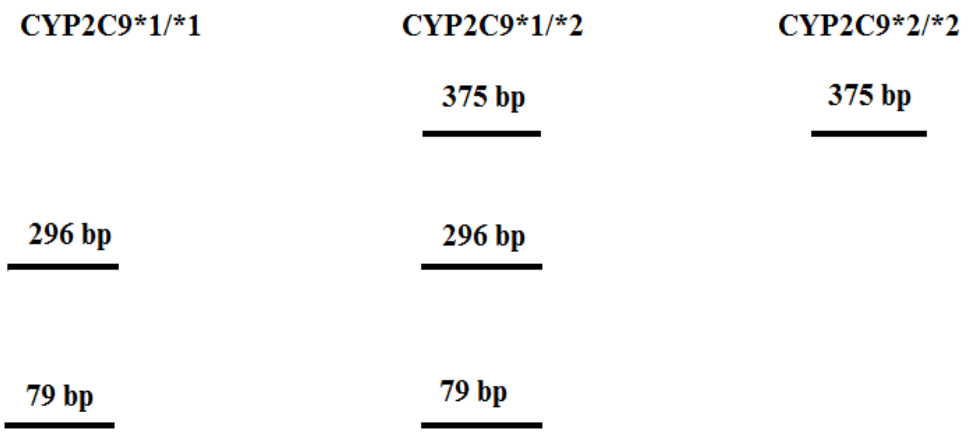


Figure 5.8: *Ava*II RFLP pattern of the PCR product produced by primer set 1F 1R.

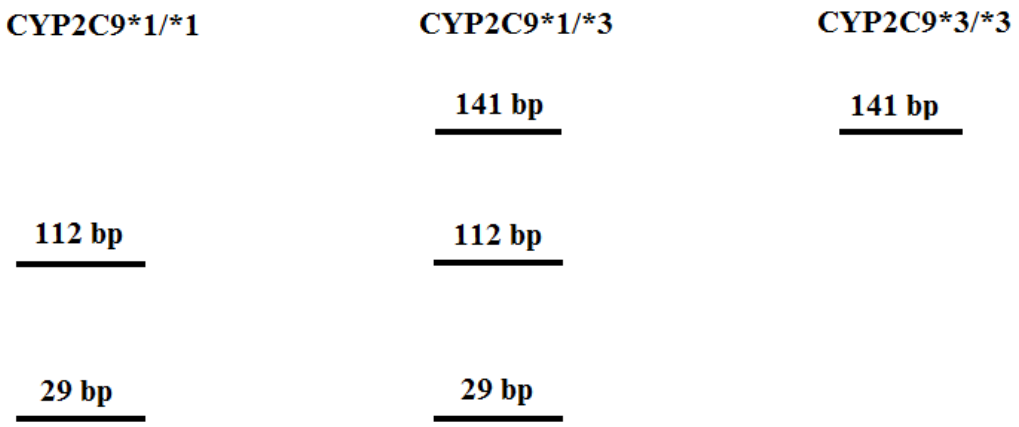


Figure 5.9: *Nsi*I RFLP pattern of the PCR product produced by primer set 3F 3R.

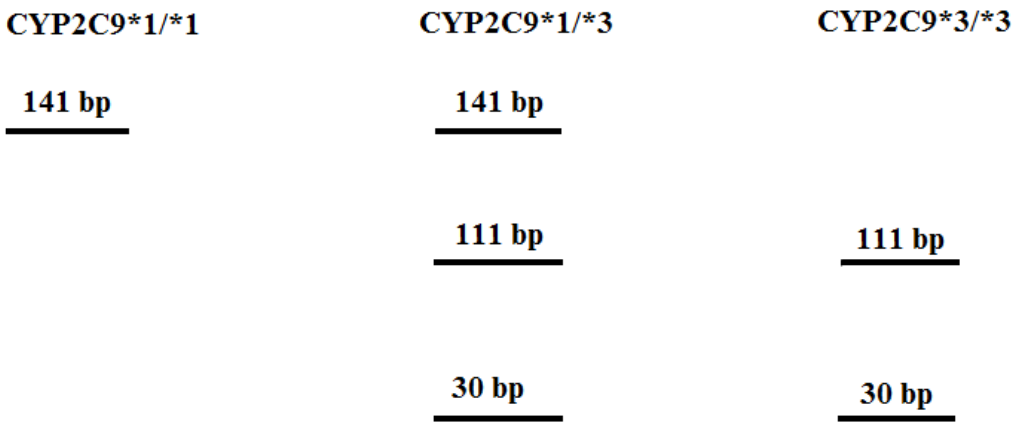


Figure 5.10: *Kpn*I RFLP pattern of the PCR product produced by primer set 4F 4R.

5.2.11 Statistical analysis

Genotype and allele frequencies were calculated according to Hardy-Weinberg equilibrium. χ^2 test was done to see whether the population surveyed were in equilibrium.

Allele frequency was calculated according to the formula given below:

Allele frequency = Genotype frequency of Homozygotes + ½ X Genotype frequency of Heterozygote (Strickberger, 1999).

5.3 Results

A total of 224 individuals were genotyped for CYP2C9 gene using exon 2 forward, intron 3 reverse primer (for detection of CYP2C9*2 allelic variant) and exon 7 forward, intron 7 reverse primer (for detection of CYP2C9*3 allelic variant) and PCR RFLP. Out of 224 individuals, 94 were pesticide exposed tea garden workers and rest 130 were considered as non-tea garden workers which included 60 control subjects, 39 smokers and 31 alcohol consumers. They were not directly exposed to pesticides and resided in areas away from the pesticide-exposed ones. The 94 pesticide exposed individuals were directly involved in pesticide spraying/mixing or tea leaf plucking. The results obtained from the analysis of CYP2C9 gene are represented in **Table 5.5 and 5.6**. 94 tea garden workers (exposed to pesticides) and 130 non-tea garden workers (not exposed to pesticides) were analysed to estimate CYP2C9 allelic variants compared to wild type. Analysis revealed the presence of all the genotypes except CYP2C9*2/*2 (**Table 5.6**).

In case of non-tea garden worker population, PCR analysis showed that CYP2C9*1 is the most predominant allele and is present at a frequency of 0.87695 (78.46% of individuals). Sequences containing CYP2C9*2 and CYP2C9*3 were also studied. 2.30% of the individuals were found to be homozygous for CYP2C9*3 with an allele frequency of 0.09615. No homozygotes for CYP2C9*2 allele were detected. Genotyping revealed that one individual was heterozygous for both mutations hence was interpreted as CYP2C9*2/*3 genotype as there is no evidence of linkage between CYP2C9*2 and

CYP2C9*3 allele on the same chromosome (Stubbins et al., 1996). The CYP2C9*2 allele was found to be least frequent with a frequency of 0.02690 (**Table 5.5**). About 0.77% of individuals were heterozygous for CYP2C9*2/*3 alleles. 4.62% of individuals were heterozygous for CYP2C9*1/*2 allele, while 13.85% of non-tea garden worker population were found to be heterozygous for CYP2C9*1/*3 alleles, respectively (**Table 5.6**). The observed and expected frequency distribution indicates that the results are in good agreement with that of the expected calculated by the Hardy-Weinberg law. χ^2 value was found to be 3.661 ($p < 0.05$).

