Chapter III
Methodology
CHAPTER III

METHODOLOGY

3.1. Selection of Subjects

1. Subjects were limited to healthy, young and non-smoking males.

2. Subjects are limited to only male gender due to the possibility of the aforementioned relationship between estrogen and antioxidant properties.

3. All subjects should confirm having a physical exam within the past 5 years.

4. Upon review of the medical history questionnaire persons with cardiovascular and other contraindications to exercise as outlined in the American College of Sports Medicine’s guidelines (ACSM) (Whaley, 2006), is excluded from the study.

5. Participation in the study was voluntary. A medical history questionnaire completed, and informed consent signed. (in Appendix A&C)

3.2. Selection of Subjects

1. Random sampling method is used in the present study.

2. The subjects selected from 8000 ordinary students from Azad University, Khoramabad Branch, and Iran.

3. The Khoramabad University human subjects committee approved all experimental procedures.

4. The total number of subjects is 221 ordinary university students who are unaccustomed to the selected exercises of this study.

5. Only male subjects aged between 20-25 years are selected after a thorough medical check-up.

3.3. Study Timeline

1. The experiments of this study were done within six months.

2. Four weeks are required for getting the necessary permission from the university officials and recruiting the volunteers and 2 weeks for
instructing and selecting the subjects and preparing the required materials.

3. The rest needed to carry out the experiment.

4. Each subject visited the Exercise Physiology Lab at University Exercise Sciences Center on four occasions.

5. The first visit included a complete explanation of the study protocol, completion of the medical history questionnaire and consent form and familiarization of the testing equipment voluntary procedure.

6. The second visit consisted of an oxygen consumption test on a treadmill (GXT) to determine VO₂ peak.

7. At least 24 hours after the GXT on the third visit, subjects had a blood draw, a baseline evaluation of oxidative stress and delayed onset muscle soreness (DOMS) symptoms, performed a test, and performed a downhill treadmill run protocol (EP).

8. The fourth visit was 24 hours after the EP in order to perform the final blood draw, evaluate oxidative stress and delayed onset muscle soreness (DOMS) symptoms.

9. Duration of visit was 1 to 2.5 hours.

3.4. Treatment protocol

Protocol design for various exercises (the control of intensity for different exercises).

Many people are unable to determine the percentage of their VO₂max. This has caused restrictions on maximum oxygen consumption test. For this reason, they cannot base their exercise intensity should be based on a percentage of VO₂max.

With regard to the relationship between age, gender and activity levels, (David Swain in 1994) discussed the relationship between % MHR and% VO₂max estimate is based on the following equation. \( %\text{VO}_2\text{max} = (\%\text{HRmax} – 37)/.64 \): If we write the above equation in terms of %HRmax, The equation is as follows: \( %\text{HRmax} = (%\text{VO}_2\text{max} \times 0.64) + 37 \) For example if we train at 75% of VO2 max, when the VO₂max percentage is placed into the re-engineered equation, the value
reflects the actual percentage of HRmax being used for work. % HRmax = 75 * 0.64 + 37 → % HRmax = 85

So if someone works at 75% VO2 max, he must hold his heart rate at 85% of heart rate max. We easily calculate the heart rate max with this equation: 220-age = HRmax

![Figure 3.1, The linear relationship between % MHR and % VO2max activity during walking, running and cycling (swinan et al 1994)](image)

3.4.1. Swimming

Exercise sessions consisted of 5 min of stretching (prior to water warm-up during the swimming session) and a 5 min warm-up at a low-intensity (10-15%MHR) followed by 15, 25 and 35 min of swimming at a moderate intensity (75% MHR) based upon maximal HR. Furthermore, as maximal HR is shown to be 9-13 bpm less during maximal swimming (McArdle, et al., 1971), HR was adjusted accordingly to ensure intensity was equivalent between exercises. All sessions were supervised by the study investigator. Prior to the onset of exercise, participants were instructed to exercise continuously, limiting breaks to fewer than 30 seconds when necessary. Participants wore a Polar HR transmitter across their chest throughout exercise to allow for measurements of HR every 5 minutes ensuring that exercise intensity was maintained and swimming velocity could be adjusted if necessary. Feedback was provided throughout the session to ensure that each subject remained within the prescribed exercise intensity, with swimming pace adjusted if necessary. Water temperature was maintained between 26.5°C and 27.0°C during each swimming session.
3.4.2. Skipping (roping)

