CHAPTER II

MATERIALS AND METHODS

Materials	Model	Sources
1. Spectrophotometer	DU 605	Beckman, U.S.A.
2. Spectrophotometer	7850	Jasco, U.S.A.
3. Analytical balance	NA-164	Olertling, U.K.
4. Analytical balance	AG 204	Mettler Toledo, Switzerland
5. Analytical balance	HF-3000	AND, U.S.A.
6. Gas Filter Correlation	Model 48	GFC, U.S.A.
7. Automatic cell counting	model T540	COULTER, U.S.A.
8. Automatic blood chemistry analyzer	SYNCHRON CX7	BECKMAN, U.S.A.
9. NOVA CRT ElectrolyteAnalyzer	NOVA 4 Plus	Nova Biomedical, U.S.A.
10. Homogenizer	Glas-Col	TERRE HAUTE, U.S.A.
11. Automatic pipette 0.5-10 µl		Gilson, France
12. Automatic pipette 2-20 µl		Gilson, France
13. Automatic pipette 10-100 µl		Gilson, France
14. Automatic pipette 10-200 µl		Gilson, France
15. Automatic pipette 100-1,000 µl		Gilson, France
16. Automatic pipette 1,000-5,000 µl		Gilson, France
17. Centrifuge	H7000	Kokusan Enshinki,
18. Stereomicroscopy	Olympus	VMZ-4SA-2W, Japan

Reagents	Sources
The chemicals and reagents are analytical grade.	
1. Pentobarbital sodium	
2. Sodium chloride (NaCl)	E.Merck, Germany
3. Formalin	
4. Glacial metaphosphoric acid	E.Merck, Germany
5. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Fluka, Switzerland
6. di-potassium hydrogenphosphate trihydrate ($K_2HPO_4 . 3H_2O$)]	Sigma Chemical Co., U.S.A.
7. 5,5'-Dithio-bis (2-Nitrobenzoic acid) (DTNB)	Sigma Chemical Co., U.S.A.
8. Sodium citrate	Unilab, Australia
9. Glutathione free acid standard	Sigma Chemical Co., U.S.A.
10. Sodium hydroxide (NaOH)	E.Merck, Germany
11. Trichloroacetic acid (TCA)	E.Merck, Germany
12. Hydrochloric acid (HCl)	E.Merck, Germany
13. Thiobarbituric acid (TBA)	Sigma Chemical Co., U.S.A.
14. 2-Amino-2-hydroxy methyl-1,3-propanediol (Tris)	Sigma Chemical Co., U.S.A.
15. Tetramethoxypropane (TMP)	Fluka, Switzerland
16. Cytochrome *c* (TypeVI)	Sigma Chemical Co., U.S.A.
17. 1,4-Dithio-DL-threitol solution	Fluka, Switzerland
18. Glycerol	Sigma Chemical Co., U.S.A.
19. Potassium chloride (KCl)	E.Merck, Germany

Reagents	Sources
20. Tris(hydroxymethyl)aminomethane (Trizma® base)	Sigma Chemical Co., U.S.A.
21. Sodium dithionite (NaS_2O_4)	E.Merck, Germany
22. Formic acid (HCOOH)	Ajax, Australia
23. Sulfuric acid (H_2SO_4)	E.Merck, Germany

2.1 Measurement of carbon monoxide concentration in ambient air

The instrument type gas filter correlation (GFC) Model 48 was used for measurement of carbon monoxide concentration (**Figure 15**). (Thermo Environmental Instruments Inc., 1991). The instrument was checked condition before measurement (**Table 5**). Five study sites in Chiang Mai were selected based on the traffic condition. They were Rin Kham, Khuang Sing, and Juvenile Court intersections, Nawarat bridge and Warorod market. **Figure 10 - 14** shows the measured sites for measurement of carbon monoxide concentration in Chiang Mai ambient air. The carbon monoxide concentration at each sites were measured for 7 days, 12 hours per day from 6 a.m. to 6 p.m.. Then the average concentration of carbon monoxide were used to calculate for experimental dose (Loomis, 1978; Woodward, 1996). The experimental dose was calculated from the following formula;

$$\text{Experimental dose} = A \times B \times C$$

where A is the average concentration of carbon monoxide

B is uncertainty factor of safety factor between human and animal, 10

C is intraindividual differences factor, 10

Table 5 The Gas Filter Correlation (GFC) Model 48 condition for measurement carbon monoxide in ambient air. (Thermo Environmental Instruments Inc., 1991)

