CHAPTER 3

METHODS

This study comprised the changing levels of nitrite, malondialdehyde (MDA), glutathione (GSH), total antioxidant (TAC), interleukin-2 (IL-2), and the maximal time of running before, between and after NAC supplementation at 1,200 mg daily for 7 days.

Participants

Several other research studies on NAC supplementation divided their subjects into healthy men (n = 15) (45), smokers (n = 41) (44) and COPD patients (n = 9) (53). The simple size of volunteers ranged from 8 to 50 subjects. This study consisted of thirty volunteer subjects, who gave their consent on an informed consent form after they read in detail information of this study. The research was approved by the Research Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University. (See in appendices)

Inclusion criteria

1. Healthy men aged between 19 and 24 years.

2. No supplement intake of any stimulants, vitamin C or coffee before, between or after exercise.

3. No change of eating habits throughout the period of experiments.

4. Body mass index in the normal range (18.5-24.9 $kg./m^2$, WHO and International obesity task force, 2005).

5. No athletic or regular exercise more than 3 days per week.

6. No history of blood diseases, such as anemia and thalassemia, musculoskeletal injury or cardiopulmonary organ within 6 months before the study, as well as chronic illness symptoms, e.g. cold, cough, dizziness, muscle pain or frequent alcohol consumption.

Exclusion criteria

1. Development of a disorder during the study such as heart palpitations, dizziness, syncope, leg pain or stiff or dysrhythmic heart rate pre-exercise, during, or post-exercise, according to recommendations of the ACSM guideline (2004).

2. Volunteers having any adverse effects during exercise such as severe diarrhea, headache, stomachache, vomiting or hypertension.

3. Volunteers that use the NAC supplement incompletely.

4. Volunteers who want to drop out of the study.

Chemicals & equipments

1. UV-Visible Spectrophotometer (GENESYS 10S Vis, USA)
2. Centrifuge (Xiang Yi Centrifuge Instrument Co, Ltd)
3. Micro-plate reader EL 340 (Bio-TEK Instruments, USA)
4. ELISA kit test (IL-2) (R&D system Europe, Ltd, United Kingdom)
5. Treadmill, (Turbo Fitness 3.0, Taiwan)
6. Stopwatch (Timer-5)
7. Polar F11^{TM} Fitness Heart Rate Monitor (Polar Electro Oy, FIN-90440, Finland)
8. Chemicals and reagents (See measuring protocol (page 22-24).

Independent and dependent variables

Independent variables

Independent variables are the effects of N-acetylcysteine at 1,200 mg (Fluimucil A600) on oxidative stress, inflammation and physical performance.

Dependent variables levels:

1. Oxidative stress testing (MDA, Nitrite, GSH, and TAC)
2. Inflammation testing (IL-2)
3. Maximal running time test

Procedures

1. This study was a block selected study of a supplementation (n=15) and control group (n=15). After all volunteers approved of the entire protocol and understood every detail of the study, including the risk of adverse effects from either all measurements or supplementation, they were asked to sign a consent form.

2. All volunteers had 10 ml of blood drawn from the anterior cubital vein by a medical technologist. Firstly, resting blood was separated into plasma and erythrocyte by centrifuging at 2,500 rpm for 10 min. Plasma malondialdehyde (MDA), Plasma Nitrite, Erythrocyte glutathione (GSH), Plasma total antioxidant capacity (TAC) and Plasma interleukin-2 (IL-2) were evaluated, as well as CBC screened for health status.

3. Maximal running time on a mechanical treadmill was tested, following the Bruce Protocol, and 85% of maximal heart rate was set as the end point of the target goal of exercise by monitoring with a Polar Heart Rate Monitor (F11, Finland). After exercise had stopped for 20 min, all volunteers had blood drawn again to determine all the parameters Plasma malondialdehyde (MDA), Plasma nitrite, Erythrocyte glutathione (GSH), Plasma total antioxidant capacity (TAC) and Plasma interleukin-2 (IL-2).

