Caffeine Supplementation and Moderate Intensity Exercise Modulates the Cytotoxic Lymphocyte Subset (CD+8) in NaIve and Tolerant Individuals

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CAFFEINE SUPPLEMENTATION AND MODERATE INTENSITY EXERCISE MODULATES THE CYTOTOXIC LYMPHOCYTE SUBSET (CD8+) IN NAIVE AND TOLERANT INDIVIDUALS

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Master of Physical Education

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Elizabeth Fedor

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CAFFEINE SUPPLEMENTATION AND MODERATE INTENSITY EXERCISE MODULATES THE CYTOTOXIC LYMPHOCYTE SUBSET (CD8+) IN NAIVE AND TOLERANT INDIVIDUALS

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Caffeine Supplementation and Moderate Intensity Exercise Modulates the Cytotoxic Lymphocyte Subset (CD8+) in Naïve and Tolerant Individuals

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December 2010

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Abstract

The purpose of this investigation was to determine the effects of caffeine supplementation on caffeine tolerant and caffeine naïve individual’s lymphocyte counts, apoptosis and migration levels. In addition, effects of exercise on post-caffeine ingestion lymphocyte counts, apoptosis and migration levels were determined. It was hypothesized that caffeine would alter the immune system cell counts, but that exercise would be able to restore the immune system to homeostasis. Seventeen Western Kentucky University students were tested (males n=7, females n=10; n=7: caffeine tolerant= 200mg or more per day group, n=9: caffeine naïve= 50mg or less per day group). In this double-blind investigation, all participants completed two exercise bouts: 30 min of treadmill running at 60-80% HRR once with a placebo drink before exercise and once with 6 mg/kg body weight of caffeine drink completed in a counterbalanced manner. Blood was taken at rest, 30 min after drink ingestion, immediately post exercise, and 60 min post exercise. Blood was stained with antibody markers (Annexin V to determine apoptotic cell counts, CX3CR1 to determine cell migration, CD4=helper T cells, CD8=cytotoxic T cells, CD19=B cells). Blood was analyzed using flow cytometry. We found that cytotoxic T cells showed significant increases following the cafféinated run in both groups combined (tolerant and naïve, p=0.001) and specifically in the naïve group on the caffeine run (p=.004). We did not see any significant changes in CD4, or CD19 cell counts. There were no significant changes in CD4, CD8 or CD19 cell migration or apoptosis. Our results showed that caffeine supplementation causes an increased effect on cytotoxic T cells counts when combined with exercise, and this effect was greater for the caffeine naïve group. The combined effects of caffeine and exercise may have elevated the plasma catecholamine and cortisol levels which are associated with immune cell function and movement. CD8 cells have a greater density of β-receptors, which are influenced by catecholamine, and may explain the increase in their cell counts compared to CD4 and CD19.
Chapter 1

Introduction

Exercise represents a physiological stress to the body. The bodily systems are equipped to adapt to exercise stressors and inducing stress through exercise to a certain point helps the body to grow stronger. The majority of effects and adaptations of exercise have been studied on the skeletal, muscular, pulmonary, and circulatory systems. However there are more systems and bodily functions affected by exercise. One of these often overlooked systems is our immune system. The immune system helps to keep us healthy by fighting off unwanted intruders, but it also serves to heal tissue and bring our bodies back into homeostasis after stress. The number of white blood cells in our circulation system gives insight to our current health. Low numbers of circulating blood lymphocytes have been shown to be a precursor for illnesses and risk factors, such as a second infarction (Lorgeril et al. 1998), Cardiovascular disease (CVD), Coronary heart disease (CHD) (Madjid et al. 2004), high body mass index (BMI) (Schwartz and Weiss, 1991), hypercholesterolemia, hypertension, diabetes, alcohol consumption, smoking and all cause mortality (Jee et al. 2005, Grau et al. 2004)

Stress caused by exercise effects our immune system. One way we can measure these effects is to study the white blood cells, also called leukocytes. Studies have shown different effects on leukocytes depending on the intensity and duration of exercise. Mooren et al. showed that leukocyte levels change significantly with intense exercise but not significantly with moderate exercise (2009). Steensberg et al. showed that blood counts of white blood cells increase then fall below resting values with intense exercise
The immune system has an important function in homeostasis and repairing damages done to our tissues. One of the ways our immune system regulates itself by altering blood cell counts is through apoptosis, which is a type of programmed cell death (Kerr et al. 1972). Manjo and Joris explain that apoptosis is ‘cell death by fragmentation’ that occurs when a genetic clock selects a given time for the death of marked cells. Oncosis is another type of cell death caused by swelling (1994). Simpson et al. proposed that cells are not dying, but rather they are migrating (also called extravasation) into other tissues (2007). Exercise causes immune system reactions such as increasing the number of circulating leukocytes. However, our body is always trying to maintain homeostasis so when leukocyte counts rise with exercise our body will bring that number back down after the stressor is removed. I propose to study the effects of apoptosis and migration rather than oncosis as there is little evidence of swelling and tissue damage in the bloodstream after exercise.

Exercise stresses our bodies but there are many other things that cause stress to our bodies, such as caffeine. Caffeine is a stimulant that can enhance mean arterial blood pressure, lactate and heart rate (Stebbins et al. 2001; Astorino et al, 2008). Recently caffeine also has been shown to increase blood leukocyte counts (Bishop et al. 2004). Caffeine affects bodily functions, as does exercise and together they have effects that are enhanced and others that may be nullified. Caffeine has been shown to have ergogenic effects with exercise, such as increased endurance (Graham and Spriet, 1995; Graham et al, 1998). It also enhances exercise effects by increasing blood pressure (BP), heart rate (HR) and lactate concentrations (Stebbins et al. 2001). Plasma paraxanthine and
norepinephrine also have been shown to increase (Graham and Spriet 1995). Some participants have experienced negative effects with caffeine. Graham and Spriet had participants with ‘mental confusion’ (1995). Astorino et al described symptoms such as: tremor, insomnia, greater energy, elevated heart rate, and restlessness (2008). Notwithstanding the many studies that have shown caffeine’s effects on hormones, muscles, and the cardiovascular system there has been very little research done on caffeine and leukocytes. One study by Bishop et al. has shown that the number of circulating leukocytes was higher with caffeine ingestion than the placebo group and a significant fall in cytotoxic T cell numbers occurred 1 hour post exercise in caffeine group only (2004). According to the current research to date there are no studies measuring the effects of caffeine ingestion on lymphocyte apoptosis and migration with exercise. More research needs to be done to determine if exercise will change blood apoptosis and cell counts.

**Statement of Purpose**

The purpose of this study is to determine the effects of caffeine on caffeine tolerant and caffeine naïve group’s lymphocyte counts, apoptosis and migration levels. A second purpose is to measure the effects of exercise on post-caffeine ingestion lymphocyte counts, apoptosis and migration levels.