Description	Design
Sample flow	1.0 Liter per minute
Test INT Button	
1. INT (> 10,000 Hz)	> 10,000 Hz.
2. INT (> 10,000 Hz)	> 10,000 Hz.
3. AGC (> 34,000 Hz)	> 34,000 Hz.
4. AGC (> 34,000 Hz)	> 34,000 Hz.
Test P/T Button	
1. PRESSURE	750 mmHg.
2. TEMPERATURE	25.0 °C
Test STA Button	
F. SCALE	
RANGE 1	50.0 PPM.
RANGE 2	50.0 PPM
SEC.	
TIME 1	12 SEC.
TIME 2	12 SEC.
Dip.1-8	
F. PANEL	
ZERO SET	
SPAN SET	
P.5007	
Test H.A. Button	
3. S/R RATIO	1.14-1.18

Figure 14. Rin Kham intersection was the first sampling area for measurement of carbon monoxide concentration.

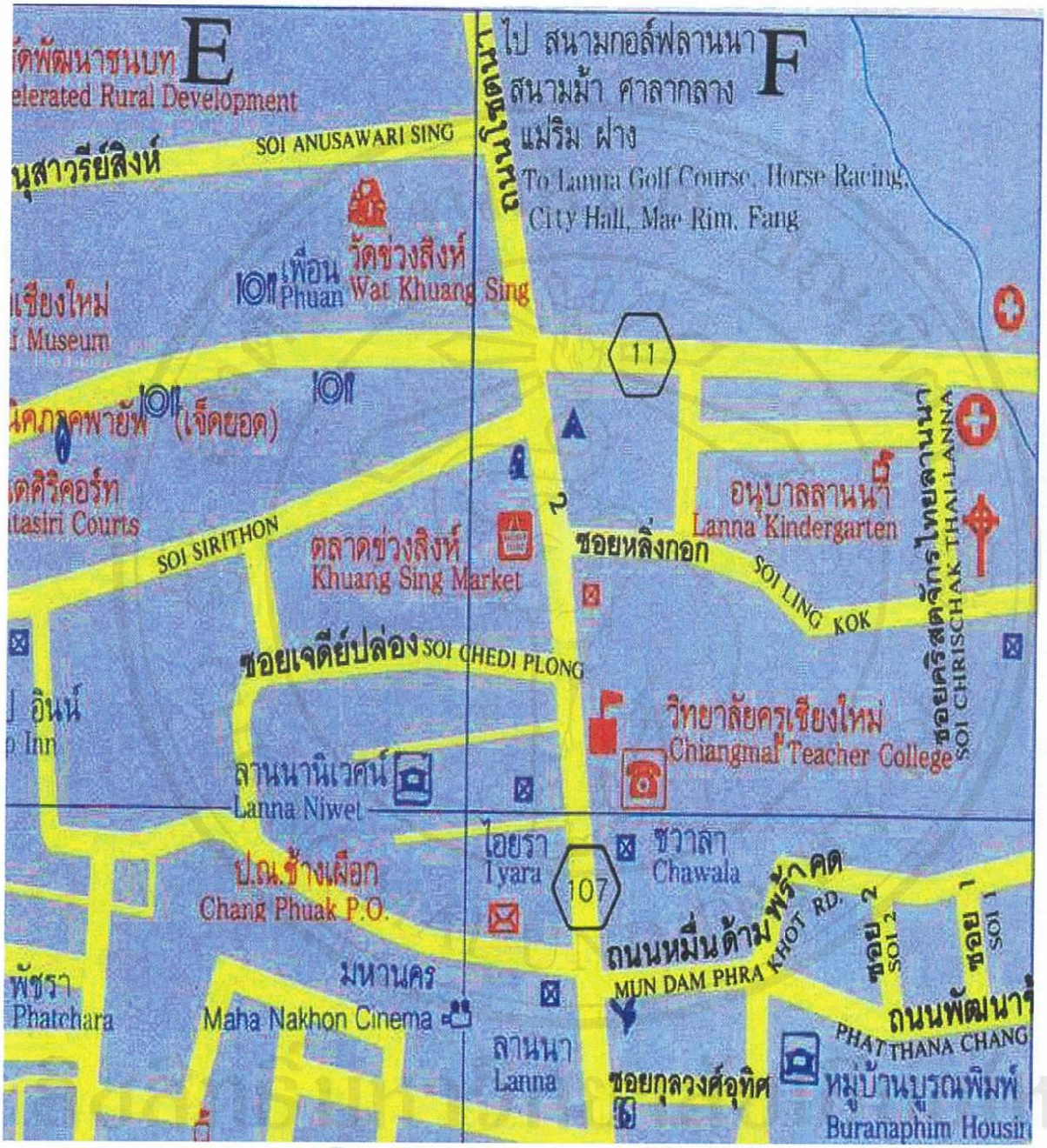

Figure 15. Khuang Sing intersection was the second sampling area for measurement of carbon monoxide concentration.

Figure 16. Juvenile Court intersection was the third sampling area for measurement of carbon monoxide concentration.

Figure 17. East of Nawarat Bridge was the fourth sampling area for measurement of carbon monoxide concentration.

Figure 18. Warorod Market was the fifth sampling area for measurement of carbon monoxide concentration.

Figure 19. Gas Filter Correlation (GFC) Model 48 was used for measurement of carbon monoxide concentration in ambient air and exposure chamber.

2.2 Design and build exposure chamber

Exposure chamber was designed and built by modifying from the chamber of Haider, *et. al.*, 1981. and Piantadosi, *et. al.*, 1997. Referring to exposure chamber of Haider's and Piantadosi's, they made of glass and plexiglass, respectively. But both were special design with many equipments, expensive, large, heavy and inconvenience to move. So, our designed chamber was only 5 mm. thick plastic as a quadrilateral cage size 45 × 75 × 40 (wide × long × high) centimeters. The cage was divided into two parts, the first part was an area for mixing air and carbon monoxide and the second part was an exposure area. Between the two parts there was an electric fan, used to evacuate mixed carbon monoxide gas and air from the first part passed into the second part. In the second part, there was another fan attached to the inside wall for remove carbon dioxide from rats exhalation. Top of the second part wall had a small hole for adding rubber sucker tube of GFC to measure carbon monoxide concentration (**Figure 19**).

2.3 Exposure experiment

