CHAPTER III

RESEARCH METHODOLOGY

3.1 Research Design

The design of this study can be classified as an experimental study. The study protocol was approved by the local ethics committee, Faculty of Medicine, Chulalongkorn University. Before entering the program, all subjects gave written informed consent to participate in the study.

3.2 Population and sample

3.2.1 Population

Target Population

The target population was defined as all coronary artery disease patients.

Sample Population

Sample population included patients with coronary artery disease who were treated at King Chulalongkorn Memorial Hospital and Thammasat University Hospital.

This study comprised of two groups of patients with coronary artery disease who were enrolled in a randomized trial, namely exercise group (n= 18, age 58 (7.0) years), and control group (n= 15, age 58 (8.5) years).

3.2.2 Eligibility Criteria

Inclusion criteria

1. All participants were coronary artery disease patients, who were diagnosed according to criteria of American Heart Association (American College of Cardiology 2000) by cardiologist.
2. All patients have New York Heart Association (NYHA) functional classification class I and II, with lower, moderate risk of American

Association for Cardiovascular and Pulmonary Rehabilitation (AACVPR) risk stratification model.

3. Patients had normal level of lipid profile (with or without lipid lowering drug) and no change of drug dosage was made during participation in this program.
4. If the patients were using ACE-I agent, there were no dosage changes during the study.
5. All patients did not have any hematological problems.
6. They were male at age of 40-70 years.
7. No one had any limitation in participating in a cardiac rehabilitation program.
8. The patients were volunteer to participate in this study.

Exclusion criteria

1. Patients who had history of acute myocardial infarction within the preceding 6 months
2. Patients who had diabetes mellitus, smoking, hemostasis disorders, and receiving anticoagulation drugs.
3. Patients who were increasing severity of CAD disease during the train program.
4. Patients who cannot be trained up to 80% of the program.
5. Patients who had a history of recent infection / inflammation within 2 weeks before phlebotomies.

3.3 Equipments and Materials

3.3.1 Physiological study equipments

The following equipments were used for determination of physical fitness characteristics, which included body height, weight, body mass index,

systolic and diastolic blood pressure, heart rate, and peak oxygen consumption.

- A weight scale (Yamato, DP-6100GP, Japan)
- A wall-mounted height measuring board
- Sphymomanometer (Baumanometer ®, Stand by and Wall Unit 33, USA)
- Stethoscope (3M ™ Littmann ™, Classic II S.E., USA)
- Cardiotachometer (Polar Accurex Plus, Polar electro, Finland)

Oxygen and carbon dioxide gas analyzer (Quinton Metabolic Cart, QMC, USA)

- Cardiac stress testing equipment (Quinton instrument CO, Q4500, USA)
- ECG monitor (SpaceLabs, USA)

3.3.2 Materials for Fibrinolytic testing

- Reagent kit for t-PA activity (COASET® t-PA, CHROMOGENIX, Italy)
- Reagent kit for t-PA antigen (COALIZA® t-PA, CHROMOGENIX, Italy)
- Reagent kit for PAI-1 activity (COATEST® PAI-1, CHROMOGENIX, Italy)
- Reagent kit for PAI-1 antigen (COALIZA® PAI-1, CHROMOGENIX, Italy)

3.4 Measurement

3.4.1 Anthropometric measurement

Body weight and height of subjects were measured by using a weight balance scale (Yamato, DP6100GP, Japan), and a wall mounted height-measuring board.

Body Mass Index was calculated according to the equation:

BMI = Body weight (kg.)/ Height (m^2)

3.4.2 Measurement of Peak Oxygen Consumption (VO_{2peak})

The peak oxygen consumption of each subject was measured during the incremental exercise test using oxygen and carbon dioxide gas analyzer (Quinton Metabolic Cart, QMC, USA). Oxygen consumption (VO_2), Carbon dioxide production (VCO_2), minute ventilation (VE), and other derived parameters were continuously monitored breath – by - breath using a computerized system (Quinton Metabolic Cart, QMC, USA). Data on VO_2 and VCO_2 were expressed in a standard condition of standard temperature pressure dry (STPD) and VE in the condition of body temperature pressure saturated with water vapor (BTPS). The Naughton protocol for treadmill was used in this exercise test.