**Statement of Hypotheses**

Ho₁: There will be no change in blood lymphocyte counts with ingestion of caffeine.

Ha₁: There will be an increase in blood lymphocyte counts with ingestion of caffeine.
Ho₂: There will be no change in blood lymphocyte apoptosis levels with ingestion of caffeine.

Ha₂: Caffeine will cause a general increase in blood lymphocyte apoptosis.

Ha₂: Caffeine will cause an increase in blood lymphocyte apoptosis in caffeine naïve group but not in caffeine tolerant group.

Ho₃: There will be no change in blood lymphocyte migration levels with ingestion of caffeine.

Ha₃: Caffeine will cause an increase in blood lymphocyte migration.

Ha₃: Caffeine will cause an increase in blood lymphocyte migration in caffeine naïve group but not in caffeine tolerant group.

Ho₄: Exercise will have no affect on lymphocyte apoptotic or migration levels in the caffeine or placebo trials.

Ha₄: Exercise will cause apoptotic levels to move back to resting, for the caffeinated trials in both groups.

Ha₄: Exercise will cause lymphocyte migration to increase immediately post exercise but return to pre-caffeine normal levels in caffeine naïve group.

Ha₄: Exercise will cause lymphocyte apoptosis to increase in the caffeine tolerant groups.
**Definition of Terms**

- Annexin V – marker of cell apoptosis
- Apoptosis – programmed cell death
- Caffeine – a bitter white alkaloid found in coffee, tea, etc. and used as a stimulant
- CD 4 – Helper T cell marker
- CD 8 – Cytoxic/Killer T cell marker
- CD 19 – B cell marker
- CX₃CR1 – cell migration marker
- Leukocytes – Cells that help the body fight infections and other diseases. Also called white blood cells (WBC).
- Lymphocyte – White cell of the blood that is derived from bone marrow stem cells. There are two main classes of lymphocytes: B cells, which can produce antibodies, and T cells, which are responsible for both cell-mediated immunity and stimulation of B cells.

**Limitations**

- Timing off on the fixation buffer
- Participants may not be able to complete a VO₂max test
- Human error
- Equipment malfunction
- Sweat mixed with blood
• Staining not complete
• Incorrect data recorded
• Too many red blood cells in samples
• Participant cooperation and honesty
• Exercise training may skew results
• Not enough caffeine to elicit a response in caffeine tolerant group
• Not enough caffeine naïve participants available
• Not enough caffeine tolerant participants available
• Nervous reaction to blood draw
• Stress causing fluctuations in lymphocyte blood counts
• Sickness

**Delimitations**
• WKU students (19-30) yrs old caffeine tolerant (average of 200 milligrams a day)
• WKU students (19-30) yrs old caffeine naïve (less than 50 milligrams a day)
• Participants refrain from caffeine for 48 hrs prior to running
• Survey to determine caffeine daily intake
• One control and one test run on each participant
• Test groups ingest 6 mg/kg body weight of caffeine
• 30 min waiting period for caffeine to enter blood
• \( V_{O2\text{max}} \) test to determine 60-80% HRR
• 30 min of 60-80% HRR jogging on treadmill
• Blood collected resting, 30 min after caffeine ingestion, immediately, and 60 min post exercise
• Process blood samples immediately
• Lysis buffer to separate red blood cells
• CD4 to mark Helper T cells
• CD8 to mark Cytoxic T cells
• CD19 to mark B cells
• CX3CR1 to mark cell migration
• Flow cytometer to analyze blood
• Annexin V to mark apoptosis
• Scientific scale to measure 6mg/kg caffeine
• Crystal Light lemon flavor to mask caffeine taste
• Lemon flavor for run 1 and raspberry lemon flavor for run 2
Chapter 2

Literature Review

Leukocytes

Our bodies are amazing organisms with multiple systems that work simultaneously together to allow us to move, live, and experience life. Each system is complicated and detailed acting for the whole to keep us in homeostasis which is a state of equilibrium within the organism. Our daily lives of stress, movement, and work constantly causes our body to leave homeostasis and our systems have to work to return to it. Leaving homeostasis is not a bad thing as there are things we need like exercise that pulls us completely out of homeostasis. Exercise even though it causes stress is necessary for our body to be healthy. Exercise causes great stress on all the bodily systems and stresses them in different ways. Each system adapts to the demands of exercise differently and the majority of the effects and adaptations of exercise have been studied on the skeletal, muscular, pulmonary, and circulatory systems. However, there are more systems and bodily functions affected by exercise. One of these often looked over systems is our immune system. The immune system helps to keep us healthy by fighting off unwanted intruders, but it also serves to heal tissue and bring our bodies back into homeostasis after all types of stress. Without our immune system, all other systems would become corrupted by outside intruders. Our immune system is complicated and full of many different types of cells with different functions. Neutrophils, monocytes, and lymphocytes are some of the main types and the ones that usually get measured.
Monocytes are large nucleated phagocytic cells found in the blood. When they migrate into tissues and organs they are referred to as macrophages (Wise, & Carter, 2002). They have three well defined functions: 1-removal of damaged or dying cells and cell debris, 2-interaction with lymphoid cells in certain phases of immunologic reactions, and 3- defense reactions against certain classes of microorganisms (Cline, 1975).

Neutrophils are 50-60% of all circulating leukocytes and are the first line of microbicidal defense being involved in the inflammatory response (Levada-Pries et al. 2007). They are essential for host defense, and involved in inflammatory conditions (Park et al. 2008).

When there is inflammation neutrophils and macrophages (monocytes) destroy and remove pathogens and damaged tissue cells. These cells do most of the phagocytosis (Wise, & Carter, 2002).

Lymphocytes have a quite a few types. Here are some of the different types and what they do; T lymphocytes are responsible for cell mediated immunity, and do not produce anti-bodies. They can be subdivided into T-cytotoxic (CD8 surface marker, respond to antigen complex MHC [Major Histocompatibility Complex] class I =peptides from intercellular sources) and T-helper cells (CD4 surface marker, respond to antigen complexed with MHC class II molecule = bind to peptides from extracellular sources) based on cell surface markers and function. Cytotoxic T cells - kill foreign cells. After contact, they are able to destroy cells of neoplasms and transplant tissue by apoptosis. They are also able to lyse and kill some virus infected cells. They are only effective in killing cells containing foreign antigens. Helper T cells - are required for the production
of normal levels of antibody by B cells and they aid in the development of CMI. Helper T’s have two subsets Td and Ts. Delayed hypersensitivity T (Td)-they produce several cytokines whose functions include attracting macrophages and other defensive cells. They are also involved in the rejection of transplant tissue, some allergic reaction, and immunity to neoplasms. Suppressor T (Ts)-the T cells that serve to produce cytokines and other effects that suppress the immune response. Last but not least are Natural Killer cells they are able to kill tumor and virus infected cells like cytotoxic T cells they use perforin to lyse target cells. Then there are B lymphocytes, B cells create antibodies. T-cells recognize the intruder; signal the B-cells to create antibodies to attack then Macrophages eat it up (Wise, & Carter, 2002).