4. The control group was asked to maintain an irregular food diet, and not take extra-food such as multi-vitamins or alcohol, or indulge in night entertainment within 7 days before the study. In the NAC supplement group, NAC pills (600 mg) (FLUIMUCIL ® A 600, ZAMBON Switzerland Ltd) were continually administered

twice per day after meals for 7 days; a total dose of 1,200 mg per day. During the 7 days, the researcher called to recheck the supplementation and any adverse effects such as headaches or abdominal pain.

5. After 7 days, all volunteers in both the control and NAC supplement group, were re-tested for maximal running time. Blood was taken again at pre- and post acute exercise.

Program for a test the Maximal running time

Use of the Bruce protocol (graded treadmill running) followed instructions from the ACSM Guideline 2005, as detailed below.

Step 1: Stretching the muscles; hip flexors (muscles of the hips), hip extensor (hip muscle stretch), quadriceps (knee muscle stretches), hamstring (muscles of the knee) and gastrosoleus (calf muscles) by extending and holding for 20 seconds, 3 times per set, for approximately 5 minutes.

Step 2: Five minutes warm up on a treadmill under 55% of maximal heart rate from a heart rate monitor, and the score of rate perceived exertion (RPE) had to be less than 12 (RPE 6-20 scale).

Figure 2. Image of the polar heart rate monitor.

Score	Level of fatigue
6	
7	very, very light
8	
9	very light
10	
11	fairly light
12	
13	somewhat hard
14	
15	Hard
16	
17	very hard
18	
19	very, very hard
20	

Figure 3. Fatigue value (rate perceived exertion; RPE)

(based on the ACSM Guideline, 2005).

Step 3: Bruce protocol testing by the following steps. Running on a treadmill:

Step I	velocity 1.7 mph with 0 % slope
II	velocity 1.7 mph with 5 % slope
III	velocity 1.7 mph with 10 % slope
IV	velocity 2.5 mph with 12 % slope
V	velocity 3.4 mph with 14 % slope
VI	velocity 4.2 mph with 16 % slope
VII	velocity 5.0 mph with 18 % slope
VIII	velocity 5.5 mph with 20 % slope.

Each step was performed for 3 minutes, giving a total of 24 minutes. The target heart rate (THR) was 85 % of maximal heart rate (MHR), calculated by 220 minus age (yr). If subjects ran until the THR reached 85% of MHR, the total time was recorded. Then, the velocity and slope of the treadmill were maintained until the running time was at least 20 min, when the treadmill was turned off slowly.

Step 4: The measurement of heart rate (HR) and rate perceived exertion (RPE) was less than 15 (RPE 6-20 scale). If any symptoms such as leg pain or others, e.g. dizziness or syncope reached the exclusion criteria, the exercise was stopped.

The measurement of Oxidative stress testing

1. Plasma malondialdehyde (MDA) from lipid peroxidation by TBARs (56)

The protocol was modified from the original report of Chirico (1994), which stated that 200 µL of plasma was mixed with 750 µL of Ortho-phosphoric acid (2.5%, *v:v*) (Merck KGaA, Darmstadt, Germany) and vortexed. Then, 500 µL of 2-Thiobarbituric acid (TBA) (0.2 mol/L) (Sigma, Louis, USA) in Tris (hydroxymethyl)-aminomethan z. A. Puffersubstanz (Trometamol) solution (0.14 mol/L) (Merck KGaA, Darmstadt, Germany) was added. After incubation in a water bath (90°C) for 30 min, all samples were cooled and centrifuged at 10,000 rpm for 3 min. A clear pink color of supernatant was read with a spectrophotometer at 532 nm. Plasma blank composed of plasma and ortho-phosphoric acid without TBA was used for comparison in the system. The exact yield of MDA in the sample was calculated from different concentrations after comparing with the absorbance of standard Tetramethoxypropane (TMP) (Sigma, Louis, USA) (0-50 µmol/L).