Criteria of obtaining VO_{2peak}

1) Target heart rate were calculated as individuals heart rate reserve

$$\text{Target HR} = ([HR_{max} - HR_{rest}] \times \%\text{target heart rate}) + HR_{rest}$$

2) Sign of exertion intolerance (fatigue) as chest pain and an inability to testing

Training intensity was assigned as 50 and 65%VO_{2peak}. Using this training intensity to calculate the equivalent METs in Naughton protocol. The calculated METs were used to determine grades and speeds of treadmill during the training session according to Naughton protocol shown in table 3.1.

3.4.3 Training Protocol

Intensity of exercise training used in this study was 50%VO_{2peak} in the first week and 65%VO_{2peak} in the following weeks. During training, we monitored heart rate, recorded blood pressure before and after training session, evaluated RPE. The training was 8 weeks long, 4 days per week, 30 min per session with 10 min warm up and 10 min. cool down.

Table 3.1 Naughton protocol for treadmill training

Stage	Time (min)	Speed (mph)	Grade (%)	METs
Rest	00.00	1.0	0.0	1.8
1	02.00	1.0	0.0	1.8
2	02.00	2.0	0.0	2.5
3	02.00	2.0	3.5	3.5
4	02.00	2.0	7.0	4.4
5	02.00	2.0	10.5	5.4
6	02.00	2.0	14.0	6.4
7	02.00	2.0	17.5	7.3
8	02.00	2.0	21.0	8.3
9	02.00	2.0	24.5	9.2
10	02.00	2.0	25.0	9.4

3.4.4 Measurement of Fibrinolysis.

3.4.4.1 Blood Sampling and Preparation

To avoid the diurnal variation in coagulation and fibrinolytic variables, all blood samples were collected between 7:30 AM and 10:30 AM after a 12-hour overnight fast (De Sousa et al. 1998). All phlebotomies were performed with minimal venostasis. The first 4-5 ml of blood was used for study of lipid profile. Blood for determination of t-PA antigen, t-PA activity, PAI-1 antigen, PAI-1 activity, was collected in sodium citrate tubes.

3.4.4.1.1 Blood sampling for t-PA activity

It is extremely important to follow the acidification and centrifugation steps exactly as described below in order to minimize the in vitro inhibition of t-PA by PAI-1. Blood (9 volumes) was mixed with 0.1 mol/l sodium citrate (1

volume). One ml of this mixture was immediately acidified with 1 ml (1:1) acetate buffer working solution (2-8°C) and centrifuged within 2 min at 3000 rpm for 20 minutes. Separated the plasma from the cells as soon as possible and then added 10 µl of 1 mol/l HCl to each 150 µl of acetate treated plasma. Then freezed the samples at – 80 °C.

3.4.4.1.2 Blood sampling for other fibrinolytic parameters such as t-PA antigen, PAI-1 activity, PAI-1 antigen was prepared as follows.

Blood (9 volumes) was mixed with 0.1 mol/l sodium citrate (1 volume) and centrifuged at 3000 rpm for 20 minutes. Separated the plasma from the cells. The samples were kept at – 80 °C until used.

3.4.4.2 t-PA antigen assay

The reagent kit of t-PA antigen assay (COALIZA® t-PA) is the product of Chromogenix (Italy).

Principle:

The Coaliza t-PA is a solid phase enzyme immunoassay for the detection of t-PA in biological fluids. Microplate wells coated with anti-t-PA monoclonal antibodies were incubated with samples or t-PA standard solution (1, 2.5, 5, 10, 15 and 20 ng/ml respectively). During this incubation, t-PA presented in the sample or standard solution was bound to the solid phase. Unbound substance was then removed by washing the well. Next, an enzyme-labeled anti t-PA monoclonal antibody was added and antibody-antigen complexes formed. Unconjugated enzyme was then removed by washing the wells. Finally, enzyme substrate was added. The action of the bound enzyme on the substrate produced a blue colour which turned yellow after stopping the reaction with acid.

Procedure:

Add 200 µl of sample diluent to one test well reserved as blank, add 150 µl of sample diluent to each test well reserved for specimen and standard. Add 50 µl of the appropriate specimen and standard to each well, then incubated at 37 ^{o}C for 60 minutes. After incubation washed each well 3 times with 300 µl of phosphate washing buffered solution and added 200 µl of conjugate solution, then incubated at 37 ^{o}C for 60 minutes. After incubation washed each well 4 times with 300 µl of phosphate washing buffered solutions and added 200 µl of substrate solution, then incubated at 20-25 ^{o}C for 30 minutes. Stop the reaction by adding 50 µl acid 1.5 mol/l sulphic to each well. The absorbance of each well was recorded at 450 nm. by microplate reader within 15 min after stop reaction.