**Leukocyte high and low counts**
The number of leukocyte cells in your blood stream is an indicator of immune system activity. Madjid et al. found that leukocyte count is a marker of inflammation (2004). Many studies have been done and have found leukocyte counts to be an indicator of disease. This section goes through what has been found and the risks of having high leukocyte counts.

The study of Lorgeril et al. followed up with patients of heart attacks about their diets (1998). The purpose of the study was to test if diet really reduced the complications and more incidents of heart problems. What they found was Mediterranean diet did help reduce after effects of heart attack. They also found that Leukocyte counts were a marker for increased risk of another infarction. When their leukocyte counts were greater than
9X10^9 /L their risk factor increased from 1.6 to 2.9 of major coronary events, such as cardiac death and acute infarction.

The purpose of the review by Madjid et al. was to discuss the effects of leukocyte counts on coronary heart disease (2004). They found that leukocyte counts is a risk factor for CHD, future cardiac events in people without CVD and a marker of future events in people who have CVD. Also leukocyte counts were found to be higher in patients of coronary atherosclerosis, and or CHD than people without it. High leukocyte counts were also associated with recurrence of a coronary event and total mortality. Patients with CAD have higher leukocyte counts. Leukocytosis is an independent predictor of acute MI. High counts have been shown to predict disease but low counts were found to have an adverse effect also. For patients with CAD five year survival was better for patients who had normal as compared with low relative lymphocyte counts. A few articles were cited that showed a relationship between BMI high leukocyte counts. Some linked obesity to inflammation. The conclusion is high leukocyte counts are associated with increased CHD morbidity and mortality.

The study by Schwartz and Weiss looked into host and environmental factors effecting leukocyte counts (1991). 8,635 subjects between the ages 30-74 were questioned and tested; WBC counts, height, weight, and health history were collected. Leukocyte counts were higher in current and former smokers. The greater the BMI the higher the leukocyte counts, also male sex, and white race. Leukocyte counts were negatively affected by alcohol consumption, the more drinks per week the lower the leukocyte counts. Also height and age are inversely affected.
The study by Jee et al. analyzed white blood cell counts for 438,500 Koreans (2005). Participants filled out health histories on alcohol consumption, smoking habits, and exercise. They found white blood cell count was positively associated with BMI, hypercholesterolemia, hypertension, diabetes, alcohol consumption, and smoking. WBC counts were not associated with death from cancer. High WBC counts in nonsmokers is a greater risk for mortality than in smokers, however, high WBC levels increase risk for both. In summary WBC count predicted all cause mortality.

The study by Grau et al. took 1,000 patients with peripheral arterial disease and looked at their leukocyte counts and health history (2004). They found that recurrent ischemic events increased with increases in leukocyte counts. Within one week but not earlier before an ischemic event leukocyte counts are significantly elevated over baseline. Neutrophil counts especially predict ischemic events. And these elevated counts were significant predictors in men but not in women. They also found current smoking, congestive heart failure, diabetes mellitus, hypertension, Caucasian race, high BMI >30 were independently associated with higher leukocyte counts. However, age ≥ 65yrs was correlated with lower leukocyte counts.

The research by Petitti and Kipp took 62,541 subjects black and white, current, past or non-smokers (1986). They found that leukocyte counts increased with smoking a large number of cigarettes a day in everyone. At every level, leukocyte counts are higher in women than men or the same race; and leukocyte counts are lower in blacks than whites. It was also found that smoking has a persistent, chronic, cumulative effect on
Leukocyte counts; also leukocyte count is related to time since quitting. The longer ago they quit the closer their levels are to non smokers.

**Leukocyte Apoptosis**

Apoptosis is a way for any damaged or unneeded cells to remove themselves from our bodies. The apoptotic cell literally destroys itself from within only leaving fragments for immune cells to clean up. In order to be able to see what cells are undergoing this change we need to determine ways to tell if a cell is apoptotic or not. The following articles are studies done to determine the best cell markers.

Apoptosis- a form of programmed cell death characterized by the fragmentation of nuclear DNA. Cytoxic T and NK cells killing mechanisms’ are: direct cell to cell interactions that involve interaction with Fas (CD95). The Fas ligand (FasL) is expressed on the surface of mature activated CD4+ and CD8+ T cells. Fas interaction with FasL stimulates intracellular signaling events that lead to apoptosis of the infected cell. Cytokine-mediated killing is very similar to cell-cell killing, except that it is based upon cytokines binding to their receptor on target cells, the TNF receptor. These interactions also lead to signaling mechanisms that result in death of the target cells.

When a cell is about to undergo apoptosis it expresses certain proteins on its outer surface. One of these is phosphatidylserine which moves from inside the cell to the outside. One marker, Annexin V has a calcium affinity so it attaches itself to phosphatidylserine easily, thus showing that that cell may undergo apoptosis. There are other markers such as CD95 that attach to Fas ligand expressed on a cell surface about to undergo apoptosis. The articles below discuss various ways to mark apoptosis.
They investigation by Kerr et al. names the phenomenon of apoptosis (1972). The word apoptosis is Greek and is used to describe dropping of falling off of pedals from flowers, or leaves from trees. The emphasis is placed on the last part of the word ‘ptosis’ with the ‘p’ being silent. Apoptosis is manifested histologically by the formation of small roughly spherical or ovoid cytoplasmic fragments. The structural changes of apoptosis take place in two stages: the first compromises the formation of apoptotic bodies, and the second their phagocytosis and degradation by other cells. When a cell is apoptotic there is marked condensation of nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances that form on the cell surface. The author states that apoptosis helps with homeostasis since it can delete cells without tissue disruption or inflammation, plus the cell components are re-utilized. Apoptosis plays an important role in the regulation of normal cell populations as it has occurs in sections of healthy tissues. Apoptosis is precisely controlled possibly by hormones or diffusible substances. Steroid hormones are known to affect apoptosis, by stimulating or inhibiting apoptosis.

The purpose the investigation by Koopman et al. was to test the potential for using Annexin V to mark apoptotic cells (1994). They took tonsil cells and added apoptotic conditions to create apoptosis in the cells. They then stained the cells with Annexin V and it was shown that Annexin V marked apoptosis at an early phase.

The purpose of the investigation by Iwai et al. was to study blood cells (monocytes, lymphocytes, and neutrophils) which expressed the Fas antigen cell surface molecule, (which is a pre-cursor to cell apoptosis) to see how many actually underwent apoptosis spontaneously in-vitro (2007). It was found that lymphocytes remained
relatively stable; neutrophils easily underwent apoptosis monocytes however, only had a small fraction undergo apoptotic changes. It was also found that some T-cell lines were sensitive to Fas antigen and some were not. They also checked for the presence of bcl-2 which is supposed to inhibit apoptosis and found lymphocytes expressed it intensity, monocytes had lower but detectable levels, and neutrophils had no expression of bcl-2.