2. Plasma nitrite by Griess reagent (57)

Plasma nitrite was evaluated by Griess reagent following Promega's instructions for use of the Griess reagent system (Promega protocol). Firstly, four 200 µL tubes of plasma were mixed with 500 µL of 0.1% of N-1-napthylethylenediamine dihydrochloride (NED) (VWR, Prolabo, EC) in water and left in the dark for 5 min. Then, two tubes were mixed with 500 µL of 1% sulfanilamide (Sulfa) (Fluka, Steinheim, China) in 5% phosphoric acid; whereas the other two tubes had water added and were kept in the dark again for 5 min. After that, a slightly pink color was

produced with an absorbance reading at 520 nm. Nitrate in plasma was calculated by comparing different values between the sample test and sample blank after comparison with the absorbance of standard sodium nitrite ($NaNO_3$) (0-40 μmol/L).

3. Erythrocyte glutathione (GSH) by DTNB reagent (58)

Reduced glutathione in erythrocyte was determined by following the protocol of previous work [Leelarungrayub et al, 2010]. A packed red cell was dissolved at 400 μL in 1.0 ml of deionized water and 3.0 ml of precipitating solution containing 0.2 mol/L glacial meta-phosphoric acid (Riedel-de Haen AG) 0.68 mmol/L Ethylenediaminetetra-acetic acid di-sodium salt (EDTA-Na_2) (Univar, APS Finechem, Australia) and 0.5 mol/L Sodium Chloride (NaCl) (Lab-Scan, Bangkok, Thailand). Clear supernatant at 200 μL was mixed with 500 μL of 0.1 mol/L phosphate buffer (pH 8.0) and 500 μL of DTNB solution (4%) in 0.2 mmol/L of sodium citrate, after centrifugation at 10,000 rpm for 3 min. An absorbance mixture of yellow color was read at 412 nm within 5 min. The reduced GSH was calculated by comparing with the absorbance of standard reduced GSH (Sigma, Louis, USA) (0-50 mmol/L). Reduced GSH was presented in a unit of μmol in one gram of Hb (μmol/g Hb).

4. Plasma total antioxidant capacity (TAC) by ABTs decolorization method (59)

Total antioxidant capacity of fresh plasma was assayed with ABTs cation radical decolorization (Re et al, 1999). Stock ABTs cation radical was produced by mixing 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTs) (14 mmol/L)

(Sigma, Louis, USA) and potassium persulfate (14 mmol/L) (Merck KGaA, Darmstadt, Germany) together, and leaving in the dark overnight. Working ABTs cation radical was diluted from stock ABTs with deionized water, until absorbance at 734 nm was shown between 0.68 and 0.74 before adding plasma. Ten µL of plasma was added to 990 µL of working solution ABTs cation radical in a plastic cuvette (size 1.5 ml), and gently alternated inversely by shaking 9 times before adding in the spectrophotometer. Decreased absorbance was recorded continuously every 1 min for 3 minutes, and finally calculated to ΔA/min. Total antioxidant capacity (TAC) of plasma was calculated by comparing with the ΔA/min of standard Trolox (0-10 mmol/L).

5. Plasma interleukin-2 (IL-2) by ELISA kit test (60)

The method to determine IL-2 in plasma was performed by following guidelines in the Quantikine, Human IL-2 Immunoassay (Quantikine®, R&D systems, Inc, 2010). One hundred micro liters of plasma and 50 mL of external standard IL-2 (100 pg/ml) were loaded onto an anti-IL-2 polyclonal antibody-immobilized solid phase and incubated for 2 h at room temperature. After washing 3 times with wash buffer at 400 µL, 200 µL of HRP conjugated IL-2 was added in each well before incubating for 2 h at room temperature. All solution in each well was then aspirated and washed with wash buffer 3 times before 400 µL of substrate TMB was added. The color product was developed by incubating for 20 min at room temperature. Finally, when 50 µL of stop solution (2N sulfuric acid) was added, the blue color changed to yellow and absorbance was detected at 450 nm within 30

minutes with a micro-plate reader. The concentration of IL-2 in plasma was calculated by comparing with the standard curve of standard IL-2 (31.2-2,000 pg/ml).