Similarly, CYP2C9 genotyping was performed on 94 pesticide exposed workers population. CYP2C9*1 was the most frequent allele present with a frequency of 0.87235 (77.66% of individuals). 2.13% of individuals were homozygous for CYP2C9*3 allele giving an allele frequency of 0.10105. Like non-tea garden workers no homozygotes were found for CYP2C9*2 in worker population. Genotyping revealed that one individual was heterozygous for CYP2C9*2/*3 genotype with a frequency of 1.06%. The CYP2C9*2 allele was found to be least frequent with a frequency of 0.02660. 4.26% of tea garden worker population was observed to be heterozygous for CYP2C9*1/*2 alleles and 14.89% were heterozygous for CYP2C9*1/*3 alleles (**Table 5.6**). The allele frequency in pesticide exposed worker population is comparable to those of non-tea garden worker (**Table 5.5**). The observed and expected frequency distribution indicates that the results are in good agreement with that of the expected as calculated using the Hardy-Weinberg law with a χ^2 value of 1.8 ($p < 0.05$).

Table 5.5: Allele frequency of non-tea garden worker and tea garden worker populations.

Allele	**Allele frequency**	
	Non-Worker	**Worker**
CYP2C9*1	0.87695	0.87235
CYP2C9*2	0.02690	0.02660
CYP2C9*3	0.09615	0.10105

Table 5.6: Observed and expected frequency distribution and number in non-tea garden workers and tea garden workers.

	CYP2C9 allelic variants						
	***1/*1**	***1/*2**	***1/*3**	***2/*3**	***2/*2**	***3/*3**	**Total**
Non Worker							
Observed no. (N=130)	102 (78.46%)	6 (4.62%)	18 (13.85%)	1 (0.77%)	nil	3 (2.30%)	130
Expected no. (N=130)	100 (76.92%)	6.1 (4.69%)	21.9 (16.85%)	0.7 (0.54%)	0.1 (0.08%)	1.2 (0.92%)	130
χ^2	**0.040**	**0.002**	**0.690**	**0.129**	**0.100**	**2.700**	**3.661**
Worker							
Observed no. (N=94)	73 (77.66%)	4 (4.26%)	14 (14.89%)	1 (1.06%)	nil	2 (2.13%)	94
Expected no. (N=94)	71.5 (76.06%)	4.3 (4.58%)	16.5 (17.55%)	0.6 (0.64%)	0.1 (0.11%)	1 (1.06%)	94
χ^2	**0.03**	**0.02**	**0.38**	**0.27**	**0.10**	**1.00**	**1.8**

The results are in good agreement with the expected number calculated using Hardy-Weinberg law.

5.4 Discussion

Cytochrome P450 enzyme system is known to be inhibited by certain pesticides for example parathion, carbaryl, chlorpyrifos etc. resulting in free radical production which causes DNA damage (Butler and Murray, 1997; Dinsdale and Verschoyle, 2001). The biotransformation of a huge variety of xenobiotics including the organophosphates (chlorpyrifos and parathion) is associated with CYP2C9 (Daly, 2003; Foxenberg et al., 2007). A number of exogenous compounds namely drugs, procarcinogens, alcohols etc. are catalyzed by enzymes belonging to the families CYP1, CYP2 and CYP3, while others metabolize fatty acids, prostaglandins, steroids (endogenous compounds) (van der and Steijns, 1999; Review by Rosemary and Adithan, 2007). In view of the above, a genotyping of the pesticide exposed tea garden worker population and non-exposed population was carried out to see if any particular allelic variant (CYP2C9*2 and CYP2C9*3) in addition to wild type was present at a greater frequency in any particular group (pesticide exposed tea garden workers and non-exposed individuals).

The wild type allele CYP2C9*1 was most frequent in both the populations i.e., tea garden workers and non-tea garden workers having a frequency of 0.87235 and 0.87695, respectively (**Table 5.5**) which is comparable to the findings of Seng et al. (2003) who had reported the CYP2C9*1 allele frequency to be 87.50% in Indians. The CYP2C9*2 mutant allele was least frequent in both the populations whereas, CYP2C9*3 was slightly higher than CYP2C9*2 allele in tea garden worker as well as non-exposed individuals. The results also indicated that the pesticide induced damage has no association with any of the alleles found. Our results showing allele frequency of 0.09615 and 0.10105 in the non-tea garden worker and the tea garden worker population strongly corroborate the results of Seng et al. (2003) with a frequency of 0.0815 for CYP2C9*3 allele. The

frequency of CYP2C9*2 allele was much low in the present study compared to 0.0435 reported by Seng et al. (2003) in Indian population. The high frequency by Seng et al. (2003) may be due to association with patients on warfarin treatment.

In comparison to CYP2C9*1, the alleles CYP2C9*2 and CYP2C9*3 have low metabolic activities (Lee et al., 2002). Singh et al. (2011, 2012) have shown that CYP2C9*1, CYP2C9*2 and CYP2C9*3 were not significantly associated with DNA damage related to pesticide exposure. However, CYP2C9*3 alleles may result in substantial reduction in metabolic activities, while CYP2C9*2 allele produces intermediate reduction with GSTM1 null genotypes and NAT2 slow acetylation genotypes (Singh et al., 2012).

CYP2C9 genetic polymorphism was studied in south Indian population (346 subjects) which revealed that the frequencies of CYP2C9*1, CYP2C9*2 and CYP2C9*3 were 0.88, 0.04 and 0.08, respectively. Different CYP2C9 genotypes CYP2C9*1/*1, CYP2C9*1/*2, CYP2C9*1/*3, CYP2C9*2/*2, CYP2C9*2/*3, CYP2C9*3/*3 were reported with their corresponding frequencies 0.78, 0.05, 0.15, 0.01, 0.01 and 0.00 (Jose et al., 2005) which are comparable to the results of the present study except that no homozygotes for CYP2C9*2/*2 was detected. On the other hand CYP2C9*3/*3 homozygotes were present at low frequency in contrast to the result obtained by Jose et al. (2005). Recent studies have reported the presence of CYP2C9*2 and CYP2C9*3 alleles with a frequency of 0.006 and 0.09 and 0.05 and 0.11 in the south and north Indian populations, respectively. There existed a significant difference in the CYP2C9*2 allele between the south Indian and north Indian population (Nahar et al., 2013).

