CHAPTER II

MATERIALS AND METHODS

1. Study populations

This cross-sectional study was designed to investigate the genetic damage between petrol pump workers, who were occupationally exposed to gasoline, its derivatives, air pollutant and other several genotoxic substances, through inhalation and skin contamination, and a control population with no history involved in this exposure.

The sample size was calculated using the following equation:

$$N = 2[(Z_\alpha+Z_\beta)\sigma]^2/(\mu_c-\mu_t)^2$$

where:

N	=	sample size
Z_α	=	critical Z value for alpha
Z_β	=	critical Z value for beta
σ^2	=	pooled variance
μ_c	=	mean of control group
μ_t	=	mean of experimental group

The assumptions were alpha of 0.05 (two-sided), which meant $Z_\alpha = 1.96$; beta of 0.20, which meant $Z_\beta = 0.84$. Sample size calculation referred to the average tail moment values from petrol pump workers in an Italy research (Andreoli, 1997).

$$\text{Therefore, } N = 2[(1.96+0.84)0.8772]^2/(0.94-1.90)^2$$

$$N = 13.09$$

Thirteen subjects were sufficiently sample size for each group.

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The exposed group consisted of 30 workers employed in petrol pumps from the center of Chiang Mai. They were included for the study according to the following criteria:

- Male employees, age 18 to 25 years old.
- Petrol pump workers in central of Chiang Mai.
- Subjects were occupationally exposed to gasoline at least 8 hours per day, 5 days per week for more than 3 months.
- No history of genetic diseases.
- Subjects had not been on drug treatment or chemotherapy or X-rays during 3 months before sampling.

Thirty students (all male) from the Faculty of Medicine, Chiang Mai University, who had not been currently or previously occupationally exposed to gasoline, were use as a control group. Control subjects were matched to exposed subjects by gender and age.

Information on the subject's health, medication history, lifestyle factors (such as smoking, alcohol consumption) and work history was obtained by interviewing using a questionnaire. Informed consent was obtained from all subjects according to the Informed Consent Form of the ethics committees of Faculty of Medicine, Chiang Mai University.

Blood samples were obtained by venipuncture using heparinized syringes. Immediately after sampling, the blood was kept cool on ice bath and brought to the laboratory. The same blood sample was analyzed using both methods: the chromosomal aberration and the comet assays.

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2. Chromosomal aberration analysis

For chromosomal aberration analysis, metaphase chromosomes were prepared from lymphocyte cultures.

Lymphocyte cultures were established by adding 0.25 ml of blood sample to 5 ml. of RPMI 1640 medium (pH 7.2), supplemented with 20% fetal bovine serum, 100 µl phytoheamagglutinin (PHA) and antibiotics (streptomycin and ampicilin). The cultures were incubated for 48 hour at 37°C and after that 0.1 µg/ml. of colcemid was

added and they were incubated for another 30 minutes. Preparation of chromosomes was carried out according to conventional methods (IAEA, 1986). Briefly, the lymphocytes were exposed to 0.075 M KCl hypotonic solution. Methanol: acetic acid (3:1) was used for the fixation of cell membranes, and an air drying technique was used for chromosome spreading (fig. 4.).

The metaphase chromosomes were kept overnight at room temperature, then they were stained with the conventional Giemsa staining or non-banding technique. The metaphase chromosomes were analyzed under a light microscope. From each subject, a total of 100 randomly cells at metaphase were analyzed according to Forni (1996), Hagmar et al. (2004) and Tompa et al. (2005).

The analysis of structural chromosome aberrations included chromatid gap, chromatid break, chromatid exchange, chromosome gap, chromosome break, acentric fragment, dicentric chromosome and ring chromosome. The number of aberrations was recorded using a spread sheet.