PAI-1 antigen assay

The reagent kit of PAI-1 antigen assay (COALIZA® PAI-1) is the product of Chromogenix (Italy).

Principle:

The Coaliza PAI-1 is a solid phase enzyme immunoassay for the detection of PAI-1 in biological fluids. Microplate wells coated with anti-PAI-1 monoclonal antibodies were incubated with samples or PAI-1 standard solution (5, 10, 25, 50, 75 and 100 ng/ml respectively). During this incubation, PAI-1 presented in the sample or standard solution was bound to the solid phase. Unbound substances were then removed by washing the well. Next, an enzyme-labeled anti PAI-1 monoclonal antibody-antigen complexes formed in previous step. Unconjugate was then removed by washing the wells. Finally, enzyme substrate was added. The action of the bound enzyme on the substrate produced a blue colour which turned yellow after stopping the reaction with acid.

Procedure:

Add 220 µl of sample diluent to one test well reserved as blank, add 200 µl of sample diluent to each test well reserved for specimen and standard. Add 20 µl of the appropriate specimen and standard to each well, and then incubate at 37 ^{o}C for 60 minutes. After incubation washed each well 4 times with 300 µl of phosphate washing buffered solution and added 200 µl of conjugate solution, then incubated at 37 ^{o}C for 60 minutes. After incubation washed each well 4 times with 300 µl of phosphate washing buffered solution and added 200 µl of substrate solution, then incubated at 20-25 ^{o}C for 30 minutes. Stop the reaction by adding 50 µl 1.5 mol/L sulphic acid to each well. The absorbance of each well was recorded at 450 nm. by microplate reader within 15 min after stop reaction.

t-PA activity assay

The reagent kit of t-PA activity assay (COASET® t-PA) is the product of Chromogenix (Italy).

Principle:

Plasminogen is activated to plasmin by t-PA. The activation rate is makedly increased in the presence of the t-PA stimulator. The amount of t-PA is determined by measuring the amidolytic activity of plasmin on the chromogenic substrate S-2251. The release of p-nitroaniline (pna) is determined at 405 nm. The correlation between the change in absorbance and the concentration of t-PA is linear within 0.25 – 10.0 IU/ml plasma.

Procedure:

I. Standards

1) 10 IU/ ml

One hundred microliter of t-PA/PAI depleted plasma, 200 µl acetate buffer working solution, 20 µl 1 mol/l HCl were added into the test tube, then mixed and incubated at 20-25 °C for 10 minutes. After incubation, 20 µl 50 IU/ml t-PA were added then mixed well. One hundred microliters of the mixture were transfer to 3.5 ml sterile water and mixed well.

2) 0 IU/ml

One hundred microliter of t-PA/PAI depleted plasma, 200 µl acetate buffer working solution, 20 µl 1 mol/l HCl were added into the test tube, then mixed and incubated at 20-25 °C for 10 minutes. After incubation 20 µl Tris buffer working solution were added then mixed well. Two hundred microliters of the mixture were transferred to 7.0 ml sterile water and mixed well.

Standard solutions at various concentrations were prepared as follows Table 3.2.

Table 3.2 Standard solutions of concentrations t-PA activity

High range 1.5-10 IU/ml			Low range 0.25-2.5 IU/ml		
t-PA IU/ml	standard 0 IU/ml	standard 10 IU/ml	t-PA IU/ml	standard 0 IU/ml	standard 10 IU/ml
1.5	850 µl	150 µl	0.25	975 µl	25 µl
2.5	750	250	0.5	950	50
5.0	500	500	1.0	900	100
7.5	250	750	1.5	850	150
10	-	1000	2.5	750	250

II. Samples

The acidified plasma was thaw rapidly at 37°C and kept on ice.

III. Assay

A fresh mixture of 1 volume of plasminogen, 1 volume of S-2251, 3 volumes of Tris buffer working solution were made prior to the assay. The solution was stable for 1 hour at 2-8 °C.

