CHAPTER II

RESEARCH DESIGNS AND METHODS

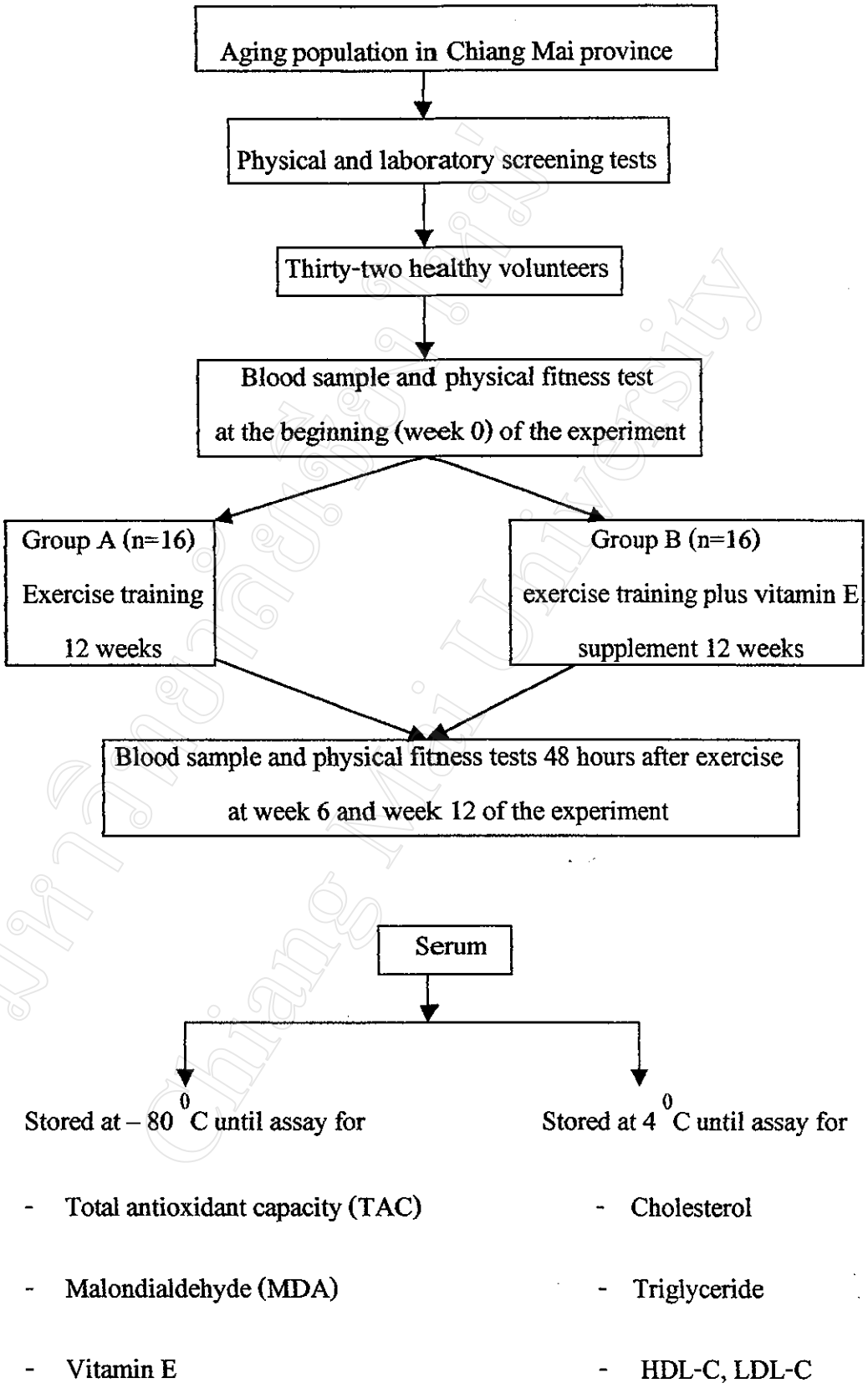
I RESEARCH DESIGNS

The elderly volunteers in this study lived in Chiang Mai province, aged between 60–75 years. The physical examination, blood chemistry, complete blood count, urinary analysis, chest radiograph, electrocardiogram (EKG) and exercise stress test (EST) were examined. The experimental protocols were clearly explained to thirty-two subjects who had passed the initial screening test from fifty-three volunteers and had given their written form of consents were noted prior the experiments. Blood samples were taken for blood chemistry tests and physical fitness tests were done at the beginning of the experiment (week 0). Then, they were divided into two groups as follows:

Group A: given exercise training for 12 weeks

Group B: given exercise training for 12 weeks plus vitamin E supplement of 800 international unit (IU) per day

At the end of the 6^{th} week and 12^{th} week of the experiment, blood samples and physical fitness tests were repeated. The serum samples from group A and group B subjects were kept at $-80^{\circ}C$ before they were analyzed for total antioxidant (TAC), malondialdehyde (MDA), and vitamin E levels and were kept at $4^{\circ}C$ for analysis of cholesterol, triglyceride, HDL- cholesterol and LDL- cholesterol.


