CHAPTER III

MATERIALS AND METHODS

A. Materials and Equipments

The following equipments were used to measure physical performance and to analyze blood samples.

1. Physical performance test

   1.1 Electrically braked bicycle ergometer (Corival 400)

   1.2 Heart rate monitor (Polar Sport Tester; Polar Electro Oy FIN-90440, Finland)

   1.3 Oxygen and carbon dioxide gas analyzer (Quinton metabolic cart ,QMC ,USA)

   1.4 A noninvasive blood pressure monitor (Quinton instrumen CO, model 412)

   1.5 A Weighting scale (Yamato DP- 6100 GP, Japan)

   1.6 A scale for height

2. Blood analysis

   2.1 Spectrophotometer (Spectronic 601 Milton Roy)

   2.2 An automated analyzer (COBAS MIRA S, F. Hoffman La Roche Ltd. Co Diagnostica, Basel, Switzerlnd)

   2.3 Dynac II centrifuge (Clay Adams , Division of Becton, Dickinson and Company)

   2.4 A vortex mixer

3. Reagents

   3.1 NaH_2PO_4 (E.Merck., Darmstadt, Germany)

   3.2 $Na_2HPO_4 .2H_2O$ (E.Merck., Darmstadt, Germany)

   3.3 Heparin Leo 5,000 i.u/u.i./ml 5ml(25,000 i.u./u.i.) (Leo pharmaceutical products Ballerup Denmark)

3.4 Ethylenediamine tetraacetic acid dipotassium salt dihydrate. (Fluka AG, Chemische Fabrik CH- 9470 Buchs.)

3.5 HCl (E.Merck., Darmstadt, Germany)

3.6 Chloroform (APS Finechem., Australia)

3.7 Cyclohexane (APS Finechem., Australia)

3.8 Methanol (J.T. Baker. USA)

3.9 NaCl (E.Merck., Darmstadt, Germany)

3.10 Tri- sodium citrate. (BDH., BDH Limited Poole, England)

3.11 Control Precinorm L. (Roche Diagnostics. Gmbh, D- 68298 Mannheim, Germany. Lot 153 891 –02)

B. Methods

1. Subjects

The study group comprised of 62 sedentary volunteers (26 men and 36 women).The subjects were recruited through announcement and advertisement at

- department of physiology , Faculty of Medicine, Chulalongkorn University.
- King Chulaongkorn Memorial Hospital,The Thai Red Cross Society.
- department of Physical therapy ,Faculty of Allied Health Sciences,
- Krung Thai Bank Public Company Limited (Head Office).

The subjects had to fulfill the following criteria:

1.1 Inclusion criteria

1.1.1 All of them are healthy and no regular medication, age 20-50 yr.

1.1.2 Sedentary life-style

1.1.3 Frequency of regular conditioning exercise for two times per week or less

1.1.4 Body mass index is between 20-25

1.1.5 No psychological disorder

1.1.6 Written informed consent

1.2 Exclusion criteria

1.2.1 Exerciser

1.2.2 Have the disease and history of regular medication

1.2.3 Hypertension

1.2.4 Regularly alcoholic drinking

1.2.5 Weakness or illness on the test day

1.3 Entry to the study

The study protocol will be reviewed and permitted by the local ethics committee Faculty of Medicine, Chulalongkorn University. The project was clearly explained to the subjects and written consent was obtained from each subject prior to experiment

2. Study Designs

The protocol was divided into 2 sections

Section I Every subjects was tested for estimation of peak oxygen uptake (VO_{2peak}) and then they continuously performed test after at least 2 days

Section II The protocol was investigated the effect of moderate exercise on lipoprotein dine conjugation which represented the marker of oxidative stress.

Each subject was requested to fill a questionnaire concerning his personal and educational background, medical history, health status and inform consent prior to the experiment.

Experiment protocol

First visit at the lab for VO_2 peak assessment baseline.

DO not perform exercise for at least 2

Second visit on the day of exercise

Blood sample collection at pre exercise for LDL- DC baseline

Ergometric cycling at 50% VO_{2peak} for 30 min with monitoring vital sign record

Blood sample collection immediately when exercise was complete.