Chaudhary et al. (2016) genotyped CYP2C9 gene in 89 epileptic children in order to see the association with drug phenytoin. CYP2C9*1, CYP2C9*2 and CYP2C9*3 allelic frequencies were 85.4%, 4.5% and 10.1%, respectively. CYP2C9*3 allelic group showed

significantly higher serum phenytoin compared to wild variant. In their study also CYP2C9*3 was more common than CYP2C9*2 which is similar to the result observed in the present study. Earlier in a similar study Kesavan et al. (2010) had reported the association of CYP2C9*3 with higher risk of phenytoin induced toxicity in epileptic patients (Kesavan et al., 2010). Swar et al. (2016) have also shown a significant association between phenotype and genotype with respect to flurbiprofen metabolism and reported comparatively high frequency of CYP2C9*3 allele (9.5%), while CYP2C9*2 was 4.3%. The CYP2C9*1 and CYP2C9*3 frequencies reported by Swar et al. (2016) corroborate the findings of the present study. The discussion indicates that in the Indian population CYP2C9*1 is most frequent and other two alleles exist in variable frequency.

Studies from other workers have yielded variable results. Genotyping of CYP2C9 in Caucasian population of clinical trial volunteers have shown highest to lowest frequency in order of CYP2C9*1 > CYP2C9*2 > CYP2C9*3 where the CYP2C9*1 frequency though lower but comparable to the result of the present study (Stubbins et al., 1996). Sullivan-Klose et al. (1996) had reported that Cys^{144} (CYP2C9*2) and Leu^{359} (CYP2C9*3) alleles are rarer in Caucasian-American and African-American population which also supports the rarer frequency of CYP2C9*2 and CYP2C9*3 allele observed in the present study. Among Asian, Cys^{144} (CYP2C9*2) was not found in Chinese-Taiwanese population (Sullivan-Klose et al., 1996). Other studies have also shown that CYP2C9*2 and CYP2C9*3 alleles are in lower frequency in different population namely Japanese population (Nasu et al., 1997), Swedish population (Yasar et al., 1999) and unrelated Turkish subjects (Aynacioglu et al., 1999).

The observed and expected frequency distribution of pesticide exposed tea garden worker population and non-tea garden workers are in accordance with Hardy-Weinberg law

(**Table 5.6**) and the allele frequencies are comparable between the two groups (**Table 5.5**). It can be assumed that none of the alleles is apparently associated with the pesticide exposure and related toxicity.

CHAPTER 6

Conclusion

Studies based on the enzyme assay, micronucleus test and comet assay indicated that the pesticides are capable of exerting toxic effects on the pesticide-exposed individuals evidenced by the lower levels of acetylcholinesterase and butyrylcholinesterase activity and increased DNA damage compared to the non-exposed (controls), smokers and alcohol consumers.

Significantly lower activity of acetylcholinesterase and butyrylcholinesterase in the pesticide-exposed tea garden workers indicated marked inhibition of the enzymes. Acetylcholinesterase and butyrylcholinesterase activities were not significantly reduced in any particular age group in the present study, however slightly lower activities observed in the 15-30 years age group reflect that these individuals might be engaged in the activities with greater risk. The gender has very insignificant or no effect on acetylcholinesterase and butyrylcholinesterase activity.

Higher frequency of micronuclei and other cell death parameters (cells with nuclear bud, binucleated cells, karyorrhectic cell, pyknotic cell, karyolytic cell) in the pesticide exposed individuals than control subjects indicated greater extent of genome damage induced by pesticides. Marked increase of micronucleus and other cell death parameters in the tea garden workers compared to the smokers and alcohol consumers indicated that smoking and alcohol consumption may act synergistically with the pesticides to cause increased DNA damage. Age has no significant effect on the frequency of micronucleus and other cell death parameters. Significantly higher frequencies of micronucleus, nuclear bud and binucleated cells in male workers than females clearly indicated a sex bias which could be due to the sum of different factors like smoking, alcohol consumption, the intensity of pesticide exposure which is not uniformly distributed between sexes.

Significantly longer comet tail length, tail moment and olive tail moment in the pesticide-exposed individuals compared to the controls, smokers and alcohol consumers suggested higher level of DNA damage induced by the pesticides.

CYP2C9 allele frequency and the related genotypes were not found to differ significantly between the pesticide-exposed tea garden workers and non-tea garden workers.

However, from the study of different biomarkers, it can be concluded that the tea garden workers are at very high risk of occupational exposure.

APPENDIX

(I) PUBLICATIONS

1. Dutta S, Bahadur M (2016) Cytogenetic analysis of micronuclei and cell death parameters in epithelial cells of pesticide exposed tea garden workers. *Toxicology Mechanisms and Methods* 26(8): 627-634.

2. Dutta S, Bahadur M. Effect of pesticide exposure on the cholinesterase activity of the occupationally exposed tea garden workers of Northern part of West Bengal, India. Communicated to the journal *Biomarkers*: 14th August, 2018.

(II) PAPERS PRESENTED AT SYMPOSIA AND SEMINARS

1. Susmita Dutta and Min Bahadur, 2018. Cholinesterase activity and buccal micronucleus cytome assay as an indicator of pesticide-induced toxicity in the occupationally exposed worker population from Terai region of North Bengal, India: National conference on "recent trends in biological research and future prospects" organized by Department of Zoology, Sikkim University, Gangtok.

2. Susmita Dutta and Min Bahadur, 2018. Cytogenetic and biochemical analysis in pesticide exposed tea garden workers in Northern part of West Bengal, India: National conference on recent trends in Zoological research in North-East India, organized by Department of Zoology, North-Eastern Hill University, Shillong and Zoological Society (Kolkata).

3. Susmita Dutta and Min Bahadur, 2015. Butyrylcholinesterase activity and W.B.C. count among the workers from Nischintapur Tea Estate: National conference on applied Zoology in sustainable development: an update organized by Department of Zoology, University of North Bengal.

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RESEARCH ARTICLE

Cytogenetic analysis of micronuclei and cell death parameters in epithelial cells of pesticide exposed tea garden workers

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ABSTRACT

Buccal micronucleus cytome assay was carried out in 47 exposed (sprayers and leaf harvesters), 47 non-exposed (controls) to determine the extent of damage working in the tea plantations of Terai region of West Bengal, India. As the pesticide exposed male workers were found to consume alcohol and smoked cigarettes/bidis, 35 smokers and 30 alcoholics were also included for comparison. Results showed a significant difference in micronuclei (9.91 ± 2.74, $p \leq .001$), nuclear bud (4.98 ± 1.31, $p \leq .001$), binucleate (6.26 ± 2.84, $p \leq .001$), karyorrhectic (8.36 ± 2.28, $p \leq .001$), pyknotic (5.62 ± 1.78, $p \leq .05$) as well as karyolytic (6.81 ± 3.00, $p \leq .001$) nuclei compared with control. Comparison also revealed a higher frequency of micronuclei (6.11 ± 2.55, $p \leq .01$), nuclear bud (4.06 ± 1.97, $p \leq .05$), binucleate (4.34 ± 1.85, $p \leq .001$), karyorrhectic (6.83 ± 2.12, $p \leq .001$), and karyolytic (6.20 ± 2.54, $p \leq .001$) nuclei except pyknotic cell in the smoker than control. Frequency of binucleate (3.80 ± 1.73, $p \leq .05$), karyorrhectic (5.57 ± 2.34, $p \leq .05$), pyknotic (5.50 ± 1.36, $p \leq .05$), and karyolytic (6.30 ± 2.71, $p \leq .001$) nuclei was higher in the alcoholics than control (non-alcoholics), whereas the micronuclei and nuclear bud were found to be non-significant compared with the control. Our analyses also revealed a higher proportion of the micronucleus and the cell death parameters in the pesticide exposed males than females, which indicated that pesticide, smoking, and alcohol may act synergistically to cause more damage to the buccal epithelial cells. However, age and the exposure duration have no influence on the micronucleus and other cell death parameters.