Aging population in Chiang Mai province
Physical and laboratory screening tests
Thirty-two healthy volunteers
Blood sample and physical fitness test
at the beginning (week 0) of the experiment
Group A (n=16)
Exercise training
12 weeks
Group B (n=16)
exercise training plus vitamin E
supplement 12 weeks
Blood sample and physical fitness tests 48 hours after exercise
at week 6 and week 12 of the experiment
Serum
Stored at – 80 0 C until assay for
- Total antioxidant capacity (TAC)
- Malondialdehyde (MDA)
- Vitamin E
Stored at 4 0 C until assay for
- Cholesterol
- Triglyceride
- HDL-C, LDL-C

II METHOD

1. Subject protocol

Thirty-two of 60 to 75 years-old-male volunteers living in Chiang Mai province participated in this study. All subjects were healthy, with normal height-to-weight ratios (body mass index between 20 –25 kg/m^2). Subjects were non-smokers or had stopped smoking for more than 5 years and had not been engaged in regular aerobic exercise. All subjects had normal medical histories with the following results of health status screening tests.

1. Normal physical examination
2. Fasting blood sugar (FBS) was between 70 –110 mg/dl
3. Normal complete blood count: hemoglobin was between 10 - 16 g/dl, hematocrit 40 - 50%, WBC 5000 - 10000 /cu.mm and adequate platelets.
4. Blood urea nitrogen was between 7 –18 mg/dl, creatinine 0.6 -1.3 mg/dl
5. Electrolyte : Na^+ was between 133 -148 mEq/L, K^+ 4.0 - 5.5 mEq/L, Cl^- 98 - 108 mEq/L and CO_2 22 - 33 mEq/L
6. Liver function test of total protein was between 6.4 - 8.3 g/dl, globulin 2.5 - 3.5 g/dl, AST 10 - 42 S.F.unit, ALT 10 - 40 S.F unit, alkaline phosphatase 32 – 92 U/L, direct bilirubin 0 - 0.3 mg/dl, total bilirubin 0.2-0.3 mg/dl
7. Normal results of urinary examination
8. Normal resting electrocardiogram
9. Exercise stress test had no evidence of acute myocardial ischemia, defined as ST segment depression of more than 0.2 mV lasting for 0.8 second or ST segment elevation greater than 0.1 mV

Subjects were excluded from this study if they were taking vitamins or diet supplements. The Committee on Human Rights Related to Human Experimentation, Chiang Mai University, had approved this protocol (see Appendix D).

The experimental protocols were clearly explained to the subjects and their written consent was obtained prior to attending the experiment. The subjects were divided into 2 groups and each group consisted of 16 males. The two groups were as follows:

Group A: Exercise group, subjects were assigned to exercise for 30 minutes per day, 3 days per week for 12 weeks.

Group B: Exercise and vitamin E supplemented group, subjects were assigned to exercise the same as those of group A and took 800 IU of vitamin E (Medicap, Samutprakarn, Thailand. Reg. No. 1A 1724/29) one 400 IU capsule before breakfast and at dinner per day , for 12 weeks.

2. Experiment protocol

A face to face interview for food consumption was conducted with every participant and a detailed food frequency questionnaire was completed in order to obtain information about their dietary habit starting one week before the experiment began and continue throughout the project. The dietary information was estimated by a semiquantitative scoring system (Appendix B) (87,88). The protocol was divided into two parts. The first or part I included the general physical fitness testing such as vital signs, body weight, height, percentage of body fat, lean body mass, maximum oxygen consumption (VO_2 max), and the second or part II included the investigation of biochemical effects of exercise and/or vitamin E supplementation. Total antioxidant

capacity, lipid profile level, vitamin E level and markers of lipid peroxidation, malondialdehyde (MDA) were determined at the beginning (wk. 0), the end of week 6 and at the end (week 12) of the experiment.

Part I : General physical fitness testing

1. Percentage of body fat and lean body mass

Body weight and height of each subject were measured followed by the estimation of the percentaged body fat (%BF). Lean body mass (LBM) was estimated by using Lange skinfold caliper to measure the subcutaneous fat at 4 sites i.e., biceps, triceps, subscapular and suprailliac. These were done on the dominant side of each subject in a relaxed standing position (figure 1). The detail of four selected sites were as follows (25).

1. Biceps: mid point of muscle belly with the arm hanging in anatomical position.

2. Triceps: The vertical fold over the belly and the distance between the acromion and olecranon process with arm hanging vertically.

