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.

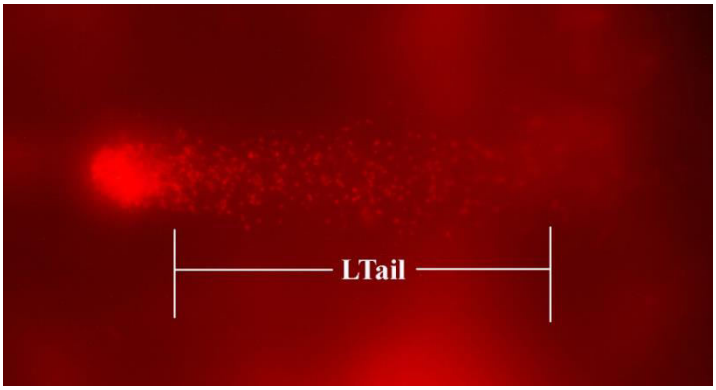


Figure 4.1: Photograph showing length of tail (LTail).

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**).

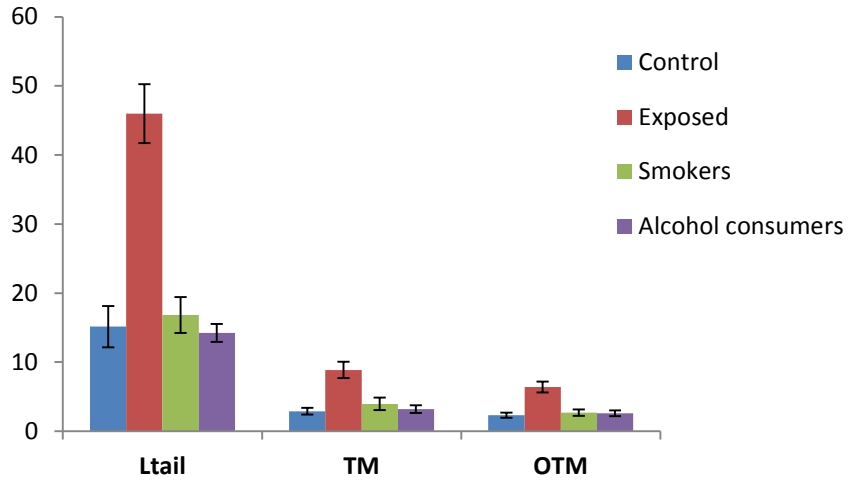
In order to see the effect of gender (sex) on damage parameters (LTail, TM, OTM), both control and the tea garden workers (exposed) were categorized into male and female groups. The results showed a mean LTail, TM and OTM of 17.20 ± 4.07 µm, 3.25 ± 0.62 and 2.59 ± 0.47 in males and 9.93 ± 2.11 µm, 1.99 ± 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.

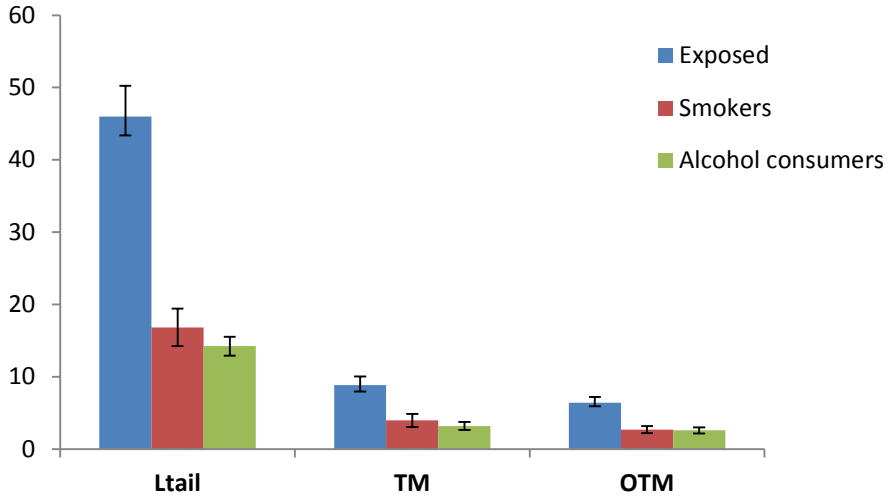
	LTail	TM	OTM
Control(N=60)	15.14 ± 2.99	2.89 ± 0.48	2.32 ± 0.36
Exposed(N=95)	45.98 ± 4.25***	8.86 ± 1.18***	6.41 ± 0.78***
Smokers(N=39)	16.83 ± 2.60	3.96 ± 0.89	2.69 ± 0.48
Alcohol consumers(N=31)	14.24 ± 1.32	3.19 ± 0.55	2.60 ± 0.42



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.