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Introduction

The chemical pesticides are one of the important measures for controlling pests infesting agricultural crops, but they also represent a potential source of occupational risk to human (Benedetti et al., 2013; Brunetti et al., 1988; Kausar et al., 2014) and is hazardous to the environment. In general, people in developing countries are at high risk of chronic exposure to pesticides because of poor working conditions and their lack of knowledge about the potential hazards associated with the manufacturing and application of these chemicals (Baker et al., 1978). Population biomonitoring has become an extremely powerful approach to determine the effect of environmental mutagens or occupation on human populations (Benites et al., 2006; Bolognesi et al., 1993, 2011; Celik et al., 2003; Chakraborty et al., 2006; Pastor et al., 2003). Workers in a variety of occupational settings are potentially exposed to hazardous substances present in the occupational environment in the form of gases, vapors, fumes, or particles (Benites et al., 2006; Celik et al., 2003). The primary route of exposure to these substances is through inhalation; however, exposure also takes place through dermal absorption or oral ingestion (Benites et al., 2006).

The buccal micronucleus cytome assay is a non-invasive method for studying DNA damage, chromosomal instability, cell death, and the regenerative potential of buccal mucosal tissue, which is widely used in biomonitoring studies (Bonassi et al., 2011; Celik et al., 2003; Thomas & Fenech, 2011). The oral epithelium maintains itself by continuous cell renewal where new cells produced in the basal layer by mitoses migrate to the surface, replacing those that are shed (Ten Cate et al., 1998). The basal layer contains the stem cells that may express genetic damage (chromosome breakage or loss) as micronuclei (MN) during nuclear division. The daughter cells, which may or not, have MN, eventually differentiate into the stratum spinosum cell layer and the keratinized superficial layer, and then exfoliate into the buccal cavity. Some of them may degenerate into cells with condensed chromatin, fragmented nuclei, pyknotic nuclei, or karyolytic cells, without nuclear material. In rare cases, some cells may be blocked in a binucleated stage or may exhibit nuclear buds, also known as broken eggs, a biomarker of gene amplification. Above biomarkers can be observed in buccal cells and thus provide a comprehensive assessment of genome damage caused by exposure to genotoxic substances (Kashyap & Reddy, 2012; Suspiro & Prista, 2011). MN has also been used to detect the toxic effect of other pollutants on the buccal epithelial cells (Celik et al., 2003; Gonsebatt et al., 1997; Martínez-Valenzuela et al., 2015).

Human biomonitoring studies use micronucleus (MN) assay as a biomarker of effect for an early stage

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carcinogenesis and other chronic diseases associated with chromosomal aberrations (CA) and exposure to the clastogenic and aneugenic agents (Bonassi et al., 2003, 2007; Chakraborty et al., 2006; Chen et al., 2015; Fenech, 1993; Ghosh et al., 2008). Micronuclei are formed from acentric chromosomal fragment when a part or whole chromosome is left behind during mitotic cellular division.

The use of pesticides without the necessary protection may lead to alterations in genetic material and possible health consequences. Agriculture workers, either in open fields or greenhouses, get exposed to pesticides or are in constant risk of exposure by accident. Moreover, the consumption of the pesticide contaminated food by the populations of urban and suburban sites makes them prone to pesticide exposure (Bolognesi et al., 1993; Falck et al., 1999). Although there are several reports on cytogenetic biomonitoring studies from different parts of the world, each one is unique because of differences in the levels of exposure, type of pesticide mixtures, geographic, and meteorological characteristics of the agricultural areas etc. (Bolognesi et al., 1993, 2011; Pastor et al., 2003).

Tea is a main agroindustry of Terai and the Dooars regions of the northern part of West Bengal in India. A large number of pesticides are used by tea planters to control the arthropod pests (Gurusubramanian et al., 2008) resulting in the exposure of non-target organisms including humans to pesticides. Residual pesticide contamination of water, sediment and fish from the river flowing through the tea gardens of Terai region of West Bengal, India has been shown by Singh et al. (2015). Bishnu et al. (2009) estimated the residues of organophosphorous (ethione, chlorpyriphos), organochlorine (heptachlor, dicofol, α-endosulfan, β-endosulfan, endosulfan sulfate) and synthetic pyrethroid (cypermethrin and deltamethrin) pesticides in made tea, fresh tea leaves, soil, and the water bodies from selected tea gardens in the Dooars and the hill regions of West Bengal. In tea industry, tea leaf plucking is carried out by females, whereas pesticide spraying is done by the males and, therefore, these workers have the risk of occupational exposure to pesticides. There is no report on the effect of this occupation on cytogenetic biomarkers among the workers from the Terai region of the Darjeeling foothills. The major goal of our study is to acquire information on the effect of this occupation on the exfoliated buccal cells using MN test so that a proper health risk assessment can be done that will be helpful for developing effective health care strategies.

Materials and methods

Characteristics of the participants

A total of 159 individuals consisting of 47 (35 males and 12 females) pesticide exposed tea garden workers, 47 control (22 males and 25 females) pesticide non-exposed, non-smoker, and non-alcoholics, 35 smokers, individuals smoking a minimum of six or more cigarettes/day (males different from the exposed) and 30 alcoholics, individuals consuming 60–120 ml alcohol/day (males different from the exposed) were analyzed. A participant was considered as pesticide exposed if he or she was involved in either pesticide spraying or tea leaf plucking for atleast 8 h/d for 6 d/week for atleast a year. The age of the pesticide exposed participants varied between 23 and 61 years. The pesticides commonly used are ethione, chlorpyriphos, heptachlor, dicofol, α-endosulfan, β-endosulfan, endosulfan sulfate, cypermethrin, and deltamethrin (Bishnu et al., 2009; Singh et al., 2015). Duration of occupational exposure ranged from 1 to 36 years. All the pesticide exposed male individuals had smoking habits (<10 cigarettes or bidi, a bidi is a thin, Indian cigarette filled with tobacco flake) and consumed alcohol too. The control subjects comprising of 47 individuals did not handle pesticide, neither smoked cigarettes nor consumed alcohol. As the pesticide exposed males smoked and consumed alcohol, for comparison, 35 smokers who smoked 6–30 cigarettes/day and 30 individuals who were in the habit of alcohol consumption, were included in the study chosen randomly from the population located far from pesticide exposed areas. Participants were asked a structured questionnaire to obtain personal information with regard to exposure period, smoking habits, alcohol consumption, and health status. After obtaining consent, samples were taken from the buccal mucosa of healthy individuals who were not under any medication or X-ray exposure during past 6 months. This study was approved by the Institutional Ethics Committee of the University of North Bengal, Siliguri, District-Darjeeling, West Bengal, India [Ref. no. Zoo/9114(i)/2015, dated: 22 September 2015].