Exercise sessions consisted of 5 min of stretching (prior to roping warm-up) and a 5 min warm-up at a low-intensity (10-15% MHR) followed by 15, 25 and 35 min of roping at a moderate intensity (75% MHR) based upon maximal HR. Prior to the onset of exercise, participants were instructed to exercise continuously. Participants wore a Polar HR transmitter across their chest throughout exercise to allow for measurements of HR ensuring that exercise intensity was maintained and roping velocity could be adjusted if necessary. Feedback was provided throughout the session to ensure that each subject remained within the prescribed exercise intensity, if necessary.

3.4.3. Jogging

Subjects performed jogging on the treadmill. Treadmill speed was such that the participants do not have to run. The subjects followed 15, 25 and 35 min of jogging at a moderate intensity (75% MHR) based upon maximal HR. Prior to the onset of exercise; participants were instructed to exercise continuously. Participants wore a Polar HR transmitter across their chest throughout exercise to allow for measurements of HR ensuring that exercise intensity was maintained and roping velocity could be adjusted if necessary. Feedback was provided throughout the session to ensure that each subject remained within the prescribed exercise intensity, if necessary.

3.4.4. Running downhill protocol (Eccentric Treadmill Run Protocol)

Subjects performed to 15, 25 and 35-minute run on a treadmill at a speed equivalent to 75% of their VO₂peak as determined by the GXT. The grade of the treadmill was -10 percent. This intensity and grade has been sufficient in other studies to yield metabolic stress and mechanical damage without serious injury to the subjects (Lee, et al., 2002; Malm, et al., 2004). Intensities above this range may suppress neutrophil activity through increased elevation of immunosuppressive hormones, adrenocorticotropic hormone, and cortical (Pyne, D. 1994). Before the EP each subject walked and running at a self selected pace for about 5 minutes to become accustomed to downhill running.
3.4.5. Running uphill protocol

Subjects performed to 15, 25 and 35-minute run on a treadmill at a speed equivalent to 75% of their VO2peak as determined by the GXT. The grade of the treadmill was 10 percent. Before the Running up hill each subject walked and running at a self selected pace for about 5 minutes to become accustomed to uphill running.

**H1:** There will be a threshold for the induction of oxidative stress and muscle soreness through exercise.

**H2:** The threshold for the induction of oxidative stress and muscle soreness is the same for different exercise activities.

Eighty subjects have been assigned to 16 groups of 5 people. Fifteen 5-member groups asked to (swimming, jogging, skipping, run on a downhill treadmill and uphill treadmill) for 15, 25 and 35 minutes (75% HRMAX) respectively while the sixteenth group will be the control group. Indicators of oxidative stress and muscle soreness compared in all the sixteen groups to determine what exercise activity and what duration can induce oxidative stress and muscle soreness optimally.

**Table 3.1, Grouping and process hypotheses**

<table>
<thead>
<tr>
<th>Duration (Minutes)</th>
<th>Swimming</th>
<th>Skipping</th>
<th>Jogging</th>
<th>Downhill Treadmill Running</th>
<th>Uphill Treadmill Running</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
</tr>
<tr>
<td>25</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
</tr>
<tr>
<td>35</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
<td>5 subjects</td>
</tr>
</tbody>
</table>

**H3:** Shorter but more vigorous exercise can induce the same level of oxidative stress and muscle soreness than longer but milder exercise.

After determining the type of exercise which can induce oxidative stress and muscle soreness better, twenty one subjects assigned to 3 groups of 7 subjects to determine whether mild and long exercise or shorter and vigorous exercise induced oxidative stress and muscle soreness. One group is made to do the selected exercise
(based on the results of H2) mildly for a longer period (50 % \( VO_2 \) MAX, 35 min) and the second group was made to do the same exercise vigorously for a shorter time (85% \( VO_2 \)MAX, 15 min). The third group was the control group. The indicators of oxidative stress and muscle soreness compared in the three groups.

