CHAPTER I

INTRODUCTION

1. Statement and significance of problem

It is now recognized that there is a lot of pollution present in the environment. Humans are exposed to a large number of environmental toxic agents. These agents enter the environment from many sources. Many chemicals or physical agents in the environment can be mutagenic or carcinogenic or both, that is cancer inducing. Exposure to these agents can cause a variety of health hazards, and may contribute to development to pernicious illness. They may induce mutations in germ cells or damage the DNA of somatic cells, thereby causing malignant transformation and cancer (Farnsworth, 1988).

In addition, humans are exposed to physical and chemical genotoxic agents in occupational and non-occupational settings (non-occupational exposure populations but living in heavily industrialized or polluted areas) (Rahman et al., 2003). There are numerous subjects in the general population as well as in specific occupational groups who might be exposed to genotoxic agents. Several epidemiological and environmental studies have been conducted on human populations exposed to genotoxic agents in their workplaces. These studies have shown that there is an increased frequency of DNA and chromosomal damage. In Egypt, Cairo has a large population of about 12 million. There is a high number of automobiles and polycyclic aromatic hydrocarbons that contribute to air pollution. Furthermore, the incidence of lung cancer in Cairo has increased, which is mostly related to air pollution. Higher exposures to engine exhausts may occur in some occupations such as traffic policemen. Anwar (1994) evaluated chromosome aberrations in traffic policemen in Cairo. They found that the percentage of chromosome aberrations was significantly higher among the traffic policemen than in the control group. A corresponding the study in Dhaka city, Bangladesh, was performed for PAH-DNA adducts in white

blood cells as a marker of environmental and occupational polycyclic aromatic hydrocarbons (PAH) exposure in 46 three-wheelers drivers and 48 non-drivers (comparison group). The study showed that drivers had a significantly higher white blood cell PAH-DNA adducts level than the non-drivers. The results suggest that urban residents who are occupationally exposed to traffic pollution are at a potentially higher risk of health effects those not exposed to it (Rahman et al., 2003).

Martino-Roth et al. (2003) used the micronucleus (MN) test and the comet assay applied to cells of the buccal mucosa in order to evaluated the genotoxic risk associated with occupational exposure of 10 storage battery renovation workers, and 10 car painters, who were exposed to lead paint, solvents and benzene. Highly significant effects of occupational exposure were found with both the MN test and the comet assay ($P<0.001$). They suggested that occupational exposure may contribute to the development of pernicious illnesses through mechanisms that involve chromosomal changes. Thus, the genotoxic evaluation is necessary to guarantee environmental quality and occupational health, as well as to orient workers to reduce genetic damage and the risk of serious illness.

Petrol pump workers or gasoline station attendants are occupationally exposed to genotoxic agents because of gasoline including a large number of organic chemicals such as benzene, toluene, other monocyclic, aromatic, aliphatic and polycyclic aromatic hydrocarbons (Carere et al., 1998; Yadav and Seth, 2001). Benzene is especially a major constituent of gasoline (Waidyanatha et al., 2001). Moreover, benzene is an important substance widely used in industry. It has been detected in cigarette smoke and it is a ubiquitous environmental contaminant (Waidyanatha et al., 2001). It is well known that gasoline contain a variety of mutagenic and carcinogenic agents.

Several studies have shown that benzene causes genetic changes and especially induce various forms of genetic damage including chromosome aberrations, sister chromatid exchanges (SCE), micronuclei formation and DNA damage (Chung and Kim, 2002). Consequently benzene, is classified as a human carcinogen and mutagen widely found in the work-place and environment, is an established cause of hematotoxicity, leukemia and other bone marrow disorder in humans (Carere et al.,

1998; Celik and Akbas, 2005; Chung and Kim, 2002; Waidyanatha et al., 2001; Zhang et al., 1999).

Kasuba et al. (2000) used chromosome analysis and sister chromatid exchanges to assessed peripheral blood lymphocytes of 36 workers in the shoe industry occupationally exposed to benzene. They found that the incidence of dicentric chromosomes was significantly higher in those exposed than in the control group ($P<0.05$). These results confirm the potent genotoxic effect of benzene as an inducer of chromosomal aberrations in somatic cell of human. This study corresponds to the study of Tunca and Egeli (1996) that indicating that differences in the frequencies of chromosomal damages between the exposed and non-exposed groups were significant.