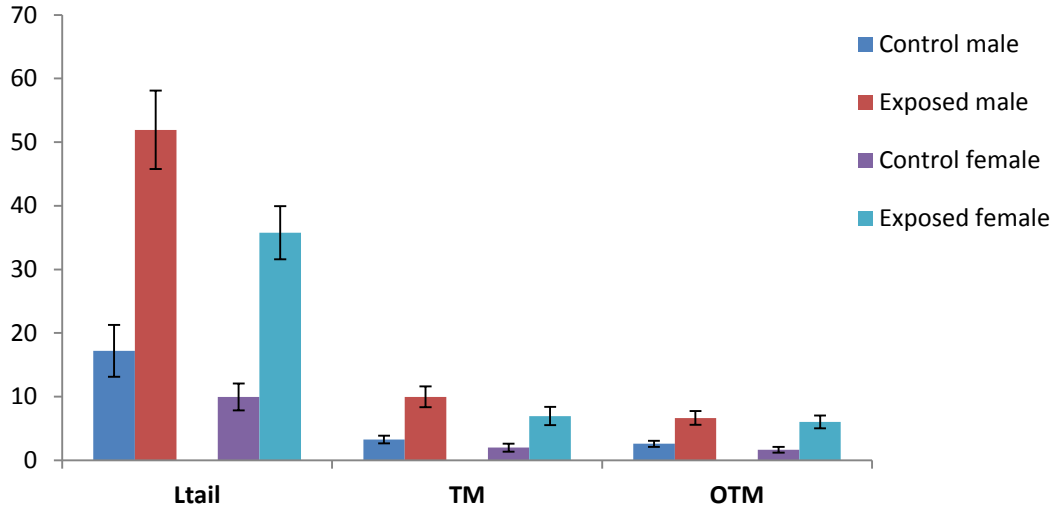
	LTail	TM	OTM
Exposed(N=95)	45.98 ± 4.25	8.86 ± 1.18	6.41 ± 0.78
Smokers(N=39)	16.83 ± 2.60***	3.96 ± 0.89*	2.69 ± 0.48**
Alcohol consumers(N=31)	14.24 ± 1.32***	3.19 ± 0.55*	2.60 ± 0.42**