3. Subscapula: Just below the inferior angle of the scapula 45° to the vertical plane.

4. Suprailiac: The fold was lifted to follow the natural diagonal line just above the hipbone.

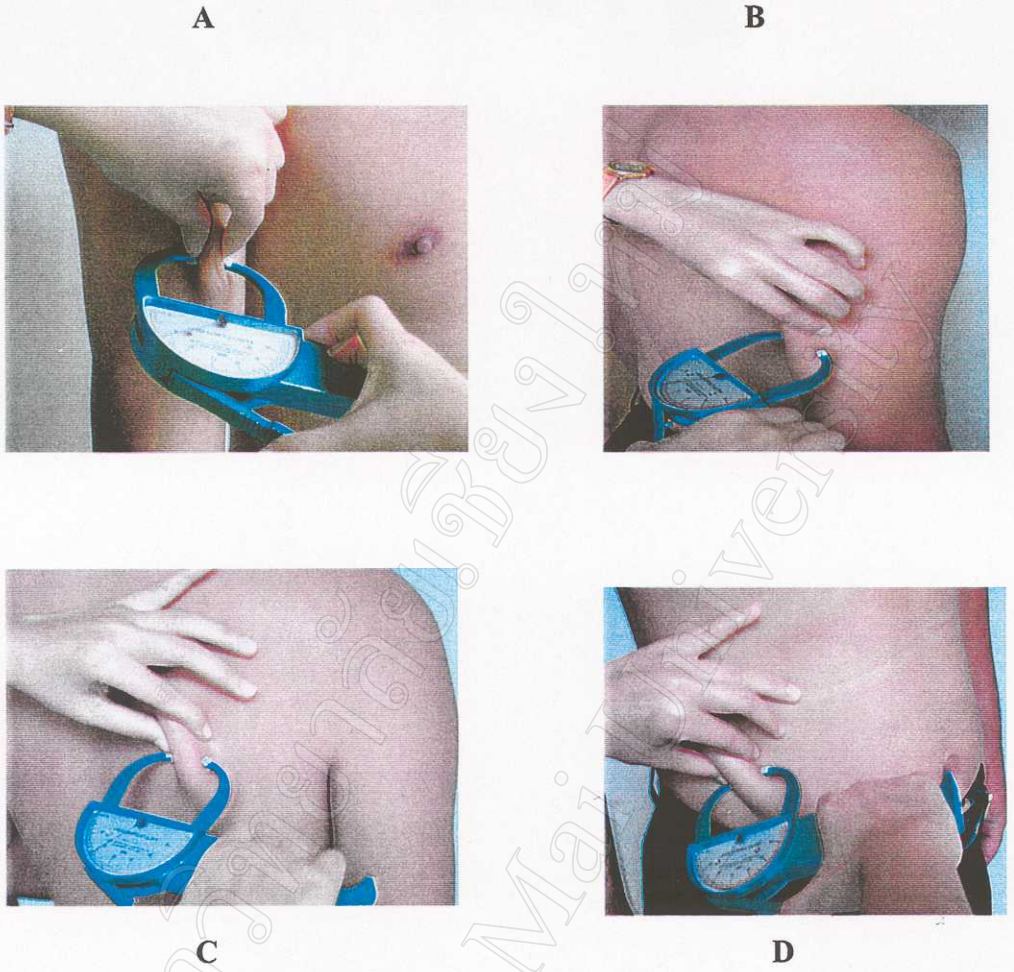


Figure 8 Measure of skinfold thickness using Lange caliper.

Anatomic location of skinfold sites: **A**:triceps, **B**:Biceps, **C**:Subscapular, **D**:suprailiac.

The following skinfold equations by Durnin and Wancesley (89) were used for the calculation of body density (BD) and %BF

$$BD = 1.1715 - 0.0779 \times \log A$$

$$\%BF = [(4.95/BD) - 4.50] \times 100$$

log A = log of sum of skinfold thickness measured in millimeters from 4 sites.

The following equation was used to calculate lean body mass (LBM)