Blood sample collection at 2hr post exercise

Section I Test for (VO_{2max}) (McArdle et al 2000)

VO_{2max} represents a fundamental measure in exercise physiology and serves as a standard to compare performance estimates of aerobic capacity and endurance fitness. This tasks activate muscle groups with sufficient intensity and duration to engage maximal aerobic energy transfer. However, this project adopted the term " peak oxygen uptake" (VO_{2peak}) described the highest oxygen uptake value during the test due to the untrained subjects cannot be able to do this task. Then in VO_{2peak} this project was defined and described following criteria (Wasserman et al., 1994; Poole and Richardson, 1997):

1) The increase of VO_2 was less than 2 ml/kg/min in the last 2 minutes (for constant VO_2 despite increment increase)
2) Maximum heart rate(HR_{max}) near theoretical $HR_{max} \pm 10$ beats ($HR_{max} = 220 - age$)
3) Respiratory exchange ratio exceeded 1.10

Procedure

1) Subjects were prepared and they were detected by heart rate polar on chest wall of the subject for monitoring subject's heart rate during exercise and he was clearly undersupervise throughout experiment.
2) The Quinton metabolic cart machine was set up. (Figure 3.1)
3) Subject was allowed to sit on saddle of the cycle ergometer with a suitable position.
4) Subject was performed rhythmical pedaling at 50 rpm.
5) Work load of bicycle ergometer is regulated by Q4500 machine used ordiramp test protocol. It attributed free load for 3 min and then increased work load for every 25 watt both men and women untill they weren't able to perform exercise (Figure3.2).

6) We also correctly detected the heart rate during exercise by manual method.

7) The subject was cheered up to do the best he can and he can give up every process when he felt tired.

After completion of section I, we recorded and confirmed the completeness of the data.

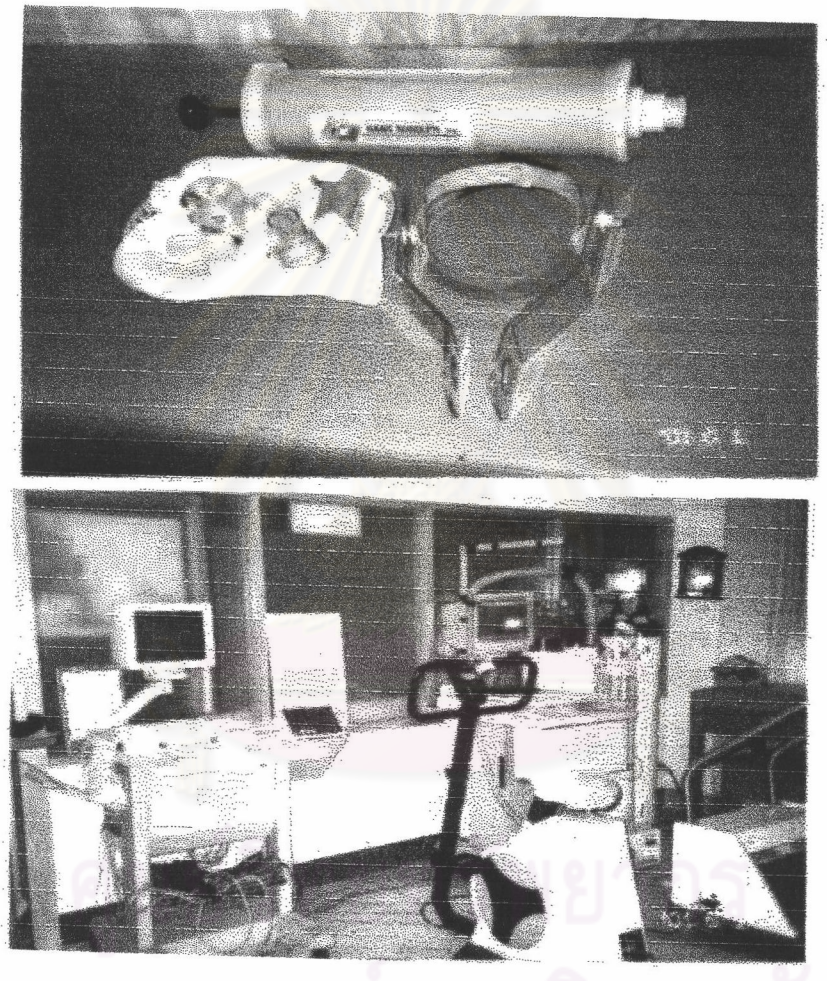

Figure. 3.1 Set up the Quinton Metabolic Cart.