Spraque- Dawley rats (initial weight 150-200 g) were purchased from The National Laboratory Animal Center, Mahidol University, Salaya campus, Nakorn Pathom, Thailand. The rats were divided into 4 groups, seven animals in each group, and were housed individually. The temperature (20-25 °C) and lighting (12 hours day / night cycle) were constantly controlled. The rats were acclimated for one week before starting the experiment.

Control groups were seven male and female rats exposed to only air (**Figure 20**). Experiment groups were also seven male and female rats exposed to carbon monoxide gas (from the in house carbon monoxide generator) and air (**Figure 21**). Carbon monoxide gas was released from carbon monoxide generating system (**See Appendix A**) through rubber tube into the first part of the chamber. And the carbon monoxide gas was blown through the second part of the chamber by a small electric fan. Carbon monoxide concentration was measured in the second part of the chamber using carbon monoxide analyzer. The concentration of carbon monoxide in the chamber was adjusted to achieve the final concentration. All rats were continuously exposed to carbon monoxide for 12 hours per day (6 a.m. – 6 p.m.) for three months.

After 3 months experiment, all rats were sacrified (**See Appendix B**). The rat blood was collected from the carotid artery into glass tube which had ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The EDTA bloods were collected for determination of completed blood cell count (CBC), carboxyhemoglobin (COHb) and glutathione (GSH). Five milliliters blood was collected from the carotid artery into a glass tube without anticoagulant. The clotted blood was centrifuged at 3,000 rpm for 10 minutes and separated serum for determination of malondialdehyde and clinical chemistry values. After the blood was collected, artery was flushed with 0.9% normal saline solution (NSS) until the color of livers and lungs were changed from red to white. The other organs of the rats, e.g. brain, lung, heart, eye, liver, spleen, stomach, intestine, kidney, adrenal gland, testis, uterus, ovary and muscle were cut and kept for determination of cytochrome *c* reduction and pathology study. The lung and brain were divided to a little pieces as soon as possible, placed in ice cold 50 mM Tris-KCl and kept at –80 °C for cytochrome *c* reduction determination (Bagchi, *et al.*, 2002). For pathology study, the other organs were cut and kept in 10% formalin.