1) Sample activity

One hundred miriliters of diluted test plasma or standard (2-8 °C), 100 µl of Plasminogen/ S-2251/ Tris buffer (2-8 °C) , 50 µl of t-PA stimulator working solution (20-25°C) were added into each well of microplate, then mixed and incubated at 37°C for 2 hours 15 min.The absorbance of each well were recorded at 405 nm.

2) Blank activity

One hundred microliters of diluted test plasma or standard (2-8 °C) , 100 µl of Plasminogen/ S-2251/ Tris buffer (2-8 °C) , 50 µl Tris buffer working solution of (20-25°C) were added into each well of microplate, then mixed and incubated at 37°C for 2 hours 15 min. The absorbances of each well were recorded at 405 nm.

PAI-1 activity assay

The reagent kit of PAI-1 activity assay (COASET® PAI) is the product of Chromogenix (Italy).

Principle:

A fixed amount of t-PA is added in excess to undiluted plasma where part of it rapidly forms an inactive complex with the fast inhibitor PAI-1. Plasminogen is then activated to plasmin by the residual t-PA in the presence

of a stimulator. The amount of plasmin formed is directly proportional to the residual t-PA activity and hence inversely proportional to the PAI-1 activity in the sample. The amount of plasmin is determined by measuring the amidolytic activity of plasmin on the chromogenic substrate S-2403. The release of p-nitroaniline (pna) is determined at 405 nm.

Procedure:

I. Standards

1) 0 AU/ml: Twenty-five microliters of 40 IU/ml t-PA were added to test tube. Then 25 µl PAI depleted plasma, 4.00 ml of sterile water were added to each tube then mixed well and kept at 2-8 °C.
2) 40 AU/ml: Twenty-five microliters of Tris buffer working solution were added to test tube. Then 25 µl PAI depleted plasma, 4.00 ml of sterile water were added to each tube then mixed well and keep at 2-8 °C.

Different standard concentrations are obtained by mixing these two standards according to the following Table 3.3.

Table 3.3 Standard concentrations of PAI-1 activity

PAI-1 level in plasma AU/ml	40 AU/ml PAI-1 µl	0 AU/ml PAI-1 µl
40	1000	-
30	750	250
20	500	500
10	250	750
0	-	1000

II. Samples

Twenty five microliters of 40 IU/ml t-PA (20-25 °C), 25 µl of sample (20-25 °C) were added to test then mixed and incubated for exactly 10 minutes at 20-25 °C. Four ml sterile water were added then mixed well and kept at 2-8 °C.

III. Assay

1) Assay mixture: mix 1 volume of Plasminogen, 1 volume of S-2403, and 3 volumes of Tris working solution kept on ice at 2-8 °C.
2) One hundred microliters of incubated and diluted test plasma or standard (2-8 °C), 100 µl of assay mixture (2-8 °C), 50 µl stimulator working solution (20-25 °C) were added into each well of microplate then mixed and incubated at 37°C for 50 minute.
3) The absorbance of each well was recorded at 405 nm.

3.4.5 Measurement of lipid profile

Lipid profiles, including total cholesterol, triglycerides, high-density lipoprotein cholesterol and low-density lipoprotein cholesterol were determined using automate analyzer (Integra 400 plus, Roche Diagnostics GmbH, Mannheim, Germany), which analyzed by the special laboratory at King Chulalongkorn Memorial Hospital. Total cholesterol level was measured by Enzymatic, colorimetric method (CHOD/PAP) with cholesterol esterase, cholesterol oxidase, and 4-aminoantipyrine. Triglyceride level was measured by enzymatic, colorimetric method (GPO/PAP) with glycerol phosphate oxidase and 4-aminophenazone. High-density lipoprotein cholesterol and low-density lipoprotein cholesterol levels were determined by homogeneous enzymatic colorimetric assay.

3.5 Data Collection

The cardiac rehabilitation program was eight weeks long. In order to study the effect of exercise at light to moderate intensity (60 - 65% $VO_{2\ peak}$) on the fibrinolysis system compare with untrained group, the data collection schedule was showed in the following diagram.