The review by Manjo and Joris gives the history and definitions of different types of cell death (1994). Glucksmann in 1950 described cell apoptosis in this way “both the nucleus and the cytoplasm…shrink by the loss of fluid…The granule loses its affinity for nuclear stains, becomes Feulgen-negative, breaks up and disappears…The degenerating cell may be phagocytosed by a neighbor…the nucleus may break up into several pycnotic granules”. They listed the key features of apoptosis which are: morphologically the cell shrinks and becomes denser, the chromatin becomes pyknotic and packed into smooth masses applied against the nuclear membrane creating curved profiles, the nucleus may also break up and the cell emits processes (the budding phenomenon) that contain pyknotic nuclear fragments. These processes tend to break off and become apoptotic bodies which may be phagocytized by macrophages or neighboring cells or remain free; however the cell may also shrink into a dense rounded mass as a single apoptotic body. Apoptosis can happen very quickly, it has been measured in 34min; which makes it unobtrusive in tissue sections. When cells undergo apoptosis they emit cellular processes described as budding this has been referred to as “cell death by fragmentation”.

Programmed cell death is also explained as separate from apoptosis. Programmed cell death is when a genetic clock selects a given time for the death of certain cells. Whereas the genetic program of apoptosis specifies the means to produce instant suicide. The
concept of necrosis is also discussed as it related to apoptosis. Necrosis is signaled by irreversible changes in the nucleus (karyolysis, pyknosis, and karyorhexis) and in the cytoplasm (condensation and intense eosinophiia, loss of structure, and fragmentation). We can safely assume these are the features of a cell’s cadaver, whatever the mechanism of the cell’s death. Another type of cell death discussed is oncosis, which is death with swelling.

Through these studies we can see which cell markers have been shown to be effective in marking whether a cell is apoptotic or not. Annexin V has proven itself while Fas antigen and bcl-2 seem to be present in many apoptotic cases they are not always present, therefore they are seldom used alone.

*Exercise and its effects on lymphocytes*

Some of the first immunological changes due to exercise were measured through cell counts. Many studies have focused on or reported the changes that exercise has on the immune cell counts. Simpson et al. reported lymphocytosis (an increase in lymphocyte cell numbers) occurs during and immediately following exercise, followed by a rapid lymphocytopenia (a decrease in lymphocyte cell numbers) in the recovery phase after cessation of exercise (2007). From these changes in counts we realized that something was happening here with the immune cell levels in the blood stream. The studies below took various circumstances such as temperature changes, exercise and diabetes to see the effects on leukocyte blood cell counts.

De Souza et al. studied the effects of jump training on neutrophil responses in rats (2008). They had control, untrained, diabetic, and trained-diabetic groups. Diabetic rats
presented lymphocytopenia when compared to non-diabetic rats. The anaerobic jump training decreased the circulating lymphocyte counts. However the opposite happened to the neutrophils, their count increased with training. Monocyte count was also reduced by physical training. In short the diabetic rats showed lymphocytopenia and neutrophilia which were accentuated by the jump training.

Peake et al. investigated the effect of body temperature on the immune system during and after exercise (2007). Ten well trained male cyclists completed three 90min at 60%V02max trials, once in 18°C, and twice in 32°C. They sat in cold water afterwards twice then once in room temperature post exercise. Blood leukocyte counts all increased significantly following cycling in both temperatures, but did not differ between conditions. Cold water immersion recovery did not have any significant effect on leukocyte count

Cunniffe et al. sampled the blood of 10 international rugby players before, 15min after, 14, and 38 hours after a game. They measured immunoendocrine and inflammatory markers including CD4 and CD8 cell counts. They found a significant decrease in CD4 and CD8 cell counts immediately post exercise. CD4 cells had a significant increase in the 14 hour post time compared to immediately after the game.

The study by Park et al. measured the effects of a triathlon on concentrations of leukocytes, neutrophils, lymphocytes, monocytes and other hormones (2008). Elite (n=7) and amateur (n=8) triathletes competed in the 2006 Tong Yung triathlon. Blood samples were taken 3 days prior, immediately, 2 hours, and 7 days post. They found the leukocytes immediately after the triathlon were significantly increased and remained
increased during recovery in both groups. Neutrophils also remained increased after exercise and during recovery; however the elite group recovered after 7 days. Lymphocytes and monocytes also significantly increased after and during recovery. The lymphocyte levels increased during exercise and fell below pre exercise levels after the triathlon. Neutrophils represent 50-60% of the total circulating leukocyte pool. They are essential for host defense, and involved in inflammatory conditions.

Suzi Hong et al examined the differences in physically active and inactive participant’s lymphocyte counts after a physical or psychological stressor. Forty eight participants were divided into low and high activity groups. Each participant completed an exercise bout and a speech stressor test. They found general lymphocytosis in response to both speech and exercise stressors.

From the literature we can see that exercise has the same effect on blood leukocyte counts and is not altered because of outside changes. Stress also shows some of the same effects as exercise.

**Exercise**

The American College of Sports Medicine along with the American Heart Association have established exercise guidelines for the average person to maintain a healthy lifestyle. The guideline for healthy adults under the age 65 are “a minimum of 20min of cardiovascular exercise, exercising at 60%-80%HRR for 20-30min, excluding time spent warming up and cooling down enables most individuals to fulfill their goals… The 30-minute recommendation is for the average healthy adult to maintain health and reduce the risk for chronic disease.”
**Exercise and apoptosis**

Once we established that leukocyte levels changed because of exercise the next step is to find out why. What might be causing the change? Many are investigating the role of immune cell apoptosis induced through exercise. In this section we will review the findings and theories of why and how apoptosis influences the leukocytes during and after exercise.

Mars et al. tested the effects of high intensity exercise to see if it was related to apoptosis (1998). 11 male did a max test until fatigue, blood was taken before, immediately after, 24 and 48 hours after the test. Blood was analyzed using slides and flow cytometry. Apoptotic cells were identified by peripheral aggregation, polar nucleus, and comet tail effects. Polar clumping was significantly increased in all post exercise samples. The increase 24hrs post was significantly greater than immediately post. Microscopy suggests that approximately 50% of lymphocytes have nuclear changes present 24 hours after exercise while flow cytometry identifies more than 80% of the lymphocytes as being apoptotic. They were not clear if all these cells went on to die or if some would be salvaged. All subjects showed an increase in lymphocyte apoptosis immediately after exercise of 31-48% and of 83-88% 24h after exercise. Their results suggest that there may be accumulative effect in terms of the exercise induced apoptosis.