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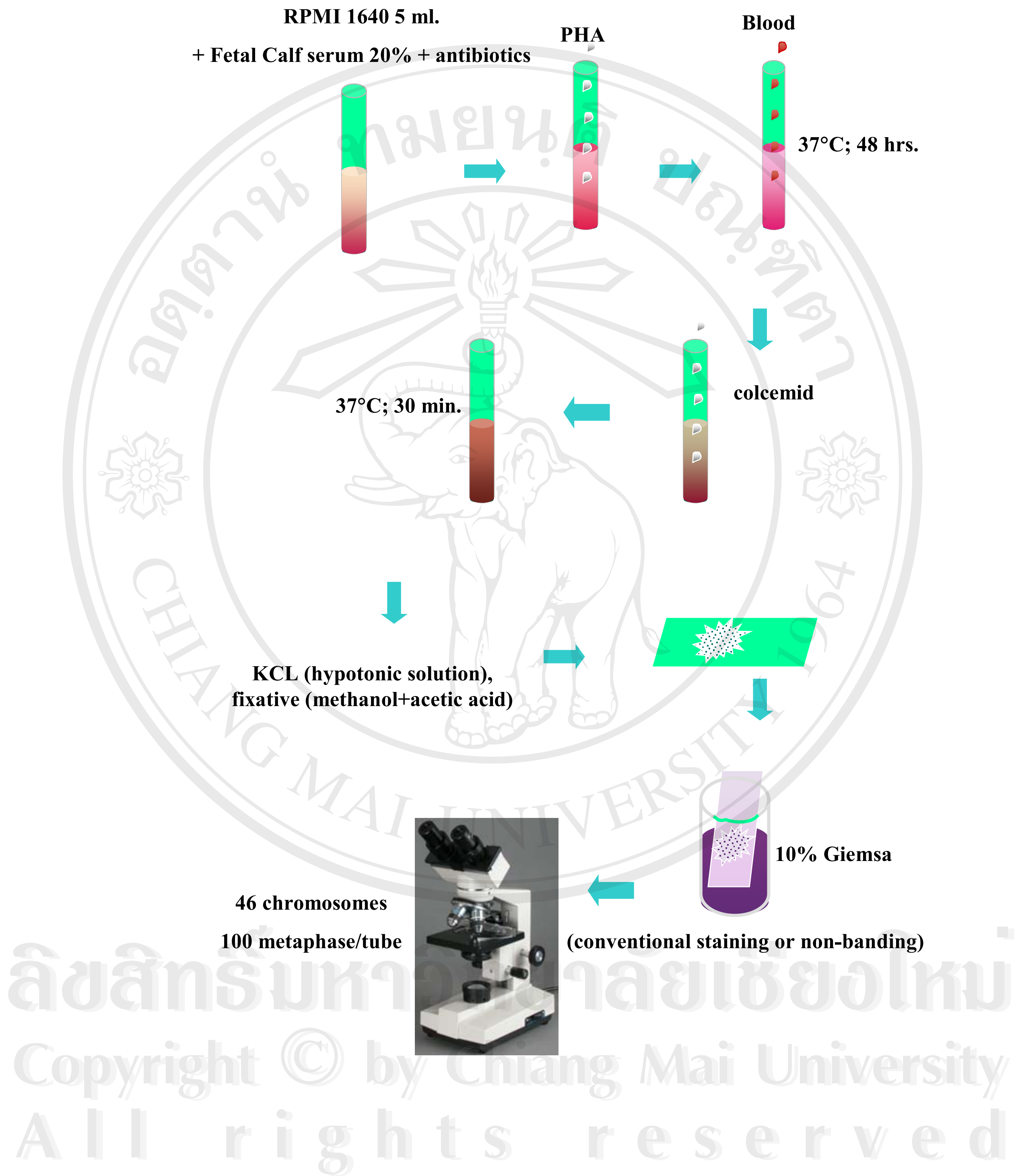

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Figure 4 Schematic representation of lymphocyte culture steps in chromosome aberration analysis.

3. Alkaline single cell gel electrophoresis (SCGE) or comet assay

The basic steps of the comet assay include (1) preparation of microscope slides layered with cells suspended in agarose; (2) lysis of cell to liberate DNA; (3) exposure to alkali (pH>13) to obtain single stranded DNA and to express alkaline labile site (ALS) as single-strand break (SSB); (4) electrophoresis under alkaline conditions; (6) DNA staining and (7) Image analysis (Fig. 8.). The experimental procedures for the comet assay were kept at the same condition throughout the study.

3.1 Cell Preparation

The peripheral blood lymphocytes were isolated by centrifugation gradients using Histopaque (Sigma), which had a gradient of 1.077 density. The lymphocytes were prepared by mixing a heparinized blood sample in a centrifuge tube with PBS in a proportion of 1:1 by volume, overlaying onto the Histopaque in a proportion of 1:1. Then, the tube was centrifugated at 1,200 rpm for 30 minutes. During the centrifugation, the erythrocytes and granulocytes were aggregated by ficoll and sedimented to the bottom of the tube. The lymphocytes separated in a ring located between the plasma and Histopaque interface. The lymphocytes were removed immediately after isolation; they were washed 3 times with PBS and centrifuged 2,000 rpm for 5 minutes each time. After washing, the lymphocytes were resuspended in 400 µl of RPMI-1640 medium supplemented with 20% fetal bovine serum and antibiotics.