**TABLE 3.2. Grouping and process hypotheses**

<table>
<thead>
<tr>
<th>Group</th>
<th>No:</th>
<th>Speed</th>
<th>Time</th>
<th>Type of protocol</th>
<th>Intensity</th>
<th>( VO_2^{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group1</td>
<td>7</td>
<td>14km/h</td>
<td>15</td>
<td>Downhill Treadmill Running</td>
<td>Shorter, but more, vigorous</td>
<td>85%</td>
</tr>
<tr>
<td>Group2</td>
<td>7</td>
<td>10km/h</td>
<td>35</td>
<td>Downhill Treadmill Running</td>
<td>Longer, but milder</td>
<td>50%</td>
</tr>
<tr>
<td>Group3</td>
<td>7</td>
<td>//</td>
<td>//</td>
<td>Control groups</td>
<td>//</td>
<td>//</td>
</tr>
</tbody>
</table>

**H4:** Commercially produced green tea extract has no attenuating effect on muscle soreness and oxidative stress caused by exercise.

1. All groups take an oxygen consumption test on day one to determine \( VO_2 \) peak.

2. At least 24 hours after the exercise, subjects tested on the indicators of muscle soreness and oxidative stress.

3. Two groups of subjects consisting of 20 subjects (10 in each group) made to do muscle-soreness and oxidative-stress-inducing type and level of exercise every other day for three times and given the maximum possible dose of GTE which is allowed for a normal person.

4. 400 mg commercially produced green tea extract capsules of a decaffeinated (< 0.86% caffeine) formulation of polyphones with catechins 82.67%, EGCG 15.7 % (Sunphenon, Taiyo International, MinneapolisMN) administered.

5. Peak plasma EGCG after consumption of 400 mg GTE (~50% EGCG) occurs approximately at 1 hour and stays elevated for several hours post consumption, with levels returning to baseline at ~24 hours post consumption (Chow, et al., 2003).

6. The indicators of oxidative stress and muscle soreness measured in the subjects 24 hours after each round of exercise and compared among the two groups.
7. After a time gap of three weeks and six weeks the same procedure followed two more times to secure the consistency of the observed results in the first run.

### TABLE 3.3, Grouping and process hypotheses

<table>
<thead>
<tr>
<th>Groups</th>
<th>No:</th>
<th>Time</th>
<th>Pre-test</th>
<th>Post-test after 24hr</th>
<th>After 3 week</th>
<th>After 6 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group1</td>
<td>10</td>
<td>15</td>
<td>Blood samples</td>
<td>Blood samples</td>
<td>Blood samples</td>
<td>Blood samples</td>
</tr>
<tr>
<td>Group2</td>
<td>10</td>
<td>15</td>
<td>Blood samples</td>
<td>Blood samples</td>
<td>Blood samples</td>
<td>Blood samples</td>
</tr>
</tbody>
</table>

H5: There is a significant difference in the effect of commercially produced Green Tea Extract, vitamin C and vitamin E on the reduction of Muscle Soreness and Oxidative stress.

To compare the effect of vitamin E and vitamin C with that of commercially produced green tea extract, two of 10 member groups involved in the same exercises described above and given vitamin E and vitamin C (maximum possible dose of vitamin E,C which is allowed for a normal person) at the same intervals described above. The indicators of oxidative stress and muscle soreness in these groups compared with those of the commercially produced green tea extract groups and the control group.

### TABLE 3.4 Grouping and process hypotheses

<table>
<thead>
<tr>
<th>Groups</th>
<th>No:</th>
<th>Type of medicine</th>
<th>Dosage before</th>
<th>Dosage after</th>
<th>Type of protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group1</td>
<td>10</td>
<td>Vitamin E</td>
<td>200 mg</td>
<td>200 mg</td>
<td>Downhill Treadmill Running</td>
</tr>
<tr>
<td>Group3</td>
<td>10</td>
<td>Vitamin C</td>
<td>200 mg</td>
<td>200 mg</td>
<td>Downhill Treadmill Running</td>
</tr>
<tr>
<td>Group3</td>
<td>10</td>
<td>Green tea</td>
<td>200 mg</td>
<td>200 mg</td>
<td>Downhill Treadmill Running</td>
</tr>
<tr>
<td>Group4</td>
<td>10</td>
<td>Control</td>
<td>200 mg</td>
<td>200 mg</td>
<td>Downhill Treadmill Running</td>
</tr>
</tbody>
</table>
H6: Dosage and frequency of commercially produced green tea extract administration do not influence its effectiveness on muscle soreness and oxidative stress.