2.4 Analytical methods

2.4.1 CBC

CBC was determined by using COULTER Model T540 (COULTER, 1991)

Procedure

1. Gently mixed the sample.
2. Held the sample up to the aspirator tip with the tip submerged toward the bottom of the sample container.
3. Pressed the WHOLE BLOOD button to initiate the operating cycle.
4. Removed the container from aspirator tip when heard the beep and be cleared of the area around the aspirator tip.
5. Reviewed the results when printed.
6. Mixed the sample gently again.
7. Aspirated the blood by capillary tube and dropped one drop of the blood on slide.
8. Spreaded the blood smear and labeled code of the sample on the slide.

Figure 20. The carbon monoxide exposure chamber used in this study.

Figure 21. The control rats were placed into the exposure chamber and exposed to only air in the animal room at 25 °C, 12 hrs light-dark cycle per day.

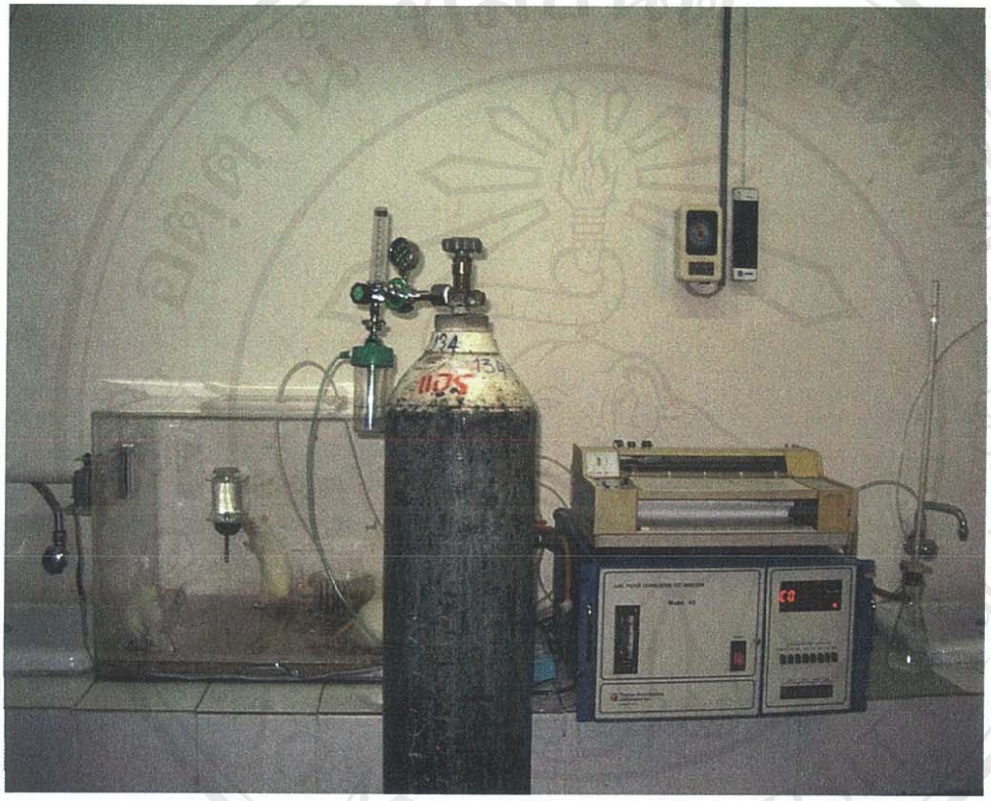

Figure 22. The experimental rats exposed to carbon monoxide in the exposure chamber. The carbon monoxide gas was produced from in-house carbon monoxide generator. The carbon monoxide level was measured.