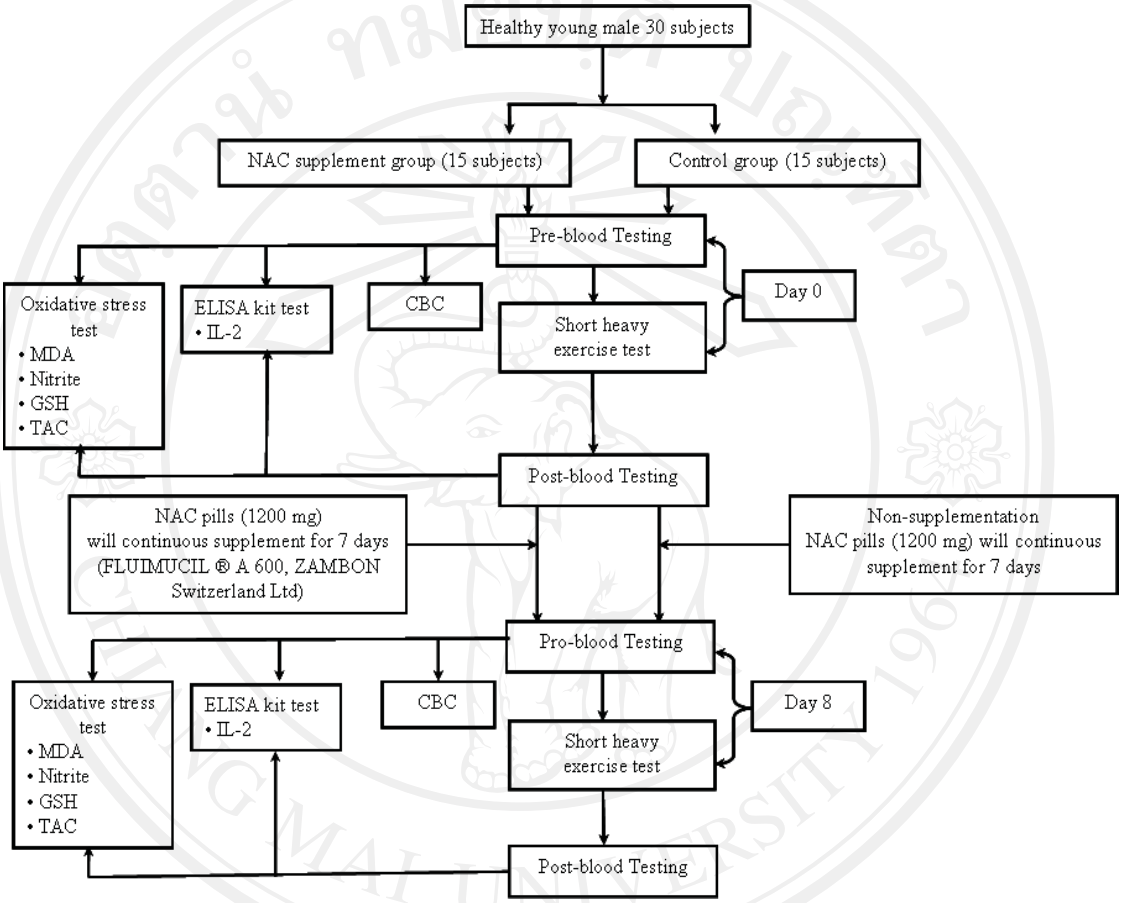

Diagram 1. Data collection procedure

Statistical analysis

1. Descriptive data analysis of age, weight, height and body mass index (BMI) was in mean, standard deviation.

2. Comparison between the levels of change in all parameters (MDA, Nitrite, GSH, TAC, and IL-2) after acute exercise and resting, and maximal running time before, between and after NAC supplementation was carried out with Two-way

ANOVA in the SPSS program (version 16). A *P*-value of less than 0.05 was considered as significantly difference.

Data collection location

The study was conducted in the Oxidative Stress and Exercise Biochemistry Laboratory on the 4th floor of the Department of Physical Therapy, Faculty of Associated Medical Sciences, Chiang Mai University.