The petrol pump workers are also often exposed to gasoline and it derivatives during fuel delivery and several other genotoxic substances and air pollution such as dust, traffic fumes, lead acetate, carbon monoxide, carbon dioxide produced by vehicles (Celik and Akbas, 2005; Yadev and Seth, 2001).

Carere et al. (1998) applied the FISH technique to evaluate the aneuploidy and hyperploidy of chromosomes 7, 11, 18, X in peripheral blood lymphocytes of gasoline station attendants in Rome, Italy. They found that the incidence of aneuploidy and hyperploidy in the occupationally exposed group were significantly higher than in the control group. Corresponding the study of Zhang et al. (1999) in China, suggested that occupational exposure to benzene is associated with increased level of chromosomal damage in human lymphocytes.

Andreoli et al. (1997) performed the alkaline single cell gel electrophoresis (comet) assay to study the occurrence of DNA damage in peripheral lymphocytes of gasoline station attendants who had occupational exposure to benzene. The results obtained show a significant excess of DNA damage in lymphocytes of exposed workers as compared to the unexposed control. The average tail moment values, based on 100 cells/individual, were 1.900 μm in the exposed and 0.936 μm in the unexposed group ($p=0.028$). They discussed also the role of peripheral blood lymphocytes as target tissue in the biomonitoring of human exposure to genotoxic agents.

Yadav and Seth (2001) conducted several cytogenetic tests on 37 petrol pump workers exposed to benzene, carbon monoxide, chlorobenzene and nitric oxide and 36 control individuals with no known exposure at work. The results showed that the mean MI in exposed workers (7.86) was significantly higher than in controls (4.6). Chromosome aberrations were observed to be elevated in the exposed group and frequency of SCE in the exposed workers (6.49) was significantly higher than in the controls (5.62). They concluded that the petrol pump workers are at a risk to cytogenetic damage at their workplaces. Similarly, Tompa and coworkers (2005) reported a higher chromosome aberrations and SCE in benzene-exposed oil refinery workers than in the control group.

Celik and Akbas (2005) investigated peripheral blood lymphocytes in 30 gasoline station attendants and 30 controls in the city of Mersin, Turkey. They found that the urinary phenol levels of exposed workers were significantly higher than those of control subjects. There is a correlation between benzene exposure and replication and mitotic indecies, benzene exposure decreases the replication index and the mitotic index. This results indicated that there are significant differences in SCE and chromosome aberrations in the exposed workers compared to the controls.

The several studies conducted on the workers exposed to benzene have shown that there are benzene induced chromosomal changes in human lymphocytes. An increased incidence of chromosome aberrations was especially correlated with a high risk of cancer, especially hematological malignancies. Several studies indicated an increased incidence of lymphopoietic cancer, lung cancer, leukemia and an excess of cancer mortality among gasoline station attendants at various sites (Carere et al., 1998; Celik and Akbas, 2005).

Chiang Mai is the third biggest city of Thailand with a large population. The National Statistical Office reported that in 1990, Chiang Mai had a population of about 1.6 million people (National Statistical Office, 2000). Additionally, Chiang Mai land transport data showed that there were of motor vehicles of about 695,172 in 2000 and increasing to 933,733 in 2004 (Chiang Mai land transport, 2004). Therefore, the demand for gasoline delivery increased. Since gasoline contains a variety of mutagenic and carcinogenic agents, the number of petrol pump workers or gasoline station attendants occupationally exposed to mutagenic and carcinogenic

agents, creates a significant impact on health status. Consequently, cytogenetics biomonitoring studies on this workers are essential. The DNA status in their lymphocytes may be a predictor of future cancer risks and may help to prevent further deterioration of the state of the health of these workers.