The article by Phaneuf and Leeuwenburgh was to review literature up to (2000) on apoptosis and exercise. It began explaining what apoptosis is and its function in the body; also the difference between apoptosis and necrosis. Exercise increased glucocorticoid secretion, intracellular calcium concentrations, and reactive oxygen species production- all of which may cause apoptosis. Mitochondria regulate apoptosis by
increasing oxidant production which may cause apoptosis because it damages the DNA. In humans lymphocyte apoptosis has been documented to occur immediately and 24 hours after exhaustive exercise. They think this occurs because during exercise there is an increase in reactive oxygen species causing DNA damage, and an increase in catecholamine levels. They concluded stating that apoptosis is a normal process to remove damaged cells and more research is needed in this area.

The purpose of investigation by Steensberg et al. was to see whether apoptosis contributes to lymphocytopenia (2002). Subjects ran at 75% VO2 max for 2.5 hours. Blood was sampled before, .5 and 1.5 hours during the run, then again at 1,2,4,8, and 24 hours post exercise. Cells were incubated four hours. Lymphocytes increased during the first 30 min then declined. Post exercise they decreased below resting values but were back to normal after eight hours of rest. They found a 60% increase in percentage of early apoptotic lymphocytes two hours post exercise, however the total number of lymphocytes did not change. The concentration of neutrophils increased with exercise but stayed high during recovery. They concluded that apoptosis does not contribute to lymphocytopenia.

The purpose of the research by Cury-Boaventura et al. was to determine if the intake of glutamine dipeptide might decrease leukocyte death (2008). They took 9 elite male triathletes and had them do two exhaustive exercise tests. Some were supplemented with maltodextrin, whey protein; glucose and dipeptide water 30min before. Blood samples were taken before and immediately after exercise, cells were marked and incubated. The MGln supplementation prevented mitochondrial polarization in
lymphocytes and neutrophils induced by exhaustive exercise. It was shown that the MGln helped to prevent apoptosis slightly.

The purpose of the study by Tanimura et al. was to determine the apoptotic effects on leukocytes through the CD95 marker on Helper T (CD4+) and Cytotoxic T (CD8+) cells after short-term high intensity exercise (2009). Kendo athletes (n=8) participated in 6 days of training, 310min each day at estimated 77-88% HR max. Blood samples were taken 2 weeks prior, during training on day 1, 3, 5 and one week after training. Flow cytometry was used to interpret data. Results: total lymphocyte counts were lower on day 3 than pre training and recovered to normal levels after that. Cytotoxic T levels were lower at day 3 than pre but Helper T counts did not change significantly during training. On day 3 the lymphocyte expression of CD95+ increased significantly, Cytotoxic T CD95+ decreased significantly, and Helper T CD95+ increased significantly. At day 5 the lymphocyte levels and CD95 expressions had returned to normal. One issue with this study is as CD95 is a precursor to apoptosis it is not a true marker of apoptosis, there needs to be another marker such as Annexin V along with it to truly measure apoptosis.

Simpson et al conducted an investigation on exercise and cell counts and possible apoptosis (2007). Eight male athletes performed three bouts of exercise; the first was 80%V02 max until exhaustion. The second was 60%V02 max for the duration of the first trial; the third was 80% V02 max on a -10 incline for the duration of their first bout. Blood was sampled before, immediately after, 1hr and 24hrs after exercise. Annexin-V and CD 95 were used to determine apoptosis. CD59 (membrane attack complex inhibiting factor, MACIF) and CD55 (decay accelerating factor, DAF) were measured.
Lymphocyte counts increased immediately after all three treadmill running protocols, with the extent of lymphocytosis being more pronounced after the two 80% protocols. Lymphocytopenia was observed 1hr after the 80% protocols only, before returning to base line levels 24hrs later. Only very low levels of apoptotic lymphocytes were found in the bloodstream, and these did not change in response to exercise. There were also no necrotic lymphocytes in the bloodstream. The number and percentages of lymphocytes expressing CD95 increased immediately after both 0% bouts, followed by a reduction of CD95 positive cells 1hr later. All lymphocyte subsets increased in number immediately post exercise with all numbers falling below the pre-exercise values 1hr after, however, for CD8 and CD56 marked cells the changes and significance were more pronounced. They observed a significant reduction in lymphocyte counts 1hr after exercise no evidence of lymphocyte apoptosis was found. In short they found no evidence to suggest that apoptosis affecting blood lymphocytes contributed to exercise-induced lymphocytopenia. They believe that mechanisms other than cell death by apoptosis are likely responsible for lymphocytopenia. Perhaps an extravasation of particular lymphocyte subsets to other body compartments is responsible.

**Lymphocytes and exercise and apoptosis**

As we noticed the changes in leukocyte counts lymphocytes were always affected. These studies focus on the lymphocytes only some look into possible gender differences, exercise intensity and duration differences. Another broke down further to determine which types of lymphocytes are affected the most by exercise induced apoptosis. Some look at the Helper T cells identified by CD4+ expression, others Cytotoxic T identified by CD8+. 
The investigation by Mooren et al. studied 17 athletes: 9 badly trained, 8 highly trained according to their VO\(_{2}\text{max}\) (2004). All participated in the 2002 Munster marathon. Blood samples were taken 2 days before, immediately after, 3 and 24 hours after the run. Blood samples were measured using flow cytometry, and marked with Annexin V and CD95. After the marathon, leukocyte counts increased and persisted 3 and 24 hours after. Lymphocyte apoptosis decreased slightly for each group immediately after exercise but by the 3 hour mark the badly trained marathoners greatly increased with their Annexin-V+ cells. By 24 hours after apoptotic levels were below resting. In the highly trained group, Annexin-V+ cells decreased steadily from resting through 24 hours. It was found that the highly trained athletes’ lymphocytes seemed more resilient to exercise induced apoptosis as compared to the badly trained group. They also had a group do a high intensity run on a treadmill and a low intensity run. From this they found high intensity/short duration exercise is equally effective in apoptosis induction than low intensity/long duration.

The purpose of the study by Navalta et al. was to determine whether differing levels of estrogen hormones affect the apoptotic effect from maximal exercise (2007). Healthy non-smoking men (n=7) and women (n=7) completed two VO\(_2\text{max}\) tests; the women in the mid-luteal and the follicular phase (at the midpoint of the cycle, one week following menstruation) and men at the same time intervals. Blood was sampled before, immediately after and one hour post exercise. Blood was stained and dried on slides. There was no difference in lymphocyte apoptotic levels between the men and women, even during different times in menstrual cycles. It was also found that two weeks was a sufficient amount of recovery time for levels to return to normal after exhaustive exercise.
The purpose of the investigation by Mooren et al. was to test lymphocyte apoptosis after exhaustive and moderate exercise (2002). 12 volunteers (7 men, 5 women) ran at about 80% V02 max, then again later at 60% v02 max. Their blood was taken before, immediately after and one hour post exercise. It was found one hour post exhaustive exercise lymphocyte levels declined below baseline and 50% increased in the percent of apoptotic cells immediately after exercise. No gender differences were found. Moderate exercise did not change levels or cells or cause apoptosis.