$$LBM = \text{Body weight } [1 - (\%BF/100)]$$

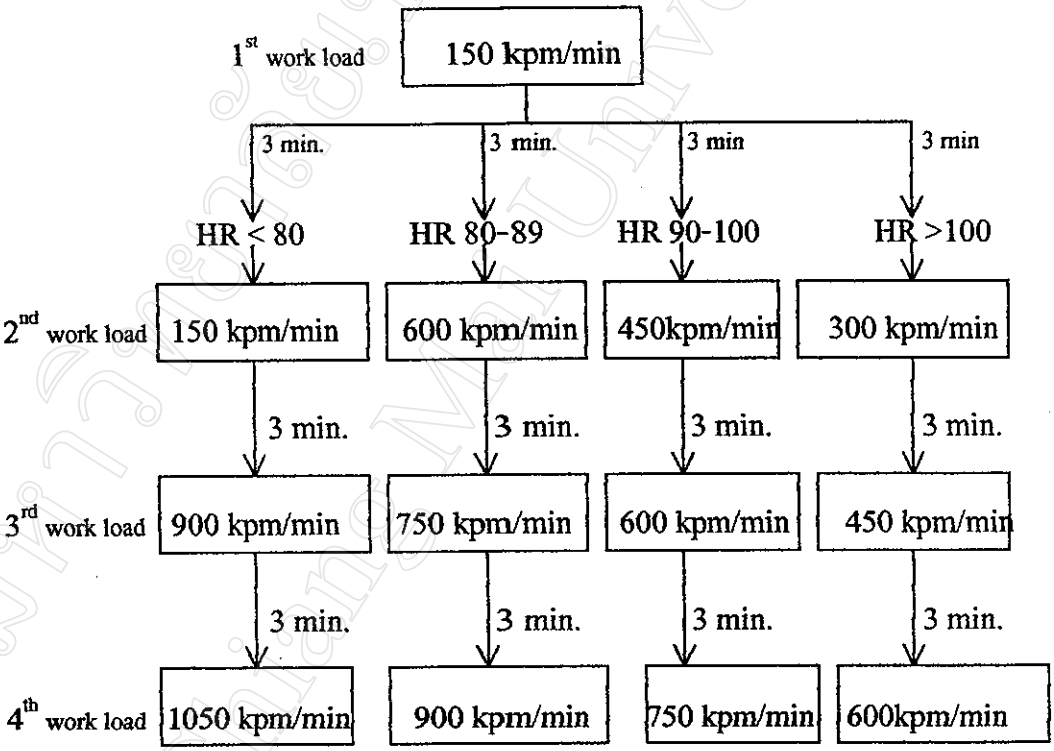
2. Maximum oxygen consumption

The maximum oxygen consumption (VO_2max) value quantitatively expressed the maximum rate at which oxygen can be used by the body during maximal work and is related directly to the maximal capacity of the heart rate to deliver blood to the muscles (25,75). In this experiment, the maximum oxygen consumption is calculated by using the indirect method of *Y's Way to Physical Fitness* (YMCA submaximal cycle ergometer test). This protocol relies on the observation that there is a linear relationship between heart rate and work rate, once a heart rate of approximately 110 bpm is reached, which requires the subject to complete one more stage passed the one causing a heart rate of 110 bpm (74). The procedure was as follows.

1. The subject was allowed to sit on the saddle of the cycle ergometer. The height of the saddle was adjusted to suit the individual subject to allow and almost completely stretched leg at the lowest pedal and the knee was in a slightly bent position.

2. Sport Tester was attached to the anterior chest wall and used to continuously measure the subject's heart rate during exercise.

3. The test began with a power output of 150-kilopound meter per minute (kpm/min). After 3 minutes the heart rate was measured and the power output was increased by an amount that was inversely related to the individual subject's heart rate, as shown in the diagram below.



4. The heart rate at each submaximum workload was considered as steady when the difference in heart rates between two consecutive readings was less than 5 bpm. The values of heart rate during the last two minutes of each workload were used for calculating the average steady heart rate.

5. The work load and the steady heart rate were used to estimate VO_2max using theYMCA plot chart, as shown in figure 8.

Part II : Exercise protocol

The exercise program was modified from that of Blumenthal, J.A., et al. (79) Tiidus, P.M., et al (90) and McArdle, W.D., et al. (34). According to the guideline for aerobic exercise prescription for healthy adults recommended by the American College of Sports Medicine (76), an intensity of 60% to 75% of HRmax, 3 days per week for at least 6 weeks is proper in developing and maintained cardiorespiratory and muscle fitness in older persons. Thus, The subjects were assigned to exercise for 30 minutes per day, 3 days per week for 12 weeks. The subjects were allowed to rest for 10 minutes before exercise. Blood pressure and heart rate were recorded as resting values. Each subject sat on the saddle of an ergometer, cycling in a comfortable position at 50% of maximum heart rate reserve [(HRmax–HRrest) 0.5 + HRrest] for 30 minutes (maximum heart rate was calculated from 220 minus age of each subject) during the first and second weeks. Subjects cycled at 60% of maximum heart rate reserve during the third and fourth weeks, and cycled at 70% of maximum heart rate reserve after fourth week through the 12^{th} week. Before starting to exercise, the subjects warmed up by cycling at 0 watt work load (friction brake 0 kpm) for 4 minutes. Then, the intensity was gradually increased every 2 minutes to obtain the target heart rate of each subject. During this exercise, the heart rate was monitored by Sport Tester, which was strapped to the chest wall. At the end of the exercise time, the subjects were asked not to stop the exercise suddenly but to gradually decrease intensity during the cool down process for 5 minutes. Vital signs were recorded during the recovery period.