2. Literature review

2.1 Structure of human chromosome

The composition of genes in the human genome is specified in the DNA of the 46 human chromosomes. Each chromosome consists of a continuous DNA double helix; that is a linear double-stranded DNA molecule (Fig. 1.). The DNA double helix exists as a complex with a family of basic chromosomal proteins called histones and with acidic nonhistone proteins. Together, this complex of DNA and proteins is called “chromatin”. There are five major types of histones that play a critical role in the packaging of the chromatin fiber. Two copies each of the four core histones H2A, H2B, H3 and H4 consitute a histone octamer, around which are 140 base pairs of DNA double helix, making just under two turns, like threads around a spool. Each complex of DNA with core histones is called “a nucleosome”. After a short (20-60 base pair) “spacer segment of DNA”, the next core DNA complex forms, and so on, giving the chromatin the appearance of “bead on a string or nucleosome fiber”. The fifth histone, H1, appears to bind to the DNA in the spacer segment of DNA. Later, the nucleosome fibers are compacted into a secondary helical structure, called “solenoid”. The solenoids are themselves packed into loops or domains attached to an acidic nonhistone protein “scaffold or matrix”, that has the appearance of chromatin fiber in interphase of the cell cycle (Fig. 1.). In the metaphase, chromatin fibers reach maximal condensation, and appear as metaphase chromosomes compose of two chromatids with a diameter of 1400 nm (Fig. 2.).