**Caffeine**

Caffeine is a naturally occurring plant methylxanthine. It has stimulating effects, pain relief, diuretic and other effects (Kantamala et al. 1990) Caffeine can enhance mean arterial blood pressure, lactate and heart rate. It has been shown to enhance exercise-induced increases in BP and decreases in systemic vascular resistance (Stebbins et al. 2001).

**Caffeine and its effects on WBC**

The article by Roberts gives some good measurements for caffeine (2003). It states that more than half of American adults consume over 300milligrams of caffeine a day, which is equal to two and a half cups of coffee. Your regular 12 ounce can of soda contains about 45milligrams of caffeine. A seven ounce cup of coffee has about 100 milligrams of caffeine. When caffeine is ingested it takes from 15-45min to concentrate in the blood stream, but its metabolic effects may last over an hour. Caffeine has different effects on different people but studies show that when it comes to exercise consuming caffeine before exercise seems to extend endurance and decrease their sense of overall exertion. However, habitual caffeine users become tolerant of the effects of caffeine
In Kantamala et al.’s study they tested the effects of chronic caffeine consumption on rat Lymphocyte B and T cells, and natural killer cells (1990). Four groups of ten rats each were the control, or ingested daily with 2mg/kg, 6mg/kg, or 18mg/kg of caffeine for 120 days. They found that in the 6mg/kg group there was a significant decrease in the cytotoxicity of splenic NK cells and the overall mitosis of all cells. The other groups did not vary much from the control group. Caffeine exerted an inhibitory effect on both B and T proliferation due to stimulation from mitogenic things (PWM, PHA-P). They hypothesized that chronic caffeine consumption could interfere with the level of intracellular calcium concentration of NK, and B cells, affecting their activities. Or chronic caffeine consumption may result in a lower level of mononuclear cells, especially K K and B cells than in the normal spleen. Caffeine at the same concentrations showed inhibitory effects on both B and T cell proliferation, which also demonstrated a dose-related inhibition.

The study of Liao et al. tested mice that drank only a tea solution or a caffeinated solution for 16 weeks (2004). At the end of the study the mice were killed and their lungs were analyzed. The percent of apoptosis was measured and it was found that cell apoptosis significantly increased from the control group to the .6% tea treated group, which created a decrease in the amount of tumors in the lungs. They determined that cell apoptosis could be another anticancer mechanism of green tea. In the caffeinated group their body weight dropped significantly, however they did not state the apoptotic level of the caffeine group because it was not significant.
Effects of caffeine on exercise

Graham and Spriet conducted research on 8 male well trained runners who completed 4 trials of 85% VO2 max till exhaustion (1995). Their subjects were asked to abstain from caffeine 2 days prior to each test and tests were conducted a week apart. Each trial blood was taken 1 hour before, and then they consumed placebo, 3, 6, or 9 mg/kg of caffeine. Waited an hour took another blood sample, and then completed the running. Blood samples and expired air were taken every 15min and close to exhaustion. They found that 6mg/kg was sufficient to produce similar effects as 9mg/kg. Plasma paraxanthine and norepinephrine increased. With the highest dose FFA concentration and glycerol were increased. Pulmonary VO2 and RER were not affected. Subjects were unable to distinguish when they were given caffeine or the placebo and were not able to distinguish between different doses of caffeine. Blood glucose and lactate increased with exercise and caffeine. They did not find any relationship between caffeine habits and optimal dose. Some of the subjects complained of mental confusion with the 9mg/kg dose. Everyone’s run increased by about 10min in the 3 and 6mg/kg as compared to placebo. The lightest caffeine users had the weak or negative responses (mental confusion) to the 9mg/kg dose.

The research by Stebbins et al. tested the effects of caffeine and body temperature and exercise (2001). Eleven caffeine naïve active men (less than 50mg/kg day) were studied at rest and during exercise after ingestion of 6mg/kg caffeine. Each subject completed two 80min protocols, once with a placebo and once with caffeine. They reported from a 12hour fast, and abstained from caffeinated foods and beverages for 4 days prior. They also abstained from exercise 24hour prior. They found that during
exercise BP, HR and lactate concentrations were greater after caffeine treatment than the placebo.

Graham et al. conducted an experiment on the metabolic and exercise effects of plain caffeine vs. coffee (1998). They took 9 young endurance runners, performed a VO2 max test and then had them run 5 trials at 85% VO2 max to exhaustion. They abstained from caffeine for 48 hours prior. Before each trial resting blood was taken, then they ingested one of the following: placebo, caffeine, regular coffee, decaffeinated coffee, or decaffeinated coffee plus caffeine. All the caffeine pills contained 4.45mg/kg. The coffee was prepared in a way that would also equal 4.45mg/kg of caffeine. One hour after ingestion and rest another blood sample was taken, they then ran their trial. They found that caffeine concentrations actually increased after the exercise bout in every instance. Endurance was increased in significantly in the trial where caffeine only was ingested. There were no significant differences in the running time of the 3 trials where coffee was ingested. When caffeine was consumed independent of coffee there was an enhancement in endurance. They concluded that some components of coffee must interfere with the ergogenic effects of caffeine. Caffeine did not affect the circulating glucose, glycerol and lactate.

Astorino et al. studied the effects of caffeine on one-rep max muscular strength (2008). 22 men, four of which were caffeine naïve, completed two trials a week apart; one placebo and one caffeine dose of 6mg/kg waited an hour then after a 5min warm up completed 1-RM for bench press and leg press. There was no effect of caffeine on either max press. Participants did react to the caffeine with symptoms such as tremor, insomnia,
greater energy, elevated heart rate, and restlessness. These were more pronounced in participants naïve to caffeine. Pre exercise HR and BP was significantly higher after caffeine ingestion, yet not significantly different during warm up as compared to placebo.

Sale et al. studied the effects of bitter orange, green tea and guarana during treadmill walking in overweight males (2006). Ten sedentary overweight males participated in each study. The first study saw the effects of these things on metabolic rate and substrate utilization after ingestion. The second study looked at the effects after 60min at 60%Vox max treadmill walking. Gx had no effect on total ATP utilization during light exercise, however it did increase the contribution of CHO to energy expenditure and decreased fax oxidation. There was no effect of HR and BP even though it contained 150mg caffeine.