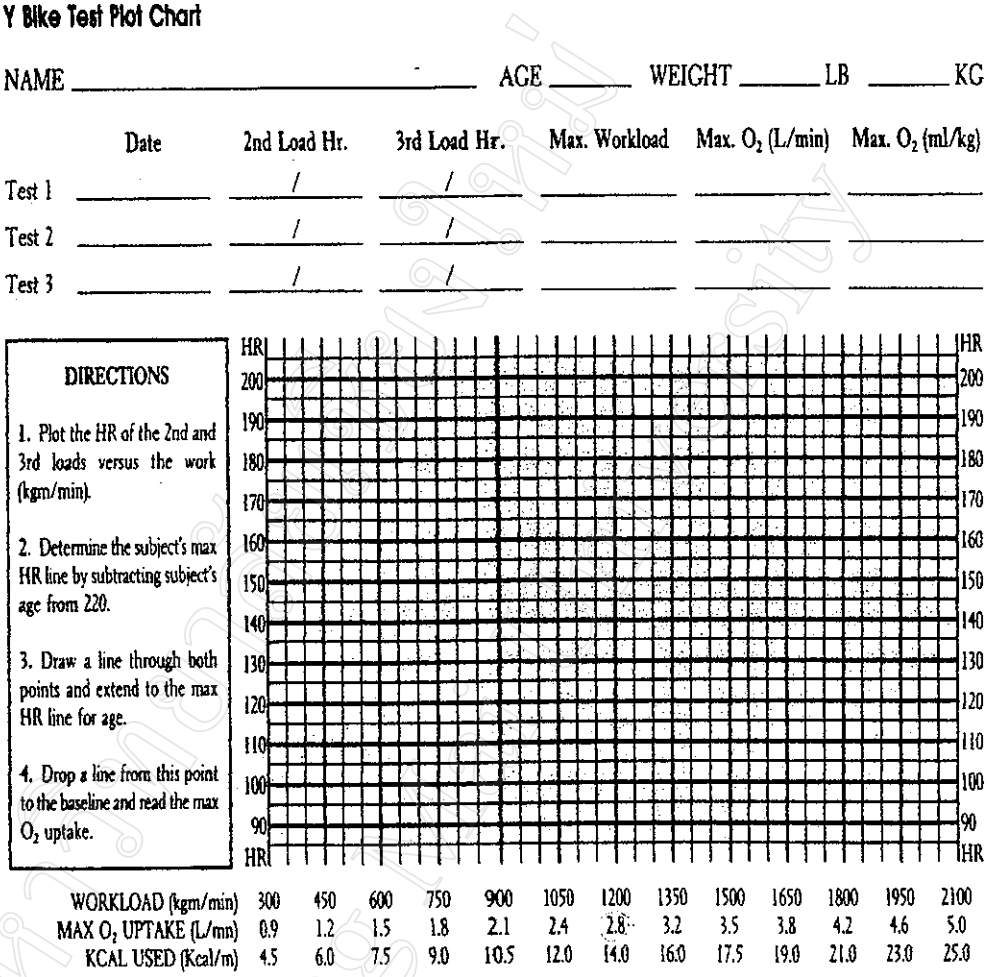
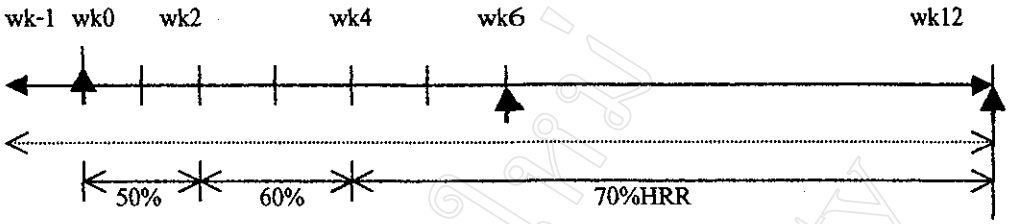


Figure 9 Chart for prediction of maximum oxygen uptake from the heart rate response to submaximal workloads (74).

Protocol of experiment



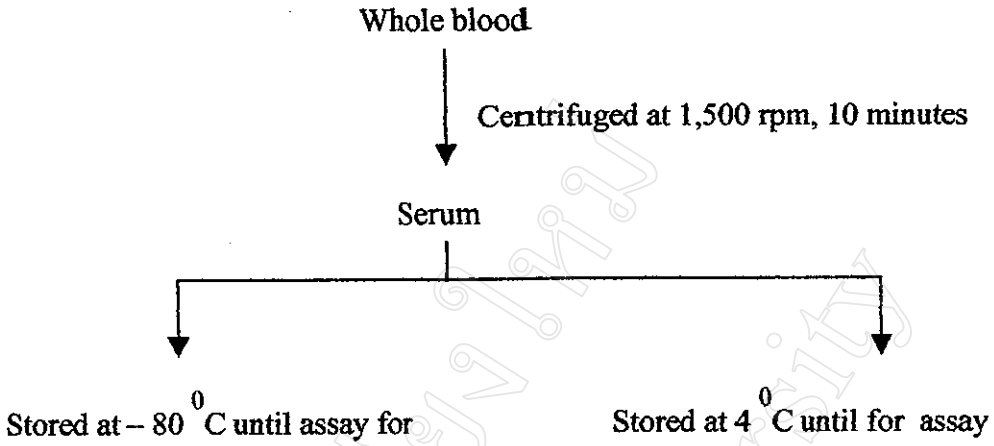
▲ = blood sample, percentage of body fat, lean body mass, maximum oxygen consumption (VO_2 max)