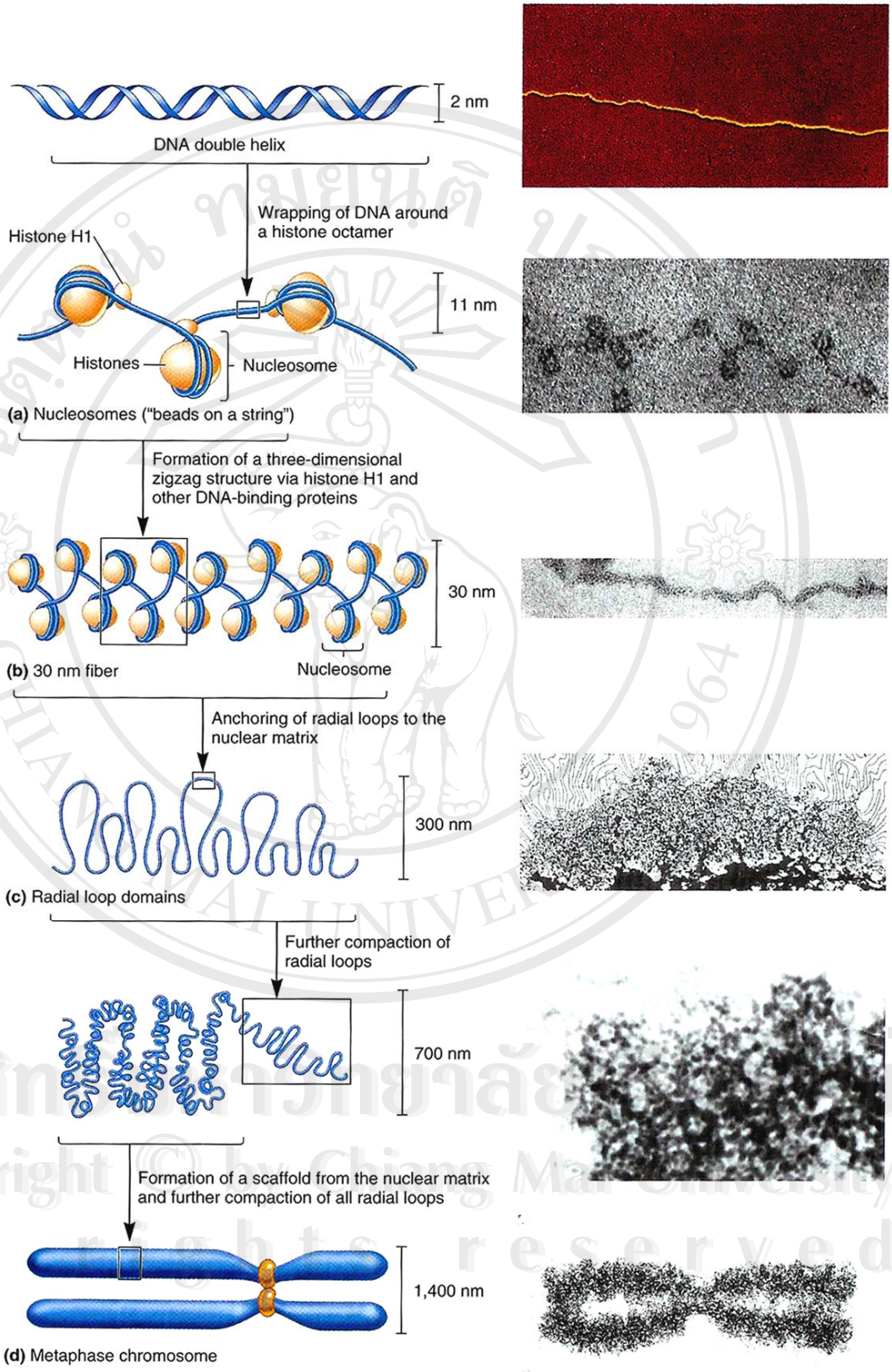


Figure 1 Structure of human chromosome (Brooker, 2005).

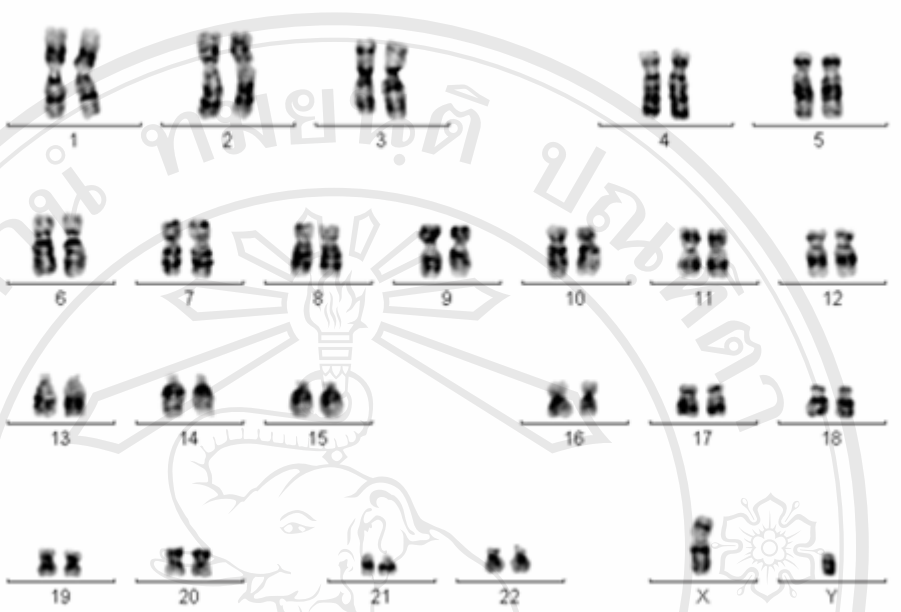


Figure 2 Normal human metaphase chromosome prepared from lymphocytes culture and stained with G-banding technique (46, XY).

2.2 DNA damage and structural chromosome aberrations

DNA damage usually arise from the cells were exposed to mutagenic chemicals or ionizing radiation, and produce the discontinuities or breaks in the DNA double helix. The damage generates different kinds of DNA lesions such as single or double-strand breaks, base damage, DNA-DNA or DNA-protein crosslinks, alkylation at base or phosphate groups intercalations and thymine or pyrimidine dimmers. These lesions may be repaired by the DNA-repairing processes to normal or may result in genome damage, on the nucleotide level in a particular gene and in the appearance of structural chromosomal aberrations. Only double-strand breaks can be seen on metaphase chromosome, and can be detected as structural chromosome aberration under light microscope.

Structural aberrations can be divided into two groups. Chromatid-type and chromosome-type aberrations, which differ from each other morphologically. Chromosome-type aberrations are those aberrations that involve both chromatids of the metaphase chromosome at precisely the same location. Chromatid-type aberrations, on the other hand, generally involve only one of the two chromatids of the metaphase chromosome (Fig. 3.).

Chromatid-type and chromosome-type aberrations have different mechanisms of formation resulting from DNA damage in different stages of the cell cycle (Fig. 3.). When double-strand breaks are formed prior to replication of the chromosome (it is functionally a single thread) in G1 and early S phases of the cell cycle, chromosome type aberrations may be formed, whereas double-strand breaks generated in chromosome replication and during the interval between chromosome replication and cell division (late S phase and G2) give rise to chromatid type aberration.