3.2 Slide preparation and lysis

Initially, the frosted part of microscope slides (Fig. 5.) were covered with 90 µl of 1% normal melting agarose (NMA) for the first layer and covered with a cover slip. Then the slides were laid on a flat surface in a tray and kept in a refrigerator at 4°C for 10 minutes to allow the agarose to solidify. After removal of the cover slips, the second layer of 90 µl. of 0.75% low melting agarose (LMA) containing 10 µl. of lymphocytes suspension was added, and again allowed to solidify in 4°C for 10 minutes. The cover slips were removed; the slides were immersed in a lysis solution for at least 1 hour in the dark.

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3.3 Alkaline (pH>13) unwinding and electrophoresis

The slides were placed in an electrophoresis alkaline buffer (pH 13) for 20 minutes to allow the unwinding of DNA and expression of alkaline-labile damage before electrophoresis. Electrophoresis was performed in an electrophoretic buffer pH 13 for 20 minutes using a power supply of 18 volt with 350 miliamperes.

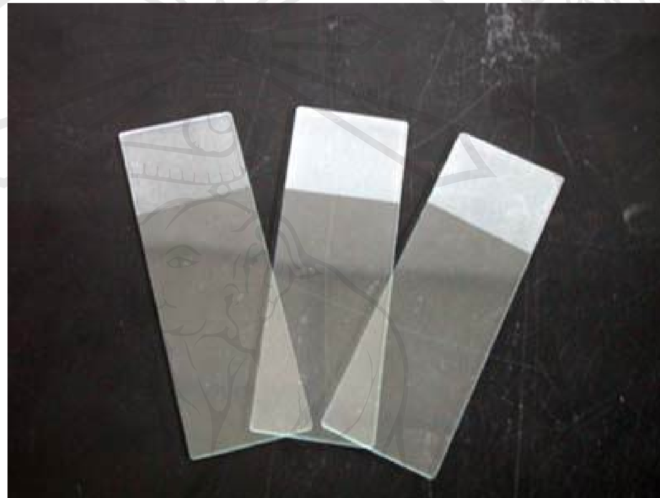

Figure 5 Shows the frosted part of microscope slides covered with microgel.

Figure 6 An electrophoresis set consisted of a power supply and electrophoresis chamber.

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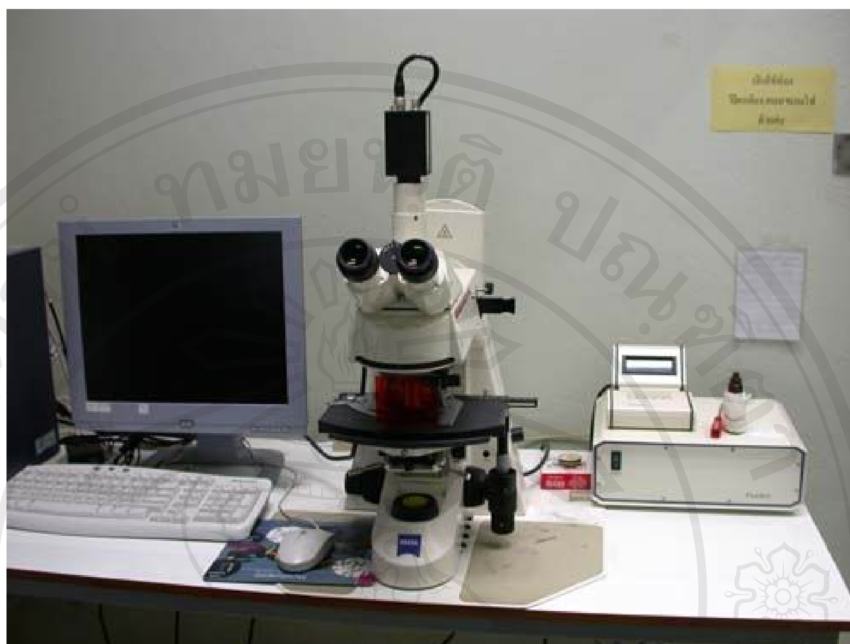

Figure 7 A fluorescent microscope equipped with a computerized image analysis system (Meta System Model "Comet Imager") (Rushmore).