..... = daily diet record

——— = intensity of exercise

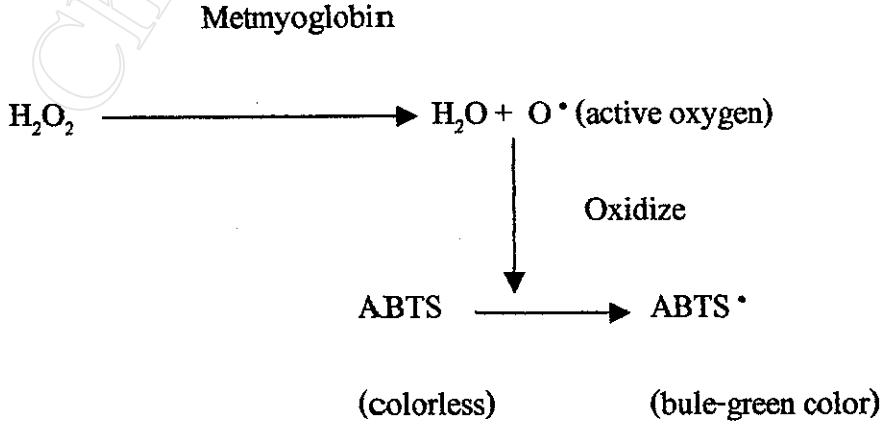
3. Blood analysis

Blood samples were obtained at week 0 (start), at the end of week 6 and week 12 of the program. Blood samples were drawn from the antecubital vein between 7.00 to 9.00 a.m. that was 12 hours after an overnight fasting and at least 48 hours after the prior exercise session. The samples were collected in clean tubes for analysis. The serum was separated by centrifugation for 10 minutes at 1,500 round per minute (rpm) to determine the total antioxidant capacity (TAC), malondialdehyde (MDA), lipids profile and vitamin E level.



3.1 Total antioxidant capacity (TAC) (91-93)

This technique utilizes the quenching of 2,2′–azinobis-(3–ethylbenzothiazoline-6-sulphonic acid) (ABTS) by oxidation of free radical cation which is formed in the presence of peroxidase according to the reaction:



Once exposed to hydrogen peroxide the metmyoglobin is activated to the ferryl state and active oxygen which will oxidize the oxidation target, ABTS to a free ABTS radical which has maximum peak absorption at 734 nm. Electron or hydrogen donors if present will reduce ferryl ion to its original state and delay the color reaction. The synthetic water-soluble tocopherol analogue, Trolox, was used as calibration agent.

Preparation of reagents for TAC (See Appendix C)

Procedure

1. Operate the UV- spectrophotometer (Shimadzu- 160A) as follows :

- MODE KINETIC
- λ = 734 nm

UPPER = +2.00A LOWER = +0.00A

LAG T = 0 SEC RATE T = 300 SEC

INTERVAL T = 60 SEC

- FACTOR = 1.000
- SAMPLE NO. = 1
- GAIN x 10 NO.

2. Pipette the following solutions into a series of polystyrene microcuvette path length 1 cm :

Reagent	Blank (µl)	Test (µl)
Serum (or standard)	-	10
Metmyoglobin	40	40
ABTS (100 µmol/l)	300	300
PBS (5 mmol/l)	510	500

3. Transfer to UV-spectrophotometer, add H_2O_2 (500 µmol/l) 250 µl and mix immediately to start the reaction.

4. Calculate %inhibition of oxidation of each serum sample and obtain the TAC equivalent to Trolox from calibration curve.

Calculation

$$\% \text{ Inhibition of oxidation} = \frac{A_B - Au}{A_B} \times 100$$

Where A_B = absorbance of blank at 5 minutes

Au = absorbance of test at 5 minutes

Calibration curve

1. Dilute stock Trolox standard 2.5 mmol/l with PBS 5 mmol/l to make various concentrations as follows.

	Trolox (mmol/l)		
	0.625	1.250	1.875
PBS 5 mmol/l (µl)	300	200	100
Trolox 2.5 mmol/l (µl)	100	200	300

2. Perform the test in the same way as serum sample.
3. Calculate the % inhibition of oxidation. Then plot the % inhibition versus concentration in mmol/l of Trolox on a graph paper.

3.2 Malondialdehyde (MDA) (94)

The sample is heated with thiobarbituric acid (TBA) at low pH. One molecule of MDA reacts with two molecules of TBA to produce a pink pigment with absorption peak at 532 nm.

$$2\text{TBA} + \text{MDA} \xrightarrow{\text{heat}} \text{TBA- MDA – TBA complex}$$

(colorless) (pink color)

Preparation reagents for MDA (See Appendix C)

Procedure

1. In 13 x 100 mm test tubes, pipette serum and reagent as follows :

Reagent	Blank (ml)	Test (ml)
Serum	-	0.1
NSS	0.55	0.45
TBA reagent	0.2	0.2
TCA reagent	1.0	1.0

2. Mix and put all tubes in boiling waterbath for 30 minutes, then cool in tap water.
3. Two milliliter of distilled water was added and shake vigorously.
4. Centrifuge to obtain the clear solution, then read the adsorbance at 532 nm against blank.
5. Obtain MDA concentration calibrate curve.