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

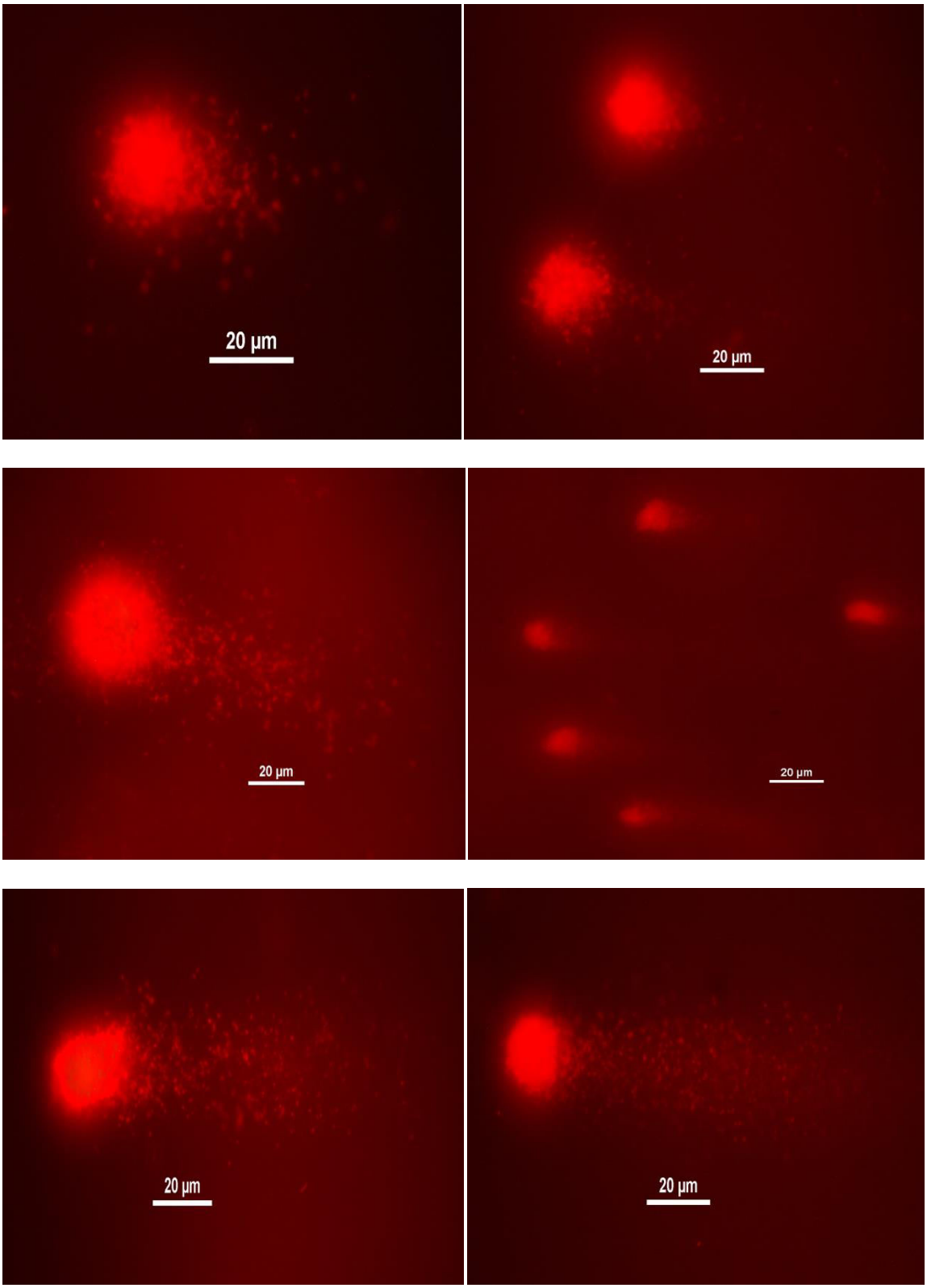


Figure 4.2: Levels of DNA damage detected in tea garden workers by comet assay. Bar = 20 µm.