Haller et al. conducted their study on the effects of caffeine and exercise (2008). Ten subjects came three times, two of the three times they received caffeine (303.8mg) containing supplement the other was a placebo. One of the caffeine times they rested the other two times they completed a cycle test to fatigue. Blood samples were taken 30, 1, 1.5, 2, 3, 4, 6, 8, and 12 hours after dosing. They found that exercise appeared to diminish slightly the effect on the dietary supplement on BP. No changes in HR were observed. Blood lactate increased in response to exercise with a peak increase 30min after exercise. Dietary supplement sessions were rated easier than placebo 83% of the time. They found that short duration; moderate-intensity exercise does not affect the pharmacokinetics of caffeine.
The study by Skinner et al. measured the dose responses of caffeine on 2000-m rowing exercise bout. Ten competitive male rowers completed the rowing bout with a placebo, 2, 4, or 6 mg/kg caffeine ingestion sixty minutes before exercise. There was no relationship between plasma caffeine concentration and rowing performance. Caffeine may peak in plasma at different times for different people, which may be why they did not find any differences in rowing performance.

**Effects of caffeine and exercise on WBCs**

The study conducted by Bishop et al. tested the effects of caffeine and exercise on lymphocyte counts and activation via CD69+ and norepinephrine, cortisol, FFAs, glucose, and lactate levels (2004). Male endurance trained athletes (n=8) were put into a placebo or caffeine group (were given 6mg/kg body mass of caffeine) and rested for one hour, then cycled for 90min at 70% V02 max. Blood was sampled before caffeine ingestion, immediately before exercise (1hr after consumption), immediately post exercise and 1 hour post exercise. The results were that the numbers of circulating leukocytes were higher in the caffeine group (CAF) than the placebo (PLA). Helper T cells marked by CD4+ showed numbers lower at pre-exercise and 1 hour post compared with pre-treatment in CAF only. Cytotoxic T cells marked with CD8+ were higher in CAF than PLA at pre-exercise. A significant fall in CD8+ numbers occurred 1 hour post exercise in CAF only. Also CD4+ and CD8+ cells expressing CD69 were higher in CAF than PLA at pre, post and 1 hr post exercise (only CD4). They stated that caffeine has the ability to activate Helper and Cytotoxic T cells; also that it influences lymphocyte recruitment and activation.
**Caffeine Naïve vs. tolerant and exercise**

Astorino et al (2008). Participants did react to the caffeine with symptoms such as tremor, insomnia, greater energy, elevated heart rate, and restlessness. These were more pronounced in participants naïve to caffeine.

As was stated earlier constant high leukocyte counts are a risk factor for many problems and diseases. Leukocytes protect our bodies through identifying, marking and destroying harmful pathogens, bacteria, and viruses. They also identify where there is damage and induce inflammation promoting healing of tissue. Exercise causes our leukocyte counts to increase but after they have completed what they were supposed to do these levels need to return to resting. There is a significant decrease in leukocyte counts one hour after exercise. Apoptosis may be one way mechanism our body uses to reduce our levels of blood leukocyte counts. Cells may also be migrating from sites of creation to damaged sites. Research has shown that apoptosis occurs in blood leukocytes after intense long exercise. It occurs more in certain types of cells than others such as, cytotoxic T cells. These seem to be prone to apoptosis following exercise than helper T cells. The reasons for the affinity of some types to apoptosis are still unknown, but are being investigated to better help us understand the mechanisms of exercise and apoptosis.

If we can harness the effects of exercise in increasing or decreasing the blood leukocyte count we may be able to use this one day to help people recover from diseases or prevent the onset of disease. What we know right now is that exercise although it causes stress to our body is a positive stress that helps us release our negative stress and trains our bodies to be able to return to homeostasis quicker than normal despite its effects on leukocyte counts.
Chapter 3

Methods

Subjects

Seventeen students from the student body at Western Kentucky University participated in this study (males = 7, females = 10). Only subjects with a ‘low risk’ status according to ACSM’s PAR-Q questionnaire (Appendix D) were allowed to participate. A power analysis was completed (effect size=.99655, alpha=.05, beta=.08) and it was determined that a total of 3 students are needed in each group (caffeine tolerant and caffeine naïve).

Each subject signed the Human Subjects Review Board approved informed consent located at the back in Appendix B. Participants completed a daily caffeine intake survey (Appendix C) to determine which group they belong to, caffeine naïve or tolerant (Caffeine tolerant= 200mg or more per day, caffeine naïve= 50mg or less per day). The study was counter balanced and double blinded.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine Naive</td>
<td>9</td>
<td>168±11</td>
<td>69±20</td>
<td>23±4</td>
</tr>
<tr>
<td>Caffeine Tolerant</td>
<td>8</td>
<td>170±9</td>
<td>68±10</td>
<td>24±3</td>
</tr>
</tbody>
</table>
**Protocol**

All subjects completed a $V_{02\text{max}}$ treadmill test (3 mph walking warm up for 3 min, the protocol began at 5.6 mph and increased .6 mph every 2 min until volitional fatigue) to determine 60-80% HRR. Heart rate (HR), rate of perceived exertion (RPE), $V_{02}$ and speed were recorded. Subjects refrained from caffeine intake for 24 hours prior to study (i.e. no coffee, tea, nicotine, chocolate, soft drinks, energy drinks, etc.).

Participants completed two exercise bouts 48 hours apart. Before each exercise bout subjects’ blood was taken, and then subjects were given a 10 oz water bottle with Crystal Light lemonade flavor for run 1 and raspberry lemonade flavor for run 2. The study was double blind and counter balanced with participants consuming water and Crystal Light during one run (placebo) and water, Crystal Light, and caffeine (6 mg/kg of caffeine, this level saturates blood caffeine (Graham, Spriet. 1995, Graham et al.1998)) during the other run. Thirty minutes after ingestion, this allows caffeine to concentrate in the blood stream (Roberts, 2003), another blood sample was taken. Subjects then warmed up for 3 min at 3 mph, next were asked to take the speed up to a normal jogging pace. HR was measured and each subject was instructed and monitored to keep their HR within their prescribed 60-80% HRR (ACSM 2006) by adjusting the treadmill speed for 30 minutes. Blood samples were taken immediately post and one hour post exercise.

Blood samples were taken through a finger stick and 200µl whole blood was collected into tubes (Sarstedt, Newton, NC). 20µl blood was placed in each of the six tubes which were prepared with standing buffer (eBioscience, San Diego, CA) and respective antibodies. Three tubes contained Annexin-V-FITC (Bio Vision, Mountain
View, CA) to measure cell apoptosis (Koopman et al. 1994), along with CD4 (Helper T cell marker), CD8 (Cytotoxic T cell marker), and CD19 (B cell marker). The other three tubes contained CX3CR1 (migration receptor) and CD4, 8, and 19. After incubation for 30min in a dark room, the samples were centrifuged, decanted and vortexed. Next 300µl Red Blood Cell Lysis buffer (eBioscience, San Diego, CA) was added, and then samples were vortexed, allowing lysising for 15min. Then 300µl PBS (Sigma, St. Louis, MO) was added and samples centrifuged for 10min. Samples were decanted, vortexed and analyzed using an Accuri flow cytometer (C6, Ann Arbor, MI) to determine cell counts, apoptosis, and migration.