9. Stained the blood smear with wright rapid stain.
10. Differentiated the WBC by microscope.

2.4.2 Blood Chemistry

Clinical chemistry values was determined by using BECKMAN SYNCHRON CX7 (BECKMAN, 1996)

Procedure

1. Pipetted the sample 1-1.5 ml and placed into the cup
2. From the MASTER Screen, pressed **F1 SAMPLE PROGRAM.**
3. From the **SAMPLE PROGRAMMING** Screen, pressed **F1 PROGRAM SECTORS.**
4. Typed the sector number to be programmed, then pressed **ENTER.**
5. Entered the sample ID.
6. If programming using panels, pressed **F1 SELECT OPTIONS** to view and selected from the list of previously defined panels. If programming individual tests in the chemistry field, cursor and **SELECT** the desired tests.
7. Pressed **F7 NEXT AVL** or **F8 NEXT CUP** to continue programming additional samples.
8. Pressed **MASTER SCREEN** or **F5 CAROUSEL STATUS** when all programming was completed.
9. Placed the sample in the sector.
10. Loaded the sector on the autoloader in the load position.
11. If system was not currently running, pressed the **START** key.
12. If the system was currently running, pressed the **LOAD** button.

Electrolyte

Electrolyte was determined by using NOVA 4 Analyzer (NOVA biomedical, 1994)

The analyzer used a microcomputer-based operating system to measure analyzes (Sodium (Na), Potassium (P), Chloride (Cl^-) and TCO_2)

Procedure

1. Tilted the sampler up into the stat position so that the probe could accept hand-held samples.
2. Selected the desired sample type.
3. Pressed **ANALYZE** if the Sample ID Input or the test selection options of both are "ON" in the Set-Up Menu, or Pressed **WORKLIST** if the Sample ID Input or the Test Selection options or both were "**OFF**" in the Set-UP Menu.
4. Program the sample as desired. Sample ID and test selection data were optional. Without test selection all available tests for the sample type would be run.
5. Pressed **ANALYZE**. Wait until the probe was fully extended and until the Position Sample screen appears. A 30 second clock starts to tick down.
6. Held the cup with the sample up to the probe. Do not allow the probe to touch the cup or to pull out of the sample.
7. Pressed **ANALYZE** again. The sample was aspirated through the probe into the electrode train where it was analyzed. A new clock starts to tick down to the end of the analysis.

2.4.3 Carboxyhemoglobin (Panzali, et al., 1987; Pantasri, *et al.*, 1998)

Principle

COHb level was measured by spectrophotometry method, used sodium dithionite as reducing agent for reduce oxyhemoglobin and methemoglobin to reduced Hb. COHb wasn't reduced because it can strongly bound with Hb. When sample was scanned with spectrophotometer in 390 and 450 nm wave lengh are show overlap normal - spectra of HHb and COHb, when mode second - derivative was used overlap spectra of HHb and COHb will clearly seperage then we can calculate COHb level. Standard curve of COHb was prepare before determine COHb concentration which used ln (natural logarithm) of A/B ratio ($A=A_{418} - A_{409}$; $B = A_{432} - A_{441}$; nm A_{418}, A_{409}, A_{432}, A_{441} are absorbance at 418, 409, 432 and 441 nm) 4 times each concentration (0, 2, 4, 6 and 10%), the average each concentration of COHb were plotted in COHb standard curve. Then placed sample in

spectrophotometer for determine second-derivative spectrum. Calculated ln A/B to compare standard curve and calculate the COHb in blood sample.

Procedure

1. Prepared standard curve of COHb
   1.1 Added 10 ml of reducing agent into test tube size 15 x 100 mm. 6 tubes.
   1.2 Added standard COHb 10 µl at 0, 1, 2, 4, 6, 8 and 10% COHb in test tube.
   1.3 Standard COHb are scanned second - derivative spectrum with spectrophotometer within 2 - 3 minutes between wavelength 390-450 nm.
   1.4 Found ln (natural logerithm) of ratio A/B ($A = A_{418} - A_{409}$; $B = A_{432} - A_{441}$ nm. by A_{418} $A_{409,}$ A_{432} and A_{441} are absorbance at wavelength 418,409, 432 and 441 respectively).
   1.5 Used average value at the other of concentration of COHb to plot standard curve.
2. Measured COHb level in blood
   2.1 By the same token 1. Used blood sample instead of standard COHb
   2.2 When get ln A/B bring to read COHb from standard curve.