3.4 Neutralization and DNA staining

After electrophoresis, the slides were washed in a neutralization buffer (pH 7.5) 3 times each for 5 minutes. Immediately before the examination, the DNA was stained with 25 µl. of the ethidium bromide (20µl/ml). The slides were kept in a humidified air-tight box at 4°C to prevent drying of the gel and analyzed within 3-4 hours. However, the agarose gel can be dehydrated by immersing the slides in absolute methanol for 5 minutes and letting the slides dry at room temperature to help to stabilize the gels during long-term storage and can be rescored at anytime.

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3.5 Evaluation of DNA damage

The samples were examined under a fluorescent microscope equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm, using 20 X objectives to explore the cell distribution. The computerized image analysis using a Meta System Model "Comet Imager" (Rushmore) was used (Fig. 7.). A total of 100 randomly

selected comet cells per slide for each sample were captured at 40X magnification according to Andreoli et al. (1997), Garaj-Vrhovac and Zeljezic (2003) and Martino et al. (2003). The computerized image analysis system was allowed to make a quantitative description of the comet using various parameters. The comet parameter was chosen to represent the data on genotoxic effects. Generally, cells with a high level of DNA damage exhibit increased comet parameters which may be expressed as tail length, % DNA in the tail (tail intensity) and tail moment (tail length X % DNA in the tail).

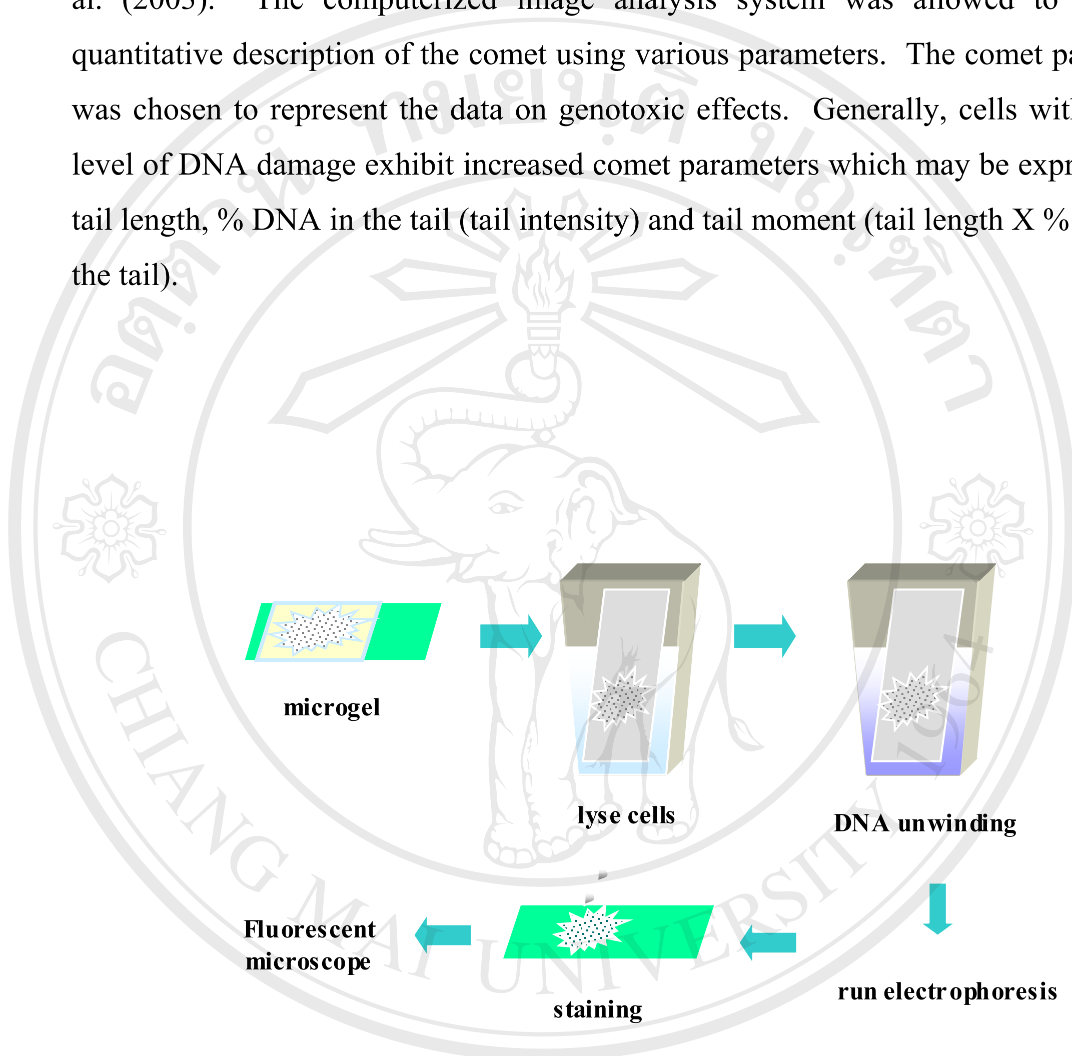

Figure 8 Schematic representation of critical steps in the comet assay.