**Statistical Analysis**

Data was analyzed using SPSS software and repeated One-Way ANOVAs comparing caffeine tolerant and naïve groups and caffeinated and non caffeinated runs. Alpha levels were accepted at 0.05.
Chapter 4

Results

Cell Counts

Cytotoxic T cells increased during the caffeinated run (p=.001). For each time:
Pre to post (p=.000), Caffeine to post (p=.003), post to 1hr (p=.000) the difference in CD8 cell counts were significant.

Both Groups Caffeinated Run CD8 + Cell Counts

Figure 1. Cytotoxic T cells counts for the caffeinated run in caffeine tolerant and naïve groups combined. There was significance between post and each time trial: a) pre to post (p=.000), b) Caffeine to post (p=.003), c) post to 1hr (p=.000).
When the groups were separated further into caffeine naïve and tolerant, CD8 cell counts displayed significant differences for the Caffeine Naïve group. Caffeine naïve group counts increased (p=.004) on the caffeinated trial. The caffeine tolerant group also increased (p=.086).

CD8 Caffeine Naïve group for caffeinated trial showed the most change (p=.004). Pre to post (p=.003), Caff to post (p=.005), post to 1hr (p=.001), showing that caffeine had a significant effect on the caffeine naïve groups Cytoxic T cell count blood levels.

Figure 2. Cytotoxic T cell counts for each group (naïve, tolerant) and each trial (placebo, caffeinated) for each time (Pre = Resting, caff = 30 min post drink ingestion, Post = immediately post exercise, 1hr = one hour post exercise). The naïve group had significance between each time [a: Pre to post (p=.003), b: caff to post (p=.005), c: post to 1hr (p=.001)].

There was not any significance in any of the other groups (placebo trial, caffeine naïve and tolerant) for Cytotoxic T cell counts.
The cell count numbers for Helper T (CD4) and B cells (CD19) both had variations for each time blood was taken, but there was no statistical significance in any of the cell counts. There was no difference between groups (caffeine naïve, and tolerant) or runs: CD4 caffeinated run (p=.411) and non caffeinated run (p=.625). CD19 caffeinated run (p=.914) and non caffeinated run (p=.241).

Figure 3. CD4 cell counts for each group (naïve, tolerant) and each trial (placebo, caffeinated) for each time (Pre = Resting, Caff = 30 min post drink ingestion, Post = immediately post exercise, 1hr = one hour post exercise).
Figure 4. CD19 cell counts for each group (naïve, tolerant) and each trial (placebo, caffeinated) for each time (Pre = Resting, Caff = 30 min post drink ingestion, Post = immediately post exercise, 1hr = one hour post exercise).

**Migration**

There was no difference in migration for any cell types (CD4, CD8, CD19) or placebo vs. caffeine runs in time (Pre, Caffeinated, Post, 1hr post).
Table 1. Cell migration. CD4, CD8, CD19 cell migration percentages ± standard deviation per 1mL blood for each time factor: Pre = resting, Caffeine = 30min post drink ingestion, Post = immediately after exercise, 1hr = One hour post exercise.

<table>
<thead>
<tr>
<th>Cell type/trial</th>
<th>Pre</th>
<th>Caffeine</th>
<th>Post</th>
<th>1hr</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo CD4</td>
<td>1.65±2.32%</td>
<td>1.75±2.65%</td>
<td>2.33±3.84%</td>
<td>2.03±2.86%</td>
<td>.916</td>
</tr>
<tr>
<td>Caffeine CD4</td>
<td>2.23±2.83%</td>
<td>3.00±4.71%</td>
<td>2.65±3.56%</td>
<td>1.81±2.99%</td>
<td>.804</td>
</tr>
<tr>
<td>Placebo CD8</td>
<td>19.83±14.37%</td>
<td>20.27±13.22%</td>
<td>25.86±14.28%</td>
<td>17.84±14.62%</td>
<td>.425</td>
</tr>
<tr>
<td>Caffeine CD8</td>
<td>24.78±15.45%</td>
<td>29.47±15.85%</td>
<td>35.54±18.44%</td>
<td>25.66±14.53%</td>
<td>.231</td>
</tr>
<tr>
<td>Placebo CD19</td>
<td>2.63±1.97%</td>
<td>1.68±2.00%</td>
<td>2.41±1.67%</td>
<td>2.79±1.76%</td>
<td>.348</td>
</tr>
<tr>
<td>Caffeine CD19</td>
<td>2.83±2.63%</td>
<td>4.68±8.36%</td>
<td>4.96±4.54%</td>
<td>2.86±2.87%</td>
<td>.503</td>
</tr>
</tbody>
</table>

**Apoptosis**

There was no increase or decrease in apoptosis for any cell types (CD4, CD8, CD19) or placebo vs. caffeine runs in time (Pre, Caffeinated, Post, 1hr post).

Table 2. Cell apoptosis. CD4, CD8, CD19 apoptotic cell numbers ± standard deviation per 1mL blood, for each time factor: Pre = resting, Caffeine = 30min post drink ingestion, Post = immediately after exercise, 1hr = One hour post exercise.

<table>
<thead>
<tr>
<th>Cell type/trial</th>
<th>Pre</th>
<th>Caffeine</th>
<th>Post</th>
<th>1hr</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo CD4</td>
<td>2.44±2.61</td>
<td>2.69±3.14</td>
<td>1.69±1.62</td>
<td>1.88±1.63</td>
<td>.591</td>
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<tr>
<td>Caffeine CD4</td>
<td>2.00±2.03</td>
<td>3.00±2.16</td>
<td>3.06±3.75</td>
<td>2.69±2.41</td>
<td>.664</td>
</tr>
<tr>
<td>Placebo CD8</td>
<td>1.60±1.06</td>
<td>2.31±2.41</td>
<td>2.19±2.37</td>
<td>2.56±2.25</td>
<td>.635</td>
</tr>
<tr>
<td>Caffeine CD8</td>
<td>2.06±1.73</td>
<td>3.75±3.17</td>
<td>5.25±7.74</td>
<td>2.00±1.83</td>
<td>.121</td>
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<tr>
<td>Placebo CD19</td>
<td>20.31±48.40</td>
<td>23.19±46.66</td>
<td>8.63±9.84</td>
<td>17.06±24.26</td>
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<tr>
<td>Caffeine CD19</td>
<td>7.88±5.18</td>
<td>10.94±13.27</td>
<td>6.75±5.74</td>
<td>8.63±6.96</td>
<td>.553</td>
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</table>
Chapter 5

Discussion

The purpose of this study was to determine the effects of caffeine on caffeine tolerant and caffeine naïve group’s lymphocyte counts, apoptosis and migration levels. In addition, this investigation was designed to measure the effects of exercise on post-caffeine ingestion lymphocyte counts, apoptosis and migration levels. It was hypothesized that caffeine would increase the lymphocyte cell counts and exercise would further increase those counts, however the hour post