2.4.4 MDA (Santos, *et al.*, 1980)

Principle

The Thiobarbituric acid (TBA) test is used for measuring the peroxidation of fatty acid. Aldehydes are produced as lipid peroxide products of metabolism in biological system. Malondialdehyde (MDA) is the intermediate substance of lipid peroxidation which is in many instances the most abundant aldehyde and interact with thiobarbituric acid (TBA) giving the pink colored product as the following pathway;

$$TBA + MDA \xrightarrow{\text{Heat}} \text{TBA-MDA adduct} + 2H_2O$$

The TBA-MDA adduct product can be detected by measuring with spectrophotometer at 532 nm.

Reagent preparation

- Trichloroacetic acid (TCA) reagent

Mixed 100 g of TCA in 0.6 M HCl 10 ml and bring to volume with distilled water in a 100 ml volumatric flask.

- Thiobarbituric (TBA) reagent

Mixed 17.298 g of TBA (Eastman, MW 144.5) in 100 ml of 0.26 M 2-amino-2-Hydroxymethyl-1,3-propanediol (Tris, MW 121.1) and bring to 1,000 ml with distilled water in a 1,000 ml volumatric flask.

- Normal saline solution

Placed 0.85 g of sodium chloride in 100 ml volumetric flask and bring to volume with distilled water.

- Stock MDA standard (100 μM)

Stock MDA standard (100 μM) was prepared by prepare stock MDA 10 mM by mixture 16.89 μl of standard Tetramethoxypropane (TMP) (MW 164.2) with 5-8 drops of concentrated HCl, and adjust volume to 10 ml with distilled water. And then preparing 1,000 μM working standard MDA by pipette 1,000 μl of stock MDA and adjusts volume to 10 ml with distilled water. The standard MDA, in the other concentration are 10, 20, 30, 40 and 50 μM, were prepared for calibration curve.

Procedure

Sample preparation and incubation

1. Added 0.45 ml of 0.85% NSS to 0.1 ml of serum.
2. Added 0.2 ml of TBA reagent and then added 1.0 ml of TCA reagent and mix.
3. Incubation in 95-100 °C water for 30 minutes in the water bath.
4. Cool the tubes on cold water and added 3 ml of distilled water.
5. Centrifuged at 3,000 rpm for 10 minutes.
6. Transferred the supernatant to a cuvette and read OD at 532 nm.

2.4.5 Glutathione (Beutler, *et al.*, 1963; Ernest, *et al.*, 1963)

Principle

A sample photometric procedure was using 5,5′-dithiobis- (2-nitrobenzoic acid) (DTNB). Only three stable reagents, including precipitating solution, phosphate reagent and DTNB reagent, are required for the reaction postulated to be as follows:

GSH + DTNB ⟶ GSTNB + 2-nitro-, 5-thiobenzoic acid (TNB)

GSH + GSTNB ⟶ GSSG + 2-nitro-, 5-thiobenzoic acid (TNB)

The first reaction product in the equation is yellow color and can be measured at 412 nm.

Reagent preparation

- Precipitating solution

  Placed 1.0 g of glacial metaphosphoric acid, 30.0 g of sodium chloride and 0.2 g disodium ethylenediaminetetraacetic acid dihydrate (EDTA) in 100 ml volumetric flask and adjust volume with distilled water.

- Phosphate solution

  Placed 34.23 g of di-potassium hydrogenphosphate trihydrate (MW 228.23) in 500 ml volumetric flask and adjust volume with distilled water.

- 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent

  Placed 1.0 g of sodium citrate in 50 ml volumetric flask and adjust volume with distilled water. Place 0.49 g of 5,5-dithiobis- (2-nitrobenzoic acid) in 100 ml volumetric flask and dissolve with sodium citrate solution and adjust volume with distilled water.

- Glutathione standard 100 μg/ml

  Placed 0.001 g of glutathione in a 10 ml volumetric flask and bring to volume with distilled water.