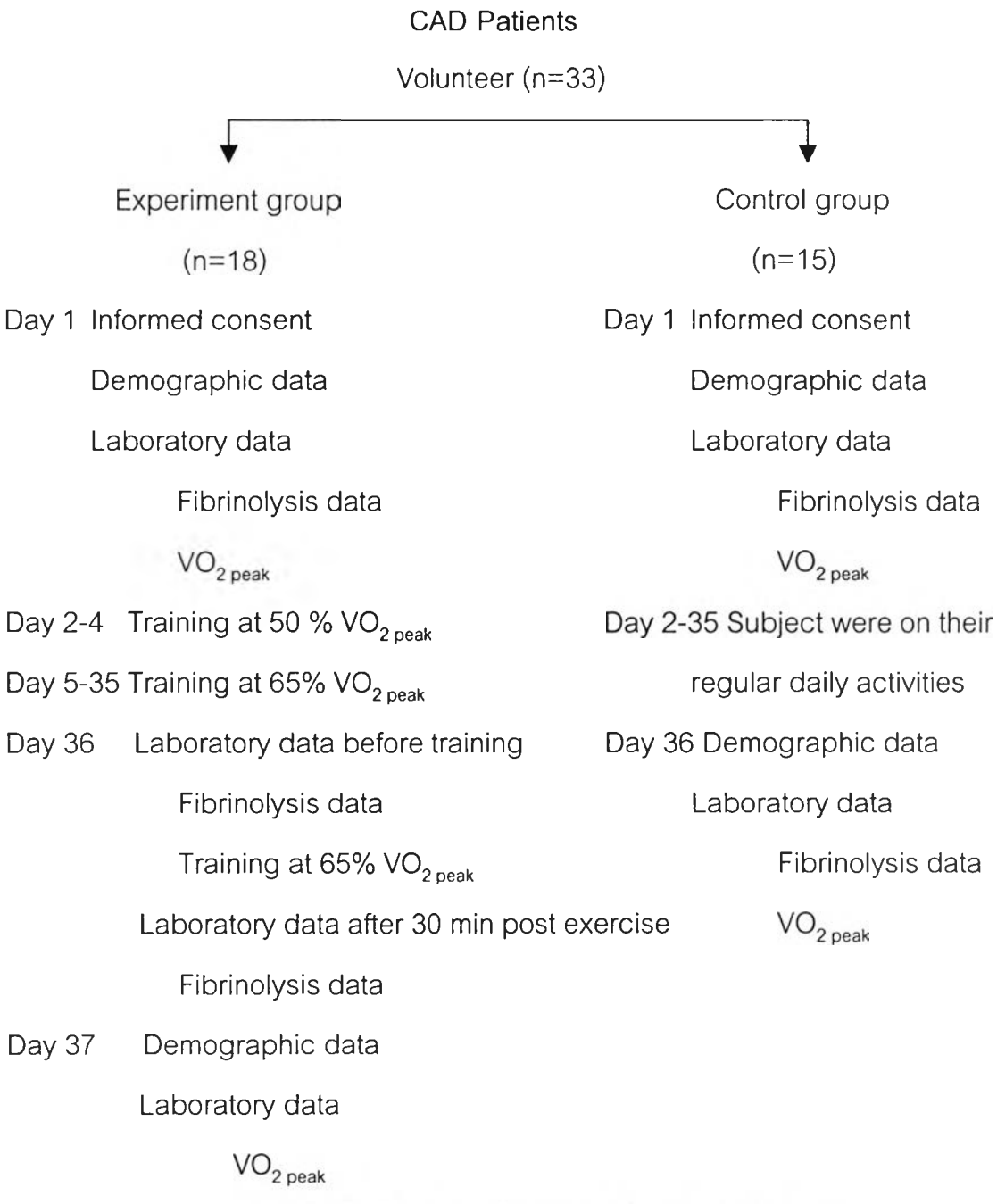

Figure 3.1 Diagram of data collection schedule.

3.7 Data Analysis

Average values and standard deviations were computed. The distribution of data was test either it was normal distribution or not. Normal distribution used paired t-test for comparing values after exercise with baseline values, unpaired t-test for comparing between control and experimental group. If data are non-normally distributed, nonparametric analysis was used, Wilcoxon Signed-Ranks test for comparing values after exercise with baseline values within group and Mann-Whitney test for comparing between control and experimental group.

All data were analyzed using the Statistical Package for the Social Science (SPSS). Differences at significance level of $p<0.05$ were considered to be significant.