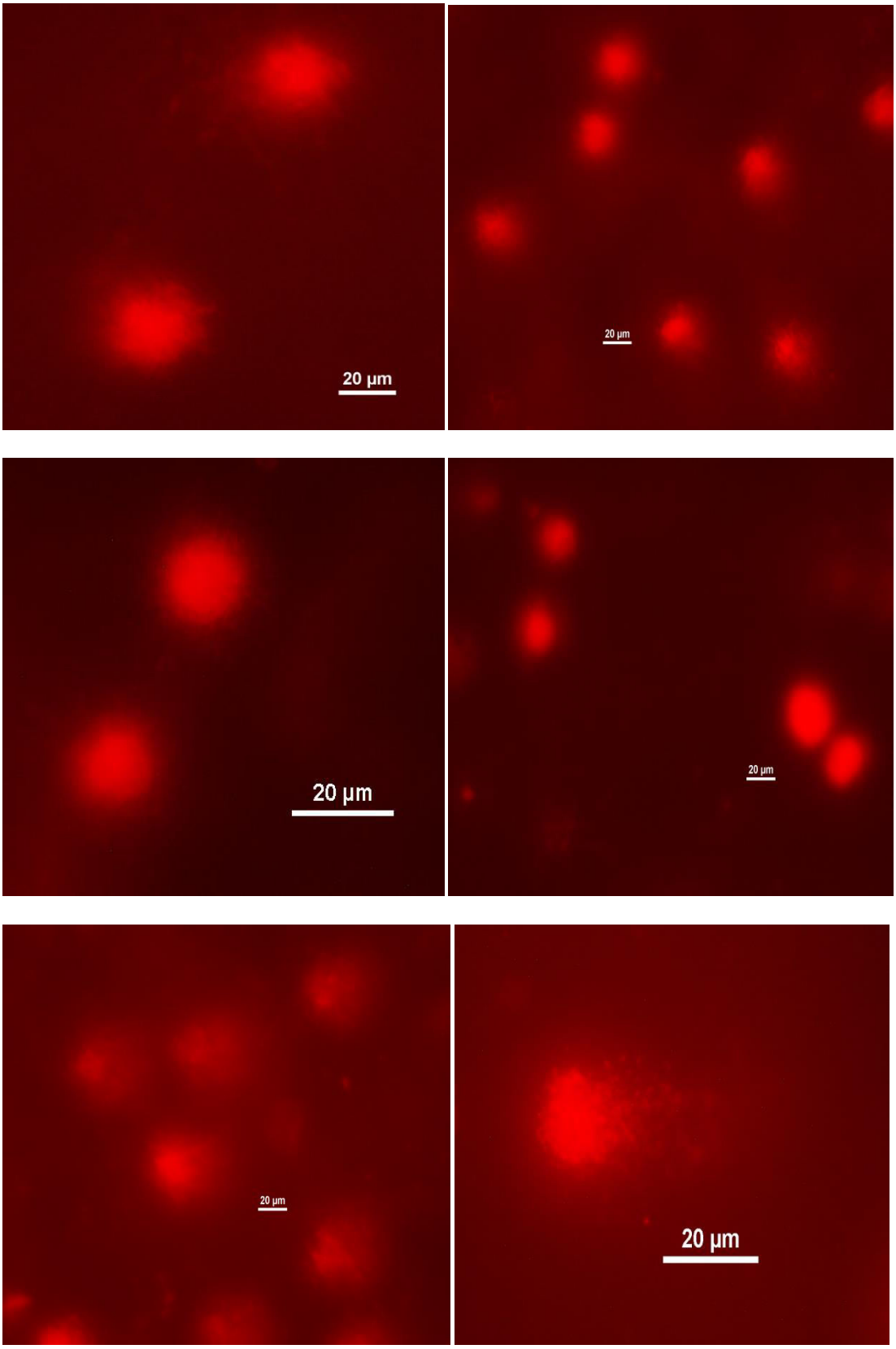


Figure 4.3: Levels of DNA damage detected in controls by comet assay. Bar = 20 µm.

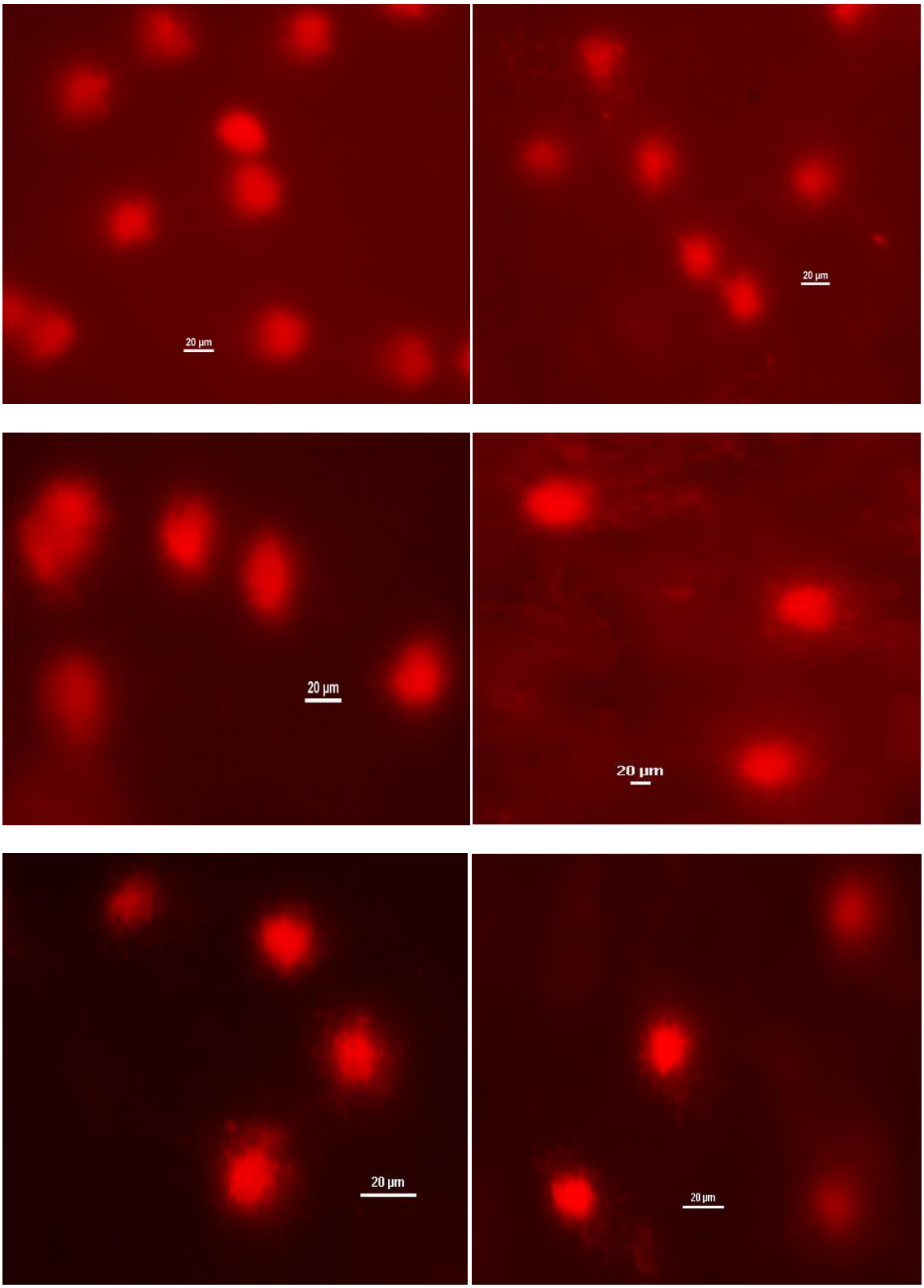


Figure 4.4: Levels of DNA damage detected in smokers by comet assay. Bar = 20 µm.