Procedure

1. Pipetted 0.4 ml of whole blood into test tube and added 1.6 ml of distilled water, mixed to hemolyze.

2. Added 3.0 ml of precipitating solution and mix.
3. Allowed to stand 5 minutes at room temperature or centrifuged at 2,000 g for 10 minutes and then filtered through coarse-grade filter paper Whatman No.1.
4. Duplicate 1 ml of supernatant from filtration and added phosphate buffer 4 ml.
5. Vortex after adding 0.5 ml of DTNB reagent and read absorbance at 412 nm within 5 minutes.
6. Calculation the GSH in blood.

$$\frac{\text{(Optical density of the test)} \times \text{(Standard GSH concentration)} \times 1.25 \text{ (Dilution factor)}}{\text{Optical density of the standard}} = \text{mg/dl of whole blood}$$

2.4.6 Cytochrome *c* reduction (Bagchi, *et al.*, 2002)

Principle

The rate constant of the reaction of $O_2^{\bullet-}$ with ferricytochrome *c* (cyt(III)) depends on the pH and ionic strength of the solution. In the present of 0.1 mM EDTA and 50 mM phosphate buffer (pH 7.8), $k = (2.6 \pm 0.1) \times 10^5\ M^{-1}\ sec^{-1}$. The formation of ferrocytochrome *c* (cyt(III)) is followed spectrophotometrically at 550 nm. ($\epsilon = 2.1 \times 10^4\ M^{-1}cm^{-1}$)

Reagent preparation

- Tris (hydroxymethyl) aminomethane solution (Trizma® base)

  Placed 6.0874 g of Trizma® base (MW 121.1) in a 100 ml volumetric flask and adjust pH with 6 M HCl to pH 7.4 and adjust volume with distilled water.

- Potassium chloride solution (KCl)

  Placed 11.184 g of KCl in a 100 ml volumetric flask and adjust volume with distilled water.

- Disodium ethylenediaminetetraacetic acid dihydrate (EDTA) solution

  Placed 0.37224 g of disodiumethylenediaminetetraacetic acid dihydrate (EDTA) in a 100 ml volumetric flask and adjust volume with distilled water.

- Tris-KCl buffer (pH 7.4)

Mixed 50 ml Tris (hydroxymethyl) aminomethane solution, 50 ml potassium chloride solution and 50 ml disodium ethylenediaminetetraacetic acid dihydrate (EDTA) solution in a 500 ml volumetric flask. Place 200 ml distilled water, 50 ml glycerol and 0.5 ml dithiothreitol solution into volumetric flask and adjust volume with distilled water.

- Cytochrome *c* solution

Placed 0.0645 g of cytochrome c (VI) in a 10 ml volumetric flask and adjust volume with distilled water.

Procedure

1. The lung and brain were homogenized in 5 ml buffer per gram using tissue homogenizer.
2. Centrifuged the fraction from 1. at 15,000 g for 30 minutes.
3. 1.0 ml reaction mixture contain 1mg protein and 100 µl cytochrome *c* , mixed
4. Incubated at 37°C 15 minutes.
5. Stop reaction by placing in ice.
6. Centrifuged at 3,000 g for 10 minutes.
7. The supernatant was measured at the absorbance at 550 nm.
8. Absorbance values were converted to nmoles of cytochrome *c* reduced per 15 min/mg protein, using the extinction coefficient $2.1\times 10^{4} M^{-1}cm^{-1}$

2.4.7 Pathology

For determine abnormalities of internal organ, after blood collected internal organ were washed with normal saline for remove excessive blood. The internal organs were examined grossly and then dissected one by one for weighing as wet weight. The organs of interest were brain, lungs, heart, liver and kidneys. These organs were dissected and fixed in 10% neutral-buffered formalin. The representative sections were selected and processed routinely. Tissue samples were embedded as paraffin blocks and cut at 4 micron in thickness. Tissue sections were stained with hematoxylin-eosin and examined microscopically.

2.5 Statistic analysis

The MDA, GSH, COHb, cytochrome *c* reduction, CBC and blood chemistry between control group and experiment group were used Mann-Whitney U test to compare difference of result. The pathology between control group and experiment group were used descriptive statistic to compare difference of result. The level of statistical significance employed in all results was $P < 0.05$.