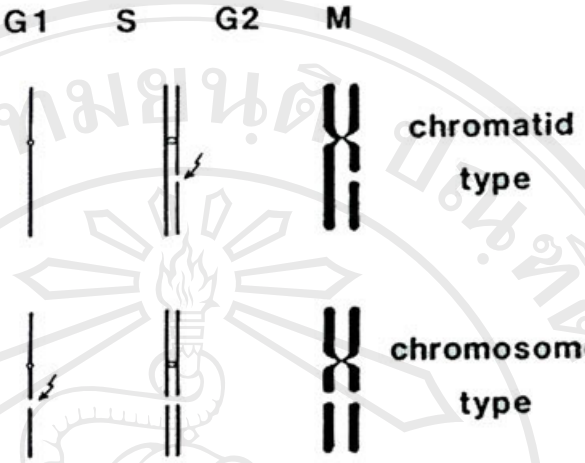


Figure 3 Shows different mechanisms of formation resulting from DNA damage in different stages of the cell cycle (Mevatee, 2005).

Furtheremore, within each group, there are several different types and a detailed classification is provide by Mevatee (2005), Rooney (2001) and Venitt & Parry (1984).

Chromatid-type aberrations

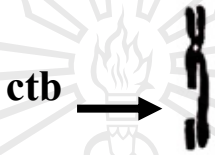
This type involves only one chromatid of a metaphase chromosome.

1. Chromatid gap (ctg)

A non-staining region on a chromatid not greater than the diameter of a chromatid.

2. Chromatid break (ctb)

   Breakage of a chromatid with displacement of the terminal fragment.



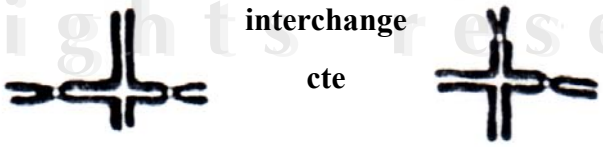
3. Chromatid deletion (ctd)

   Chromatid breaks with terminal fragment loss.

4. Chromatid exchange (cte)

   4.1 interchange (between chromosomes)

   Rejoining chromatid between two chromosome.



4.2 intrachange (within chromosome)

Rejoining between arms of one chromosome.

cte

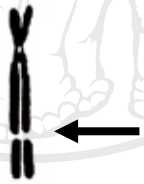
intrachange

Chromosome type aberrations

This type involves both chromatids of a chromosome at identical loci.

1. Chromosome gap (csg)

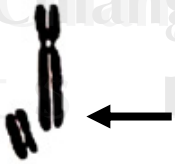
   A not-staining region on both chromatids of a chromosome at the same loci not greater than the diameter of a chromatid.



csg

2. Chromosome break (csb)

   Breakage of a chromosome with displacement of the terminal fragment.



csb

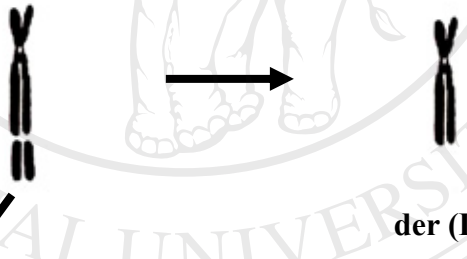
3. Acentric fragment (ace)

The fragments with no centromere.

ace

4. Chromosome deletion (csd)

Chromosome breaks with terminal fragment loss.



der (D)

loss

5. Dicentric chromosome (dic)

A chromosome with two centromeres resulting from breaks on two chromosomes and the parts containing the centromere rejoining.

dic

6. Ring chromosome (r)

A ring shape chromosome resulting from breakage on the both arms of chromosome and the broken ends rejoining.

2.3 Human biomonitoring studies

Occupational exposure to chemical mutagens and carcinogens can be monitored by measuring chemical interaction that occurs between the agent and biological macromolecules. The interaction between chemical mutagens and genetic materials may result in two of the most important type of genome damages, on the nucleotide level in a particular gene, i.e. point mutations, and in the appearance of structural chromosome aberrations. Such interaction is detectable in peripheral blood lymphocytes by cytogenetic methods.

Blood lymphocytes function as surrogated target cells for the measurement of damage in target organs. Due to lymphocytes circulate throughout the body and may come in contact with circulating carcinogens or mutagens or their metabolites (Kasuba et al., 2000). Therefore, they are easily obtained through a blood sample.