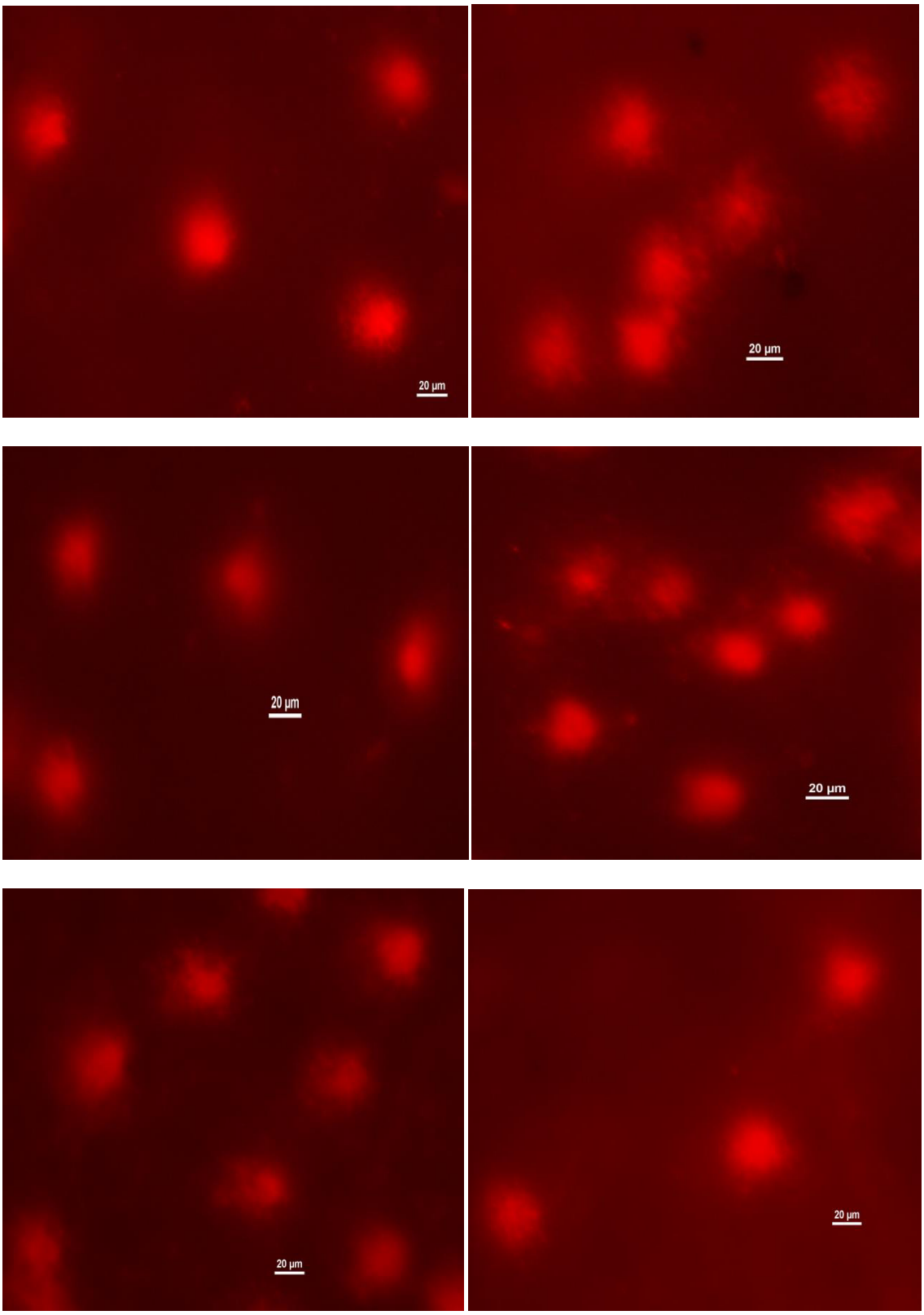


Figure 4.5: Levels of DNA damage detected in alcohol consumers by comet assay. Bar = 20 µm.

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.