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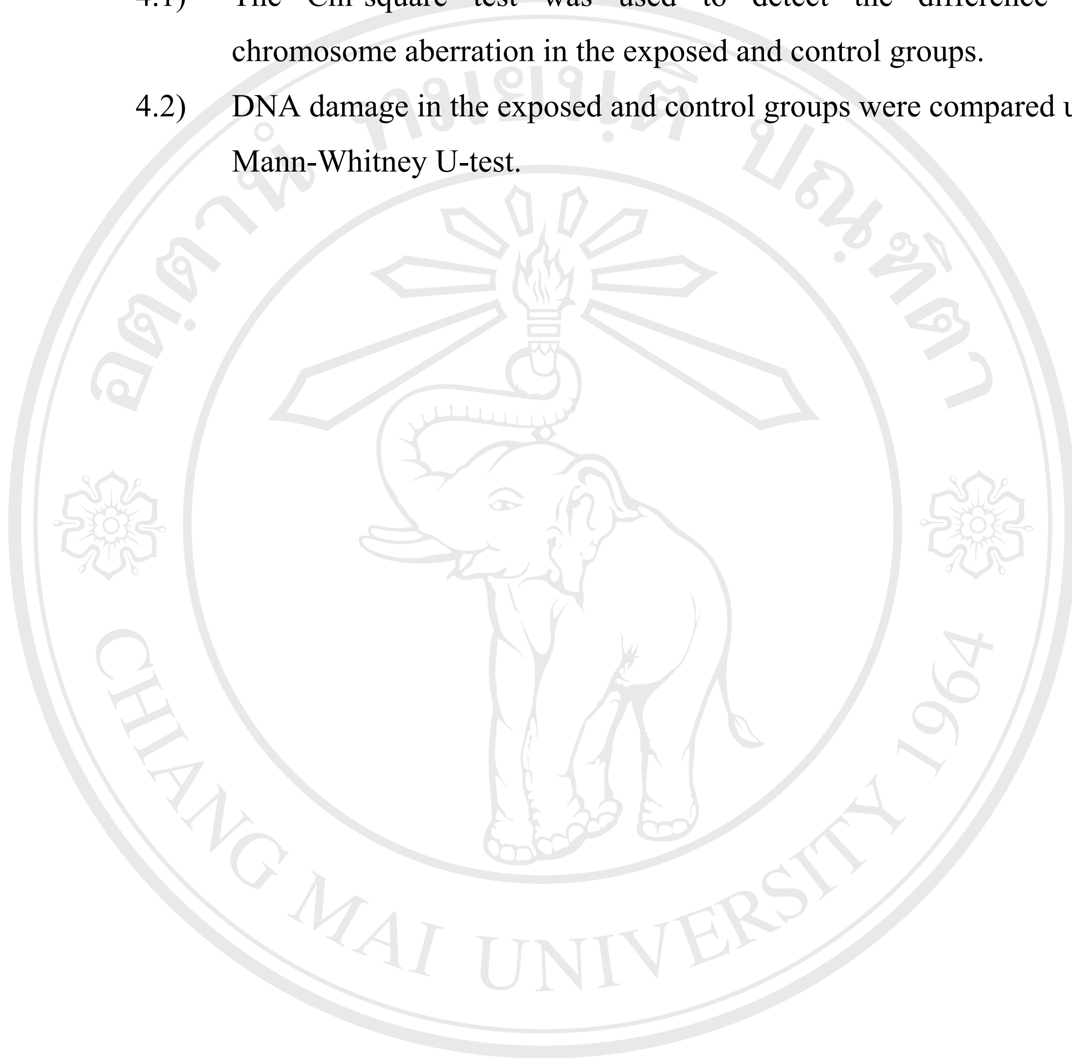
4. Statistical analysis

Analysis was carried out using the SPSS program:

4.1) The Chi-square test was used to detect the difference between chromosome aberration in the exposed and control groups.

4.2) DNA damage in the exposed and control groups were compared using the Mann-Whitney U-test.



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