Cytogenetic studies can be used to monitor populations for a range of environmental and occupational clastogen and mutagen (Rooney, 2001). These include structural chromosome aberrations, sister chromatid exchanges and micronuclei. Until recently, the most frequently used methods involved either the detection of DNA in individual cells, using the alkaline single cell gel electrophoresis (SCGE or comet) assay (Tice et al., 2000; Zeljezic and Garaj-Vrhovac, 2001).

In the present study, chromosomal aberration test and alkaline single cell gel electrophoresis (SCGE or comet) assay were used to evaluated the extent of DNA damage in peripheral blood lymphocytes of petrol pump workers.

Chromosome aberration analysis

Genetic monitoring of human populations exposed to potential mutagens or carcinogens is an early warning for genetic disease or cancer. Chromosomal changes in human lymphocytes are well-established biomarkers of occupational or environmental exposure to genotoxic agents. The most frequently used genetic endpoints are chromosomal aberrations (Celik and Akbas, 2005). Structural chromosomal aberrations in peripheral blood lymphocytes have been applied for over 30 years in occupational and environmental setting (Hagmar et al., 2004) as a biomarker of the early effects of genotoxic carcinogens.

Several studies used chromosome aberrations test to monitor populations which have been occupationally exposed to mutagenic or carcinogenic agents in their work places. For example, Tunca and Egeli (1996) performed the chromosome aberration test on 58 shoes workers in the vicinity of Bursa, Turkey who had been occupationally exposed to benzene compared to 20 healthy subjects who had not been exposed to benzene or any related physical or chemical agents and who were living in or near Bursa. The study found that the incidence of chromosomal aberrations in the study group (22.2%) was significantly higher than in the control group (2.6%).

Fatima et al. (2001) evaluated the mutagenic effects of occupational exposure to cement dust in cement industry workers by the chromosome aberrations test, comparing them with controls who had no exposure to cement dust or any known physical or chemical agents. A significant increase in the incidence of chromosomal aberrations was observed in the exposed group when compared to the control group.

The chromosome aberration test is a method for biomonitoring in occupational or environmental exposure to genotoxic agents that functions as a predictor of future cancers risk. Hagmar et al. (2004) reported that high levels of chromosome aberrations were clearly associated with an increased total cancer incidence in the Nordic cohorts and an increase total cancer mortality in the Italian cohort, this showed equally strong cancer predictability corresponding to the conclusion of Liou et al. in 1999 which indicated that chromosome aberrations are good biomarkers for the prediction of cancer development.

Comet assay

The comet assay was first used by Ostling and Johanson in 1984 (Faust et al., 2004) to detect radiation-induced DNA damage under pH neutral conditions. This version of the comet assay detects only DNA double strand breaks. Subsequently, the alkaline comet assay (pH>13) was developed by Singh et al. in 1988 (Tice et al., 2000) because of DNA is denatured and unwound at pH values above 12.0 due to the disruption of hydrogen bonds between double-stranded DNA. And at pH conditions of 12.6 or higher, alkaline labile sites are quickly transformed to single strand breaks (Tice et al., 2000). Consequently, at this pH DNA single strand breaks, alkali labile sites and DNA cross-linking in individual cell can be detect. This is the most frequently used version of the comet assay.

The steps in the methodology of the alkaline comet assay are briefty as follows: a cell suspension is embedded in agarose on a frosted part of microscopic slide and lysed by detergents and a high salts concentration in order to liberate the DNA. Afterwards, the slides are treated under alkaline conditions so that the DNA unwinds from sites of strand breakage. During electrophoresis under the same alkaline condition, DNA fragments induced by genotoxic agents migrate to the anode side of the chamber. Later, the slides are washed with a neutralizing solution and stained with fluorescent DNA binding dyes. Finally, the image of comet cells can be collected by using a computerized image analysis system. Generally, cells with a high level of DNA damage exhibit increased comet parameters, which may be expressed as tail length (the extent of DNA migration) and tail moment (the amount of DNA in the tail).

Compared with other genotoxicity assays, the advantages of the comet assay include: (1) its demonstrated sensivity for detecting low levels of DNA damage; (2) the requirement for small numbers of cell per sample; (3) flexibility; (4) low cost; (5) ease of application; (6) the ability to conduct studies using relatively small amounts of a test substances; and (7) the relatively short time period (a few days) needed to complete an experiment (Tice et al., 2000).