Sampling procedure

Cell sampling was carried out following the method of Beliën et al. (1995) with minor modifications. The individuals were asked to rinse their mouth with 0.9% saline water. Then the buccal epithelial cells were obtained from inner cheeks with the help of a sterile stainless steel spatula. The cell suspension was prepared in 0.9% saline, brought to the laboratory, washed twice in normal saline through centrifugation at room temperature for 5 min. The cells were fixed in 1:3 aceto-methanol. Slides were prepared by air-drying method.

Pap staining and scoring

The slides were stained by Papanicolaou (Pap) method as described by Mondal et al. (2011) with minor modifications. The slides were stained with Harris' Hematoxylin (Merck, Darmstadt, Germany) for 2 min after passing through graded ethanol (100–50%) and water. The slides were then rinsed in distilled water and treated in Scott's tap water ($MgSO_4 \cdot 7H_2O$:$NaHCO_3$ = 10:1) substitute for bluing, washed in running tap water, dehydrated through 50%, 70%, 90% and absolute ethanol, stained in Orange-G6 (Merck, Darmstadt, Germany, code: 60688701251730) for 20 min and differentiated in 95% alcohol. Then the slides were incubated in EA-50 (Merck, Darmstadt, Germany, code 60927201251730) solution for 20 min at room temperature, dehydrated in absolute ethanol (100%), cleared in xylene, and mounted with distrene plasticizer xylene (DPX). The slides were coded and examined under a light microscope (Nikon Eclipse E200, Nikon, Tokyo,

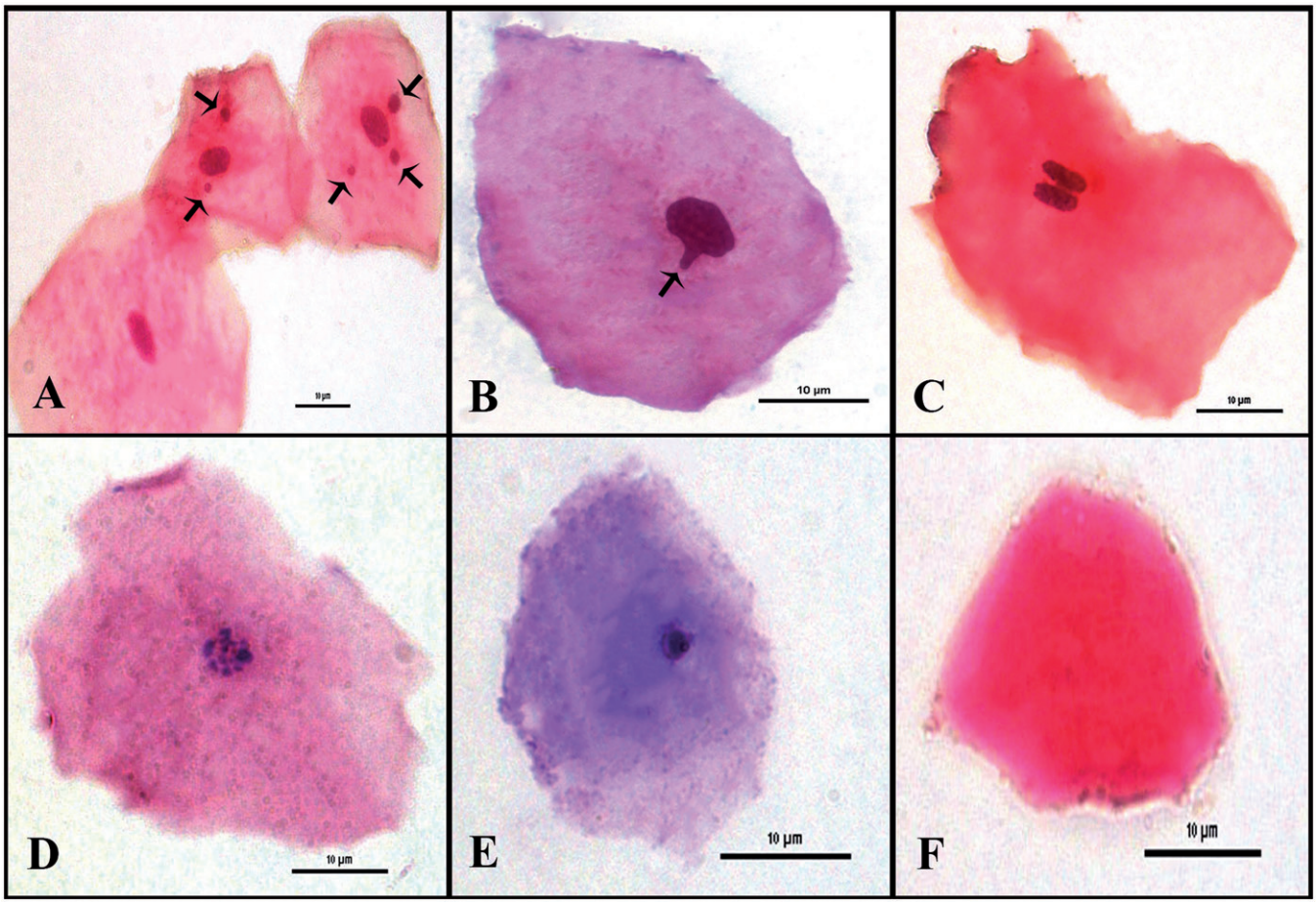


Figure 1. Buccal epithelial cells showing different types of nuclear damages in pesticide exposed workers. (A) A normal cell (below) and cells with micronuclei (arrows); (B) cell with nuclear bud (arrow); (C) binucleated cell; (D) karyorrhectic cell; (E) pyknotic cell; (F) karyolytic cell. Bar =10 µm.