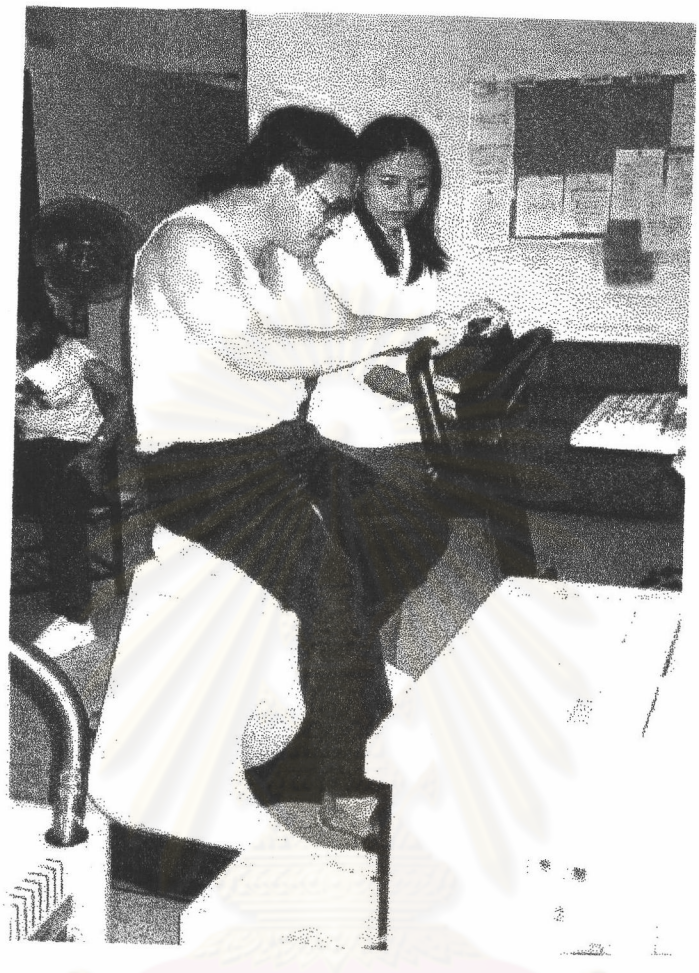

Figure. 3.2 Preparing the subjects

Section II Acute moderate exercise protocol

Each subject was asked not to perform any kind of physical exercise at least 2 day before the day of the experiment. The same breakfast for each subject was prepared on the day of the experiment.

At the pre-exercise period, the first blood sample was collected from antecubital vein and kept at room temperature prevented from UV. Each subject performed exercise by 50% VO_{2peak} work rate individually, for 30 min and his or her heart rate was detected by manual method. After he cooled down for 5 min, the second blood sample was immediately collected . Finally, the volunteer was asked to do nothing and rested for 2 hour before the last blood sample collection (Figure 3.3)

Figure 3.3. Collecting blood sample

C. Blood analysis

Whole blood were analyzed at the Endrocrinology laboratory, King Chlualongkorn Memorial hospital. We measured baseline LDL diene conjugation (LDL-DC) by a method recently validated and reported in detail.(Ahotupa et al 1996, Wieland H and Seidel D 1983, Vasankari TJ. Et l ,1998) As the test material, we used both fresh and frozen human serum which showed no differences.

Procedure

I. **Blood collection**

10mL Vacutainer tubes of blood samples

↓

allowed to stand for 30 min at room temperature

(protected from UV light)

↓

Centrifugation at 3,000 g. for 15 min

↓

The serum stored at –70'C untill assay

II. **Precipitation of Low-density lipoproteins**

Serum samples 1 mL with 1 mg/mL of EDTA

(at room temperature)

↓

Mixing with a Vortex mixer

↓

Added 7 mL. of heparin-citrate buffer*

↓

Mixing with a Vortex mixer for 10 second

↓

The suspension was allowed to stand for 10 min

(at room temperature)

↓

Centifuged 1,000g for 10 min

↓

The insoluble lipoproteins were sedimented

↓

The pellet was resuspended in 1 mL of 0.1 M Na-phosphate buffer**

(pH 7.4) and mixed

III. **Measurement of LDL Oxidation products(LDL-DC)**

Lipid were extracted from LDL

Pipette the sample(from precipitation method) 100 uL

↓

Added chloroform-methanol (2:1 Volume :Volume) 2 mL

↓

Mixing with a vortex mixer for 10 second

↓

Pipette the soluble which was extracted (Lipid soluble)

↓

Dried under nitrogen

↓

redissolved in cyclohane 1 mL

↓

measured the absorbance at 234 nm

↓

Absorbance units were converted to molar units using

the molar extinction coefficient of $2.95 \times 10^4 M^{-1} cm^{-1}$

↓

The actual level of oxidized LDL in circulation was

expressed as μmol/L

*0.064 M tri-sodium citrate +50,000 IU/L heparin, pH 5.05 with 5 NHCL

** 0.1 M Sodium phosphate +0.9% NaCl, pH 7.4

Measurement of other lipid profiles

Blood was analyzed for the rest of lipid profile by the laboratory of endrocrine at King Chulalongkorn Memorial Hospital.

D. Statistical analysis

All data were presented as mean and standard deviation. For comparison among groups, test for normality and Kruskal Wallis test was used. P-values < 0.05 were considered as significant (Appendix D).

E. Precision of the method

Control Precenorm L was used as a pool of serum for LDL precipitation average % recovery of LDL is 75-80%. LDL-DC was detectable in LDL precipitation from Precinorm L.

The coefficient of variation (CV) for within- assay precision was 5.17 % and between assay precision was 5.25 % (Appendix C).