Therefore, the comet assay is a rapid and sensitive method for analyzing single cells for DNA damage in the form of DNA strand breaks, alkali-labile sites. The

assay is particularly useful for the in vitro genotoxicity testing of chemicals and for human biomonitoring studies (Anderson et al., 1997).

There have been several studies, using the comet assay, carried out on workers who were exposed to genotoxic agents in their workplaces. Some of these studies are discussed here.

Andreoli et al. (1997) applied the alkaline single cell gel electrophoresis assay to study the occurrence of DNA damage in peripheral blood lymphocytes of subjects with occupational exposure to low levels of benzene (12 gasoline station attendants). The results obtained show a significant excess of DNA damages in lymphocytes of exposed workers, compared to unexposed controls. This result is also discussed in relation to the role of peripheral blood lymphocytes as target tissue in the biomonitoring of human exposure to genotoxic agents. The comet assay is extremely sensitive to very low levels of DNA damage and may be a useful tool in human biomonitoring, particularly in the analysis of low level exposure to environmental mutagens and carcinogens.

Zhu et al. (1999) performed the comet assay on 107 cigarette manufacturing workers who were exposed to tobacco dust. They found that the exposed workers had a larger tail moment than of the controls ($P<0.05$). They suggested that tobacco dust exposure can induce lymphocyte DNA damage and the comet assay could detect DNA single strand break and alkaline-labile sites.

Gontijo et al. (2001) performed the comet assay to detect DNA damage in nonneoplastic uroepithelial cells of smokers (patients with urinary complaints) and ex-smokers. The study showed a significant increase in DNA damage as depicted by tail moment when compare with nonsmokers ($P=0.03$). They postulated that DNA damage may trigger genomic instability, a crucial step in carcinogenesis. And classifications of individuals with a smoking history are at high risk for urinary bladder cancer.

Zeljezic and Garaj-Vrhovac (2001) used both the chromosome aberration test and the comet assay to evaluate the extent of DNA damage and DNA repair in PBL of subjects employed in pesticide production. In order to determine the possible primary genotoxic effects in workers, blood sample were taken after an 8 month long period of exposure to a complex mixture of pesticides. In the exposed group significantly

increased numbers of aberrant cells, chromatid and chromosome breaks, acentric fragments and dicentric chromosomes compared with the controls were found. And significantly increased levels of DNA damage in the comet assay in terms of tail length and tail moment were found. After the workers were removed from the pesticide exposure zone, the number of all types aberration and the comet assay end-points decreased significantly compared with the first sampling point, but it still remained significantly higher in comparison with the control group. They indicated the results of the comet assay and chromosome aberration test confirm the ability of a mixture of pesticides to caused a significant increase in the level of DNA damage that could be detected by the comet assay and chromosome aberration test.

In Chiang Mai, Thailand. Puaninta (2000) first applied the comet assay and chromosome aberration test to evaluate in vitro genotoxicity of insecticides (Lannate-L, Tamaron 600 SL and Furadan 3%G). These insecticides were widely use in Northern Thailand. The study have demonstrated that Lannate-L, Tamaron 600 SL and Furadan 3%G are genotoxic substances and the comet assay can detected DNA damage when the cells were exposed to the testing substances at lower concentrations than with the chromosome aberration test.

Later, in 2004. Mokmued used the comet assay to detected genotoxicity of extractable organic matter from total suspended particles (TSP), collected from ambient air of Warorod and Hang Dong markets in Chiang Mai. DNA damage was significantly increased after treatment of the human lymphocytes with the TSP sample. This study indicated that long-term exposure to these TSPs could induce DNA damage, which might be responsible for chronic adverse health effects among Chiang Mai's inhabitants (Mokmued, 2004).

3. Objectives

In this study, the researcher aims to

3.1 Evaluate the structural chromosome aberration in petrol pump workers who were occupationally exposed to chemical genotoxic agents using the chromosome aberration test.

3.2 Evaluate the DNA damage in petrol pump workers who were occupationally exposed to chemical genotoxic agents using the comet assay.

3.3 Evaluate the correlation between the chromosome aberration test and the comet assay.