Calibration curve

1. Dilute 10 mmol/l stock tetraethoxypropane (TEP) 1:100 with distilled water to obtain 100 μmol/L solution.
2. Make a serial dilution of working standard as follows :

	TEP (μmol/l)				
	50	40	30	20	10
TEP 100 μmol/l (ml)	0.5	0.4	0.3	0.2	0.1
Distilled water (ml)	0.5	0.6	0.7	0.8	0.9

3. Perform the test in the same way as serum sample.
4. Determine the absobance at 532 nm. Then plot the absorbance versus μmol/l of TEP on a graph paper.

3.3 Vitamin E

Tocopherol is extracted from serum with xylene while proteins are precipitated with ethyl alcohol. Tocopherol then reacts with bipyridyl and ferric chloride according to the reaction:

Tocopherol + $FeCl_3$ + α,α' -bipyridyl ⟶ Tocopherol- p- quinone

(colorless) (red color)

The intensity of red color produced was read at 520 nm. Howerver substance can also be extracted and give red color with the reagents. Factor was estimated for correction.

Procedure

1. The following reagents were added to 16 x 125 mm screw cap tubes :

Reagent	Blank	Standard	Test
Distilled water (ml)	1	1	-
Absolute ethanol (ml)	1	-	1
Standard (ml)	-	1	-
Serum (ml)	-	-	1
Xylene (ml)	1.5	1.5	1.5

2. Shake vigorously for at least 30 seconds.

3. Centrifuge at 3,000 – 4,000 rpm for 1 minutes.
4. Pipette supernatant 1.0 ml into a 10 x 75 mm cuvette, add 0.5 ml. bipyridyl reagent and mix thoroughly.
5. Read absorbance at 460 nm against distilled water within 4 minutes for carotenoid correction.
6. Add 0.1 ml of ferric chloride and mix immediately.
7. Read absorbance at 520 nm against distilled water blank at exactly 20 seconds after the addition of ferric chloride reagent.

Calculation

$$\alpha\text{- Tocopherol (mg/dl)} = \frac{Au_{520} - (\text{Factor} \times Au_{460}) \times 2}{As_{520}}$$

Where As = Absorbance of vitamin E standard

Au = Absorbance of test

2 = Concentration of vitamin E standard (mg/dl)

Calculation of factor

1. Pipette 0.05, 0.1, 0.15, 0.2 mg/dl of β -carotene in to 10 x 75 mm. cuvettes.
2. Add 0.5 ml of bipyridyl reagent and mix thoroughly.
3. Read absorbance against distilled water blank at 460 nm
4. Add 0.1 ml of ferric chloride and immediately mix.
5. Read absorbance at 520 nm against distilled water at exactly 20 seconds after the addition of ferric chloride reagent.
6. Obtain the factor value from equation:

$$\text{Factor} = \frac{\text{Absorbance at 520 nm}}{\text{Absorbance at 460 nm}}$$

The average value of all tubes will be used as factor in the calculation equation.

3.4 Serum lipids profile

3.4.1 Cholesterol

Cholesterol esterase hydrolyzes cholesterol ester to give free cholesterol and fatty acid. The free cholesterol is oxidized to cholestene-3-one and hydrogen peroxide in the presence of cholesterol oxidase. Subsequently, H_2O_2 is oxidized by peroxidase in the presence of the phenol sulfonic acid (PAS) and 4- aminoantipyrine (4AAP) to a red quinoneimine dye, which the intensity at 500 nm is directly proportional to the cholesterol concentration in the sample. The reactions of the assay are as follows:

$$\text{Cholesterol ester} + H_2O \xrightarrow{\text{Cholesterol esterase}} \text{Free cholesterol} + \text{fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholestene-3-one} + H_2O_2$$

$$2H_2O_2 + \text{PAS} + \text{4-AAP} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + 4H_2O$$

(red color)

Preparation of reagent for cholesterol (See Appendix C)

Procedure

1. Pipette 1.0 ml of reagent to test tubes and pre- warm at 37°C for about 5 minutes.
2. Add 20 µl of sample to respective tubes (for standard tube: use standard reagent 20 µl), mix and incubate at 37°C for 5 minutes.
3. Add distilled water 1.0 ml, then set zero spectrophotometer with the reagent blank at 500 nm. Read and record absorbance of all samples and standard.

Calculation

$$\text{Cholesterol (mg/dl)} = \frac{\text{Au} \times 200}{\text{As}}$$

Where Au = Absorbance of sample

As = Absorbance of standard

200 = Concentration of standard in mg/dl

3.4.2 Triglycerides

Triglycerides are hydrolyzed by lipase to give glycerol and fatty acids. The glycerol is then phosphorylated to glycerol-3-phosphate (G3P) in the presence of adenosine-5-triphosphate (ATP) and glycerol kinase (GK). G3P is converted to dihydroxyacetone phosphate (DAP) and hydrogen peroxide by glycerolphosphate oxidase (GPO). The hydrogen peroxide reacts with 4-aminoantipyrine (4- AAP) and 3- hydroxy-2,4,6-tribromobenzoic acid (TBHB) which is catalyzed by peroxidase yielding a red colored quinoneimine dye. The intensity of the color produced is directly proportional to the concentration of triglycerides in the sample when measured at 500 nm. The enzyme reactions involved in the assay are as follows:

$$\text{Triglyceride} + 3H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acid}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol-3- phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$4H_2O_2 + \text{4-AAP} + \text{TBHB} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + \text{HBR} + 4H_2O$$

(red color)

Procedure

1. Pipette 1.0 ml of reagent to test tube and pre- warm at 37°C for about 5 minutes.
2. Add 20 µl of sample to respective tubes (for standard tube: add standard reagent 20 µl), mix and incubate at 37°C for 5 minutes.
3. Add distilled water 1.0 ml, then set zero spectrophotometer with the reagent blank at 500 nm. Read and record absorbance of all tubes.