Japan) with the 100× oil immersion objective for the detection of micronuclei (MN) and the other nuclear anomalies like nuclear bud (NB), binucleate (BN), karyorrhectic (KH), pyknotic (PY), and karyolytic (KL) cells. Two slides were used for each individual. For each individual, 1000 non-overlapping, well-differentiated, uniformly stained nuclei of buccal epithelial cells were analyzed. The cells, which were considered for MN assay, have (i) round or oval shape (ii) diameter ranged between 1/3rd and 1/16th of the main nucleus (iii) same staining intensity and texture as the main nucleus (iv) MN located within the cytoplasm of the cells and (v) usually have one MN per cell, but the number could be two or more following severity of genotoxic insult. Besides MN, other nuclear anomalies like "broken egg" (cells with nuclear buds), binucleate cells, karyorrhexis (nuclear disintegration), pyknosis (shrunken nuclei), and karyolysis (dissolution of the nucleus) (Figure 1) were evaluated following the established criteria (Thomas et al., 2009; Tolbert et al., 1992).

Statistical analysis

A descriptive statistical analysis was done and data are expressed as mean ± SD. Crosstable along with Chi-square test for homogeneity of (a) four groups versus age group and (b) four groups versus gender have been done. Pearsonian product moment correlation coefficients and Spearmen rank correlation coefficient of the parameters with ages along with *t*-test for testing significance of correlation coefficients have been done, only in few cases significance of correlation coefficients have been observed. Test for normality (Shapiro–Wilks test) of the parameters (four groups, sex) have been done, in some cases, the departure from the normality has been observed. The testing of hypothesis of the differences of means across the groups, genders were carried out with the help of multivariate analysis of variance (MANOVA), multivariate analysis of covariance (MANCOVA) along with post-hoc (Tukey's test) (for multiple comparison) through generalized linear model (in case of non-normality of parameters, equivalent non-parametric Kruskal–Wallis test was performed but the results of MANOVA and Kruskal–Wallis were almost the same). No significant effect of age on parameters has been found, but in some cases, the effect of gender has been observed. The problems of confounding factors due to gender, cigeratte smoking are solved by multiple comparisons using the software SPSS version 21 (SPSS Inc., Chicago, IL). The level of significance was considered at .001, .01, and .05.

Results

In this study, the group of exposed workers included 35 males and 12 females between 23 and 61 years of age

(mean age: 33.17 ± 11.04). These individuals were directly involved in the plucking of tea leaves or application of pesticides in tea gardens under study and are exposed simultaneously to a complex mixture of pesticides during work. None of the workers in this study were found to use any kind of protective measures during pesticide application (gloves, breathing masks, protective goggles, impermeable boots, etc.) and presented symptoms related to pesticide exposure such as headache, abdominal pain, nausea, and vomiting. The non-exposed group (control) consisted of 22 males and 25 females, between 22 and 60 years of age (mean age: 27.17 ± 10.19) with no known exposure to genotoxic agents. The smoker group (smoking 6–30 cigarettes/day) consisted of 35 randomly sampled males, between 21 and 59 years of age (mean age: 31.17 ± 10.83). These individuals were divided into two groups, the first group, comprising of 18 individuals, who smoked less than 10 cigarettes per day (group I), while the second group comprising of 17 individuals, smoked more than 10 cigarettes per day (group II). The fourth group in the study included 30 alcohol consumers (consuming 60–120 ml alcohol/day) between 24 and 61 years of age (mean age: 40.53 ± 11.19) (Table 1). Figure 1 shows buccal epithelial cells with MN and different types of nuclear damages.

Evaluation of epithelial cells revealed a higher frequency of micronuclei (9.91 ± 2.74, $p \leq .001$), nuclear bud (4.98 ± 1.31, $p \leq .001$), binucleate (6.26 ± 2.84, $p \leq .001$), karyorrhectic (8.36 ± 2.28, $p \leq .001$), pyknotic (5.62 ± 1.78, $p \leq .05$) as well as karyolytic (6.81 ± 3.00, $p \leq .001$) nuclei compared with the non-exposed group (control) (Table 2). Comparison between the smokers and control revealed a higher frequency of micronuclei (6.11 ± 2.55, $p \leq .01$), nuclear bud (4.06 ± 1.97, $p \leq .05$), binucleate (4.34 ± 1.85, $p \leq .001$), karyorrhectic (6.83 ± 2.12, $p \leq .001$), and karyolytic (6.20 ± 2.54, $p \leq .001$) in the smoker group. A non-significant relation was found in pyknotic cells between the smokers and the control subjects. Results also showed a higher frequency of binucleate (3.80 ± 1.73, $p \leq .05$), karyorrhectic (5.57 ± 2.34, $p \leq .05$), pyknotic (5.50 ± 1.36, $p \leq .05$), and karyolytic (6.30 ± 2.71, $p \leq .001$) nuclei in the alcoholics compared with the control (non-alcoholics). A non-significant relation was found between micronucleus and nuclear bud when compared with the control subjects (Table 2).

The males involved in pesticide mixing or spraying have significantly higher frequencies of micronuclei (10.89 ± 2.37, $p \leq .001$), nuclear buds (5.34 ± 1.14, $p \leq .001$), binucleate (7.09 ± 2.73, $p \leq .001$) than females (leaf harvester) while the frequency of karyorrhectic, pyknotic and karyolytic in males and females were non-significant (Table 2). No significant differences were observed in the frequencies of the damage parameters between males and females in the control group (Table 2).

A comparison was also made between smokers, smoking less than 10 cigarettes per day (group I) and those smoking more than 10 cigarettes per day (group II). The group II showed a higher frequency of micronuclei (8.24 ± 1.30, $p \leq .001$), nuclear bud (5.35 ± 1.77, $p \leq .001$) as well as pyknotic (6.29 ± 2.05, $p \leq .001$), karyolytic (7.82 ± 2.46, $p \leq .001$) cells compared with group I. No significant difference was observed in the frequency of binucleate and karyorrhectic nuclei in both (Table 3 and histogram).

Table 1. Characteristic of the study population.

Variable	Control ($N = 47$)	Tea garden workers ($N = 47$)	Smokers ($N = 35$)	Alcoholics ($N = 30$)
Male	22	35	35	30
Female	25	12	0	0
Height (Feet)	5.37 ± 0.26	5.24 ± 0.45	5.60 ± 0.21	5.52 ± 0.22
Weight (Kg)	58.36 ± 5.38	47.81 ± 11.25	65.03 ± 9.82	66.37 ± 8.29
Age in years (mean ± SD)	27.17 ± 10.19	33.17 ± 11.04	31.17 ± 10.83	40.53 ± 11.19
Smoking	No	Yes	Yes	No
Alcohol	No	Yes	No	Yes

Discussion

There has been an ever increasing use of chemical pesticides in the tea plantations in India, including Terai and the Dooars region of West Bengal to curb the menace of insect pests. The chemical pesticides used in the tea plantations are a heterogeneous mixture of compounds belonging to organophosphates, organochlorines, and pyrethroids. Therefore, the workers involved in tea leaf plucking (mainly females) and pesticide spraying (males) are at a high risk of pesticide exposure through inhalation, food contamination, and skin contact. Many of these pesticides classified as "moderately hazardous" or "slightly hazardous" (WHO, 2005) are mutagens as well as "possible carcinogen to human beings" (Chen et al., 2015; Daniels et al., 1997; IARC, 1991). Different studies have indicated the genotoxic effect of pesticides on occupationally exposed human population (Costa et al., 2007; Gaikwad et al., 2015; Kausar et al., 2014; Pastor et al., 2001;

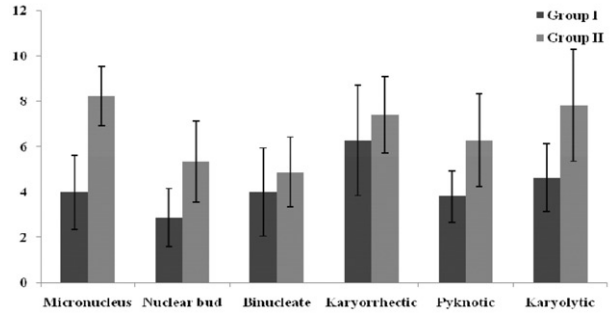
Table 2. Changes in micronucleus frequencies and other nuclear anomalies in exfoliated buccal epithelial cells of control, tea garden workers, smokers, and alcoholics and comparison of nuclear biomarkers between males and females in pesticide exposed workers and control. For each participant, 1000 cells were scored.

	Micronucleus	Nuclear Bud	Binucleate	Karyorrhectic	Pyknotic	Karyolytic
Control ($N = 47$)	4.57 ± 1.99	3.23 ± 1.74	2.72 ± 2.38	4.45 ± 1.70	4.38 ± 2.55	4.45 ± 1.73
Male ($N = 22$)	4.59 ± 1.65	3.50 ± 1.57	2.55 ± 2.34	4.45 ± 1.74	4.09 ± 2.78	4.73 ± 1.88
Female ($N = 25$)	4.56 ± 2.29	3.00 ± 1.87	3.19 ± 2.21	4.44 ± 1.71	4.64 ± 2.36	4.20 ± 1.58
Tea garden workers ($N = 47$)	9.91 ± 2.74***	4.98 ± 1.31***	6.26 ± 2.84***	8.36 ± 2.28***	5.62 ± 1.78*	6.81 ± 3.00***
Male ($N = 35$)	10.89 ± 2.37***	5.34 ± 1.14***	7.09 ± 2.73***	8.46 ± 2.39	5.71 ± 1.60	7.06 ± 3.23
Female ($N = 12$)	7.08 ± 1.51	3.92 ± 1.24	3.83 ± 1.47	8.08 ± 2.02	5.33 ± 2.27	6.08 ± 2.19
Smokers ($N = 35$)	6.11 ± 2.55**	4.06 ± 1.97*	4.34 ± 1.85***	6.83 ± 2.12***	4.97 ± 2.09	6.20 ± 2.54***
Alcohol consumers ($N = 30$)	4.67 ± 2.23	3.63 ± 1.83	3.80 ± 1.73*	5.57 ± 2.34*	5.50 ± 1.36*	6.30 ± 2.71***

Results are expressed as mean ± standard deviation.
*$p \leq .05$ compared with control using MANOVA.
**$p \leq .01$ compared with control.
***$p \leq .001$ compared with control.

Table 3. Comparison of nuclear biomarkers between smokers group I (smoking less than 10 cigarettes/d) and group II (smoking more than 10 cigarettes/d). For each participant, 1000 cells were scored.

Comparison between two groups of smokers		
	<10 cigarettes per day (group I) ($N = 18$)	>10 cigarettes per day (group II) ($N = 17$)
Micronucleus	4.00 ± 1.62	8.24 ± 1.30***
Nuclear bud	2.88 ± 1.27	5.35 ± 1.77***
Binucleate	4.00 ± 1.94	4.88 ± 1.54
Karyorrhectic	6.29 ± 2.44	7.41 ± 1.69
Pyknotic	3.82 ± 1.13	6.29 ± 2.05***
Karyolytic	4.65 ± 1.49	7.82 ± 2.46***



Results are expressed as mean ± standard deviation.
*$p \leq .05$ compared using MANOVA.
**$p \leq .01$.
***$p \leq .001$.

Scarpato et al., 1996) and the genome alteration (Rupa et al., 1991; Shaham et al., 2001; Zhang et al., 2012). The buccal epithelial cells get exposed to all the mutagenic components of pesticides as well as cigarettes, alcohol and the other toxic chemicals (Costa et al., 2007; Gaikwad et al., 2015; Kausar et al., 2014; Pastor et al., 2001; Scarpato et al., 1996). Pesticides of different categories generate reactive oxygen species (ROS) which can produce free radicals, resulting in lipid peroxidation (Panemangalore et al., 1999) leading to DNA damage. The use of the MN test to detect and quantify the genotoxic action of carcinogens and its sensitivity has already been compared with the analysis of chromatid breaks and exchanges (Adler et al., 1988). The buccal mucosal cells have been considered to be more sensitive than lymphocytes to the induction of the cytogenetic damage by the genotoxic agents in the epithelial tissue (Davis, 2003).

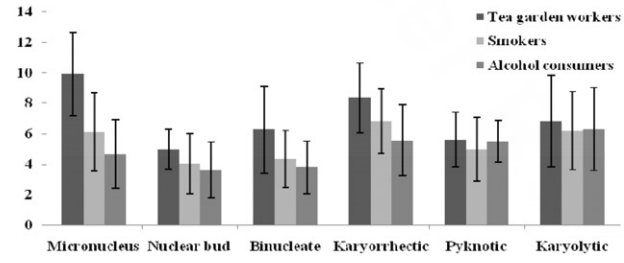
Micronucleus is a good indicator of chromosome mutation. It is non-invasive, neither require a cell culture nor metaphase preparation, has a low cost so is a good indicator of chromosome mutations (Fenech et al., 2011; Majer et al., 2001). Since the workers get exposed to a complex mixture of chemicals, the evaluation of genotoxic effect in the pesticide exposed human population in the tea gardens of the Terai regions of Darjeeling foothills is a primary health concern. Since male tea workers in this study (occupationally exposed) smoked cigarettes and consumed alcohol, we have included smokers and alcoholics for comparison. The higher frequency of micronuclei, nuclear bud, binucleate, karyorrhectic, pyknotic, and karyolytic cells in tea workers compared with the control is in agreement with the study conducted by Bolognesi et al. (1993) and Carbonell et al. (1993). Kausar et al. (2014) have shown the percentage of micronuclei, nuclear bud, binucleate cells, karyorrhectic, karyolytic, and pyknotic cells to be 1.20 ± 0.10, 0.20 ± 0.00, 0.90 ± 0.10, 2.90 ± 0.20, 1.20 ± 0.10, and 2.40 ± 0.10 in tea garden workers from Assam, India. Our results showed higher frequencies of different parameters except MN compared with the result of Kausar et al. (2014). MN frequency in grape gardens reported by Gaikwad et al. (2015) is almost similar to our result found in case of pesticide exposed males. Different studies have shown a higher frequency of MN among the pesticide exposed floriculturist (Bolognesi et al., 2011; Scarpato et al., 1996), greenhouse workers (Falck et al., 1999), agricultural workers (Ali et al., 2008), and in soybean workers (Benedetti et al., 2013) compared with control. Our results showing higher frequency of MN in the pesticide-exposed group corroborate the above and strongly support the genetic damage caused by pesticides. The frequency of MN (9.91 ± 2.74, $p \leq .001$) in the exposed group is comparable with the result of Costa et al. (2007) which is 9.03 ± 1.04, $p < .001$.

Since the male tea garden workers in the present study smoked cigarettes/bidi and consumed alcohol, we included smokers and alcoholics in our study to see the effect of cigarettes/bidi and alcohol on the buccal epithelial cells. Nersesyan et al. (2011) indicated that smoking induces MN and other nuclear anomalies (Binucleate, Broken Egg, Karyorrhexis, Karyolysis, and Condensed chromatin) in the exfoliated buccal cells in humans and that these effects depend on the types of cigarettes consumed and the tar and nicotine contents. Our results showing a significant increase in the frequency of MN (6.11 ± 2.55, $p \leq .01$), NB (4.06 ± 1.97, $p \leq .05$), BN (4.34 ± 1.85, $p \leq .001$), KH (6.83 ± 2.12, $p \leq .001$), and KL (6.20 ± 2.54, $p \leq .001$) in smokers compared with the non-smokers (control) strongly support Nersesyan et al. (2011). The increase in the micronucleus and other cell death parameters could be due to the fact that the smoke emitted from cigarettes contains ROS and tar which cause damage to buccal epithelial cells as also suggested by Valavanidis et al. (2009). Also, earlier studies by different workers reporting a higher mean frequency of MN in the smokers (Kamath et al., 2014; Kiilunen et al., 1997; Konopacka, 2003; Ozkul et al., 1997; Piyathilake et al., 1995; Sarto et al., 1987) are in concurrence with our results. However, Falck et al. (1999) detected no increasing effect of smoking on MN.

A significant increase of MN, NB, PY, and KL cells in the group II smokers (smoking more than 10 cigarettes/d) 8.24 ± 1.30, 5.35 ± 1.77, 6.29 ± 2.05, and 7.82 ± 2.46 than group I (smoking less than 10 cigarettes/d) 4.00 ± 1.62, 2.88 ± 1.27, 3.82 ± 1.13, and 4.65 ± 1.49, significant at $p \leq .001$ clearly indicated a positive correlation between the number of cigarettes smoked and cell death parameters as clearly envisaged by Wu et al. (2004). Wu et al. (2004) reported a positive relation between micronuclei and smoking intensity and have shown that the heavy smokers have a higher frequency of MN in buccal cells. The frequency of different cell death parameter observed in the pesticide exposed group in the present study is not only higher than the control but also higher than the smoker and alcoholics group (Table 4 and histogram) this suggests that there may have a synergistic

Table 4. Comparison of nuclear biomarkers among tea garden workers, smokers and alcohol consumers. For each participant, 1000 cells were scored.

	Tea garden workers ($N = 47$)	Smokers ($N = 35$)	Alcohol consumers ($N = 30$)
Micronucleus	9.91 ± 2.74	6.11 ± 2.55***	4.67 ± 2.23***
Nuclear bud	4.98 ± 1.31	4.06 ± 1.97*	3.63 ± 1.83***
Binucleate	6.26 ± 2.84	4.34 ± 1.85***	3.80 ± 1.73***
Karyorrhectic	8.36 ± 2.28	6.83 ± 2.12**	5.57 ± 2.34***
Pyknotic	5.62 ± 1.78	4.97 ± 2.09	5.50 ± 1.36
Karyolytic	6.81 ± 3.00	6.20 ± 2.54	6.30 ± 2.71



Results are expressed as mean ± standard deviation.
*$p \leq .05$ compared with Tea garden workers using MANOVA.
**$p \leq .01$ compared with Tea garden workers.
***$p \leq .001$ compared with Tea garden workers.

effect of the pesticide and the smoking and alcohol for high cell death parameters in tea workers.

The analyses of MN and different cell death parameters in the individuals taking alcohol also revealed a significant increase of BN, KH, and PY (3.80 ± 1.73, 5.57 ± 2.34, and 5.50 ± 1.36) than control (2.72 ± 2.38, 4.45 ± 1.70, and 4.38 ± 2.55, $p \leq .05$). The frequency of karyolytic cells was also found to be higher (6.30 ± 2.71) in alcoholics than control (4.45 ± 1.73, $p \leq .001$).

Age was not found to have significant effect on the frequency of MN and other cell death parameters as also shown by Sailaja et al. (2006), Costa et al. (2007), and Moura de Bortoli et al. (2009). Since the duration of pesticide exposure ranged from 1 to 36 years, the subjects were divided into three groups, 1–9 years, 10–19 years, and 20 years and above. The micronucleus and other cell death parameters did not show any correlation with duration as also shown by Moura de Bortoli et al. (2009). Significantly higher frequencies of MN, NB, and BN in male workers than female in the present study clearly indicate a sex bias which corroborates the study conducted by Pastor et al. (2002). The increase could be due to the sum of different factors like smoking, drinking, and the intensity of pesticide exposure, which are not uniformly distributed between sexes as also suggested by Pastor et al. (2002). Other studies have also reported a significantly higher proportion of MN in males than females showing sex bias in the cell death parameters due to different occupational exposures (Gonsebatt et al., 1997; Martínez-Valenzuela et al., 2015) which is consistent with our results. Moreover, male workers involved in pesticide spraying do not use any kind of protective measures against the pesticides are much more potentially exposed than females who are only tea leaf harvester which may account for the increased frequency of the cell death parameters in males. This shows that smoking as well as alcohol intake may act synergistically along with the pesticide, which is observed as the increased frequencies of the cell death parameters.

Conclusion

Our study indicated a significant damage in pesticide exposed workers compared with the control, the smokers and alcoholic groups. Age has no influence on damage in our study in any of the four groups. The males showed higher damage than the females, which indicates a gender bias that may be due to the synergistic effect of various factors like higher pesticide exposure, no use of protective measures, smoking and the alcohol intake. Moreover, the extent of damage observed in different cell death parameters among the pesticide exposed individuals than smokers and alcoholics suggests that the pesticides, alcohol, and cigarette may act synergistically with pesticide to enhance the damage.

Geolocation information

The study was carried out in the tea gardens located in Upper Bagdogra (26°68′16.73″N, 88°25′83.11″E) and Matigara (26°74′00.82″N, 88°37′83.69″E) in the Terai region of Darjeeling foothill.

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Disclosure statement

The authors do declare that there is no conflict of interest. The funding source has no direct involvement with this study.

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