To determine the optimal dosage and frequency of the commercially produced green tea extract administration for the alleviation of muscle soreness and oxidative stress, six 10-member groups made to do muscle-soreness and oxidative-stress-inducing type and level of exercise (based on the results of H1 and H2) every other day for three times and given commercially produced green tea extract capsules with different doses and frequencies. The indicators of oxidative stress and muscle soreness in these groups compared with those of the commercially produced green tea extract groups and the control group. The amount and frequency of the green tea extract capsules have been showed in table.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dosage Total</th>
<th>6hr pre exercise</th>
<th>Test</th>
<th>6hr post exercise</th>
<th>12hr post exercise</th>
<th>24hr post exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>400mg</td>
<td>200mg</td>
<td>exercise</td>
<td>-200mg</td>
<td>-</td>
<td>Blood samples</td>
</tr>
<tr>
<td>2</td>
<td>600mg</td>
<td>200mg</td>
<td>exercise</td>
<td>200mg</td>
<td>-200mg</td>
<td>Blood samples</td>
</tr>
<tr>
<td>3</td>
<td>800mg</td>
<td>-400mg</td>
<td>exercise</td>
<td>400mg</td>
<td>-</td>
<td>Blood samples</td>
</tr>
<tr>
<td>4</td>
<td>-1000mg</td>
<td>-400mg</td>
<td>Exercise</td>
<td>-400mg</td>
<td>200mg</td>
<td>Blood samples</td>
</tr>
<tr>
<td>5</td>
<td>1200mg</td>
<td>-400mg</td>
<td>exercise</td>
<td>-400mg</td>
<td>-400mg</td>
<td>Blood samples</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>exercise</td>
<td>-</td>
<td>-</td>
<td>Blood samples</td>
</tr>
</tbody>
</table>

3.5. Data Collection

3.5.1. Eccentric Treadmill Run Protocol (EP)

Subjects performed to 15, 25 and 35-minute run on a treadmill at a speed equivalent to 75% of their VO2peak as determined by the GXT. The grade of the treadmill was -10 percent. This intensity and grade has been sufficient in other studies to yield metabolic stress and mechanical damage without serious injury to the subjects (Lee, et al., 2002; Malm, et al., 2004). Intensities above this range may suppress neutrophil activity through increased elevation of immunosuppressive hormones, adrenocorticotropic hormone, and cortisol (Pyne, D. 1994). Before the EP
each subject walked and running at a self selected pace for about 5 minutes to become accustomed to downhill running.

3.5.2. VO2Peak

Expired respiratory gases collected through open circuit spirometry during running with EP for the determination of oxygen consumption (VO$_2$ peak). Expired gas analysis performed with the aid of an automated metabolic measurement cart (Parvomedics True one 2400, Sandy, UT) to assess oxygen consumption and other calculated fitness measures.

3.5.3. Delayed Onset Muscle Soreness (DOMS)

Subjects asked to choose a number on a 7 point Likert scale that represents their level of soreness, 0 being absence of pain, and 6 being severe and limiting pain.

<table>
<thead>
<tr>
<th>Likert Scale for DOMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>[10] A complete absence of soreness</td>
</tr>
<tr>
<td>[12] A moderate pain felt only when touched / a slight persistent pain</td>
</tr>
<tr>
<td>[13] A light pain when walking up or down stairs</td>
</tr>
<tr>
<td>[14] A light pain when walking on a flat surface / painful</td>
</tr>
<tr>
<td>[15] A moderate pain, stiffness or weakness when walking / very painful</td>
</tr>
<tr>
<td>[16] A severe pain that limits my ability to move</td>
</tr>
</tbody>
</table>

3.6. Assays

3.6.1. Blood collection and handling

Blood samples (10mL) were collected by venous puncture from the antecubital vein with the subject seated, after an overnight fast (8-12h), both at rest and 24 Hr after finishing the trial. No tourniquet constriction was used, to minimize potentially enhanced oxidative stress induced by an ischemia-reperfusion maneuver. Blood samples were drawn into EDTA treated vacationer tubes and non-additive serum vacationer tubes and immediately placed on ice in the dark until centrifugation. An aliquot of whole blood was separated to measure hematocrit and hemoglobin. Whole blood in serum tubes was allowed to clot for 30min at room temperature and then centrifuged at 2000 rpm for 10 minutes for serum separation. To obtain the plasma fraction, the remaining whole blood in EDTA-containing tubes
was immediately centrifuged. Erythrocytes were washed and centrifuged three times with a 0.9% sodium chloride solution and listed with ice-cold distilled deionizer water. Serum, plasma and washed erythrocytes were separated into several aliquots and frozen at -80°C for later biochemical analysis.

3.7. Biochemical analysis

3.7.1. Creatine Kinase

The serum sample (4.5 μl) was added to the reagents and was incubated for 2 minutes at 37°C. The absorbance of the sample was read each minute for 2 minutes at 340 nm using a plate reader (Spectrum 384 Plus, Molecular Devices, Sunnyvale, CA). The concentration of CK (IU/L) was calculated based on the ΔAbs./min using the following equation: \( \frac{\Delta \text{Abs/min} \times 1.845 \times 1000}{0.56 \times 6.22 \times 0.0045} \). This equation was a modification of the equation found in the assay kit.

3.7.2. Malondialdehyde

Lipid peroxidation was measured using the TBARS-MDA method. Since the measurements of MDA have been described as non-specific due to the formation of other chromagens at 532 nm, we utilized a modified TBARS-MDA assay kit that corrected for this problem (NW Life Science Specialties, Vancouver, WA). Butyrate hydroxyl toluene (BHT) was added before the TBA reagents in order to prevent amplification of peroxidation during the assay. The addition of BHT also corrects for variations in sample lipid content and the contamination due to iron content in the sample (Halliwell and Chirico, 1993). The plasma samples were thawed at room temperature and 250 μL were pipetted into a microcentrifuge vial containing 10μL of BHT. Additionally, 250 μL each of phosphoric acid and TBA reagent were added to the vial. The vials were vortexed for five seconds and incubated in a warm water bath (60°C) for 60 minutes. After incubation, the samples were centrifuged at 10,000 xg for three minutes and pipetted into the 96-well plate. Samples were analyzed on a plate reader (Spectromax 384 Plus, Molecular Devices, Sunnyvale, CA) at 532 nm. All sample values were calculated using a standard curve derived from calibration standards. Values are reported in μM MDA equivalents.

3.7.3. Lactate dehydrogenase

Lactate dehydrogenase (LDH) will also be measured as the indicators of muscle soreness in this study. LDH activities (Teco Diagnostics, USA) were
measured with Shimadzu UV/Vis 1208 spectrophotometer using commercially available kits.

3.7.4. Dietary Requirements

Subjects were required to refrain from consuming the foods and/or beverages listed in Appendix B for 3 days prior to testing (Manach, et. al., 2004; Scalbert et al. and Williamson, 2000). The foods and beverages listed possess antioxidant properties which could have potentially interfered with the green tea supplementation and confound the results. Otherwise, participants were asked to follow their normal diet. Subjects kept a three-day food intake journal prior to the EP and 24 hours post EP to ensure compliance. Subjects turned in their journals at the 24 hour visit to the lab. Dietary compliance was high, with only one subject reporting a violation: a glass of chocolate milk. Subjects were also required to refrain from using nutritional supplements at least two weeks prior to commencing the study as well as throughout the study. Multivitamins typically contain vitamins C and E that are also considered antioxidants. Studies are conflicting on the amounts and preparations of these vitamins necessary to yield alterations in antioxidant status (Bloomer, et al. 2007; Goldfarb, and McKenzie, 2006; Mastaloudis, et. al., 2006). Since multivitamins vary in the amount and preparation of vitamins C and E, as well as many brands specifically adding other antioxidants, subjects were asked to discontinue usage of their multivitamin for the duration of the study. Subjects were instructed not use anti-inflammatory drugs for three days prior to, or 24 hours post EP. Subjects were asked not to take pain relievers or use ice on the thighs for the 24 hours following the EP. Subjects were also asked not to eat food three hours prior to any exercise protocol.

3.8. Statistical analysis:

An independent statistician was consulted and his services utilized for all statistical analysis. Standard descriptive statistics for man and standard deviation were applied to all varieties measured. Differences between pre- and post test scores within the three experimental groups were determined by one –way ANOVA. In all analyses, the 95 % level of confidence (\( p < 0.05 \)) will apply as the minimum to interoperate significant differences among sets of data. All computations were performed using the statistical presentation system software (SPSS).