Calculation

$$\text{Triglyceride (mg/dl)} = \frac{\text{Au} \times 200}{\text{As}}$$

Where Au = Absorbance of test

As = Absorbance of standard

200 = Concentration of standard in mg/dl

3.4.3 HDL-Cholesterol

Apo-B containing lipoproteins including LDL-C, VLDL-C are precipitated out with phosphotungstic acid in the presence of magnesium chloride reagents. Cholesterol in the supernate is determined for the HDL-cholesterol.

Procedure

1. Pipette 250 µl of sample mixed with 50 µl of precipitating reagent.
2. Stand for 5 minutes at room temperature for complete precipitation.
3. Centrifuge at 3,000 rpm for 5 minute.
4. Pipette 20 µl of supernate into 1 ml of cholesterol reagent and mix.
5. Perform the procedure as cholesterol determination.

Calculation

$$\text{Cholesterol (mg/dl)} = \frac{Au \times 200}{As}$$

$$\text{HDL- C (mg/dl)} = \text{Cholesterol} \times 1.2$$

Where Au = Absorbance of test

As = Absorbance of standard

200 = Concentration of standard in mg/dl

1.2 = Dilution factor of serum by precipitating agent

3.4.4 LDL- Cholesterol

Cholesterol in LDL can be estimated by using Friedewald formula

LDL-C = Total cholesterol – [HDL-C + Triglycerides/5]

This formula can not be used if triglyceride level exceeds 400 mg/dl.

4. Statistical analysis

The data were test parametric statistic method and were presented as mean and standard error of means (SEM). Repeated measures analysis of variances were used to determine statistically significant difference between pre-exercise and post-exercise results for each group followed by the least significant difference test (LSD). Differences in pairs of means among groups were analyzed by unpair t-test. If the statistical probability (p-value) was less than 0.05, the difference was considered to be statistically significant.

CHAPTER III

RESULTS

In this study, the physical fitness tests and blood biochemistry were done between the 18th of March – 30th of June 2000. The results were divided into two parts. Part I showed data about general physical fitness tests and blood biochemistry between the exercise training and the exercise training plus vitamin E supplement group at the beginning of the experiment (week 0). Data obtained from part II include general physical fitness tests and blood biochemistry obtained 48 hours after exercise at the middle and the end of the experiments.

Part I: Comparison of general and physical fitness and blood biochemical data between the exercise training group (group A) and the exercise training plus vitamin E supplement (group B) at the beginning of the experiment (week 0).

1. Comparison of age and physical fitness data between group A and group B at the beginning of the experiment (week 0).

Age and Physical fitness in group A and group B at the beginning of the experiment (week 0) is shown in Table 2. The average age of group A and group B were not significantly different. Heart rate of subject in both groups found were in normal range and similar. Blood pressure and mean arterial pressure (MAP) of both groups were not significantly different. The body weight, body height and lean body mass of subject in both groups at week0 were not significant difference. There

were also no significant differences in the percent body fat and VO_2max of between the two groups.

2. Comparison of blood biochemistry data between group A and group B at the beginning of the experiment (week 0).

Table 2 shows blood biochemistry parameters including the level of serum total antioxidant capacity (TAC), lipid peroxidation product (serum malondialdehyde level, MDA), vitamin E and lipid profile between group A and group B at the beginning of the experiment (week 0).

All of subjects were in the same range of age, weight and height. Their general physical fitness parameters were relatively at the same levels (Table 2). TAC, lipid peroxidation product, vitamin E level and lipid profile were similar in both groups at the beginning of the experiment.

3. Comparison of dietary intake and plasma vitamin E levels between group A and group B in various time of the experiment.

3.1 Dietary intake

The records of daily dietary intake were obtained from each subject. There were not significant differences between the two groups and within each group throughout this experiment (Table 3).

3.2 Serum vitamin E level

Figure 10 shows the level of serum vitamin E in group A and group B at 48 hours after exercise of the beginning (week 0), the middle (week 6) and the end of the experiment (week 12). The level of serum vitamin E at resting in the group A and group B were not significantly different from each other. However, the level of serum vitamin E at week 6 and week 12 of the experiment in group B was significantly higher than group A in week 6 and week12 of the experiment, respectively. When compared within each group, serum vitamin E level at week 6 and week 12 of the experiment in group A were 0.74 ± 0.10 and 0.76 ± 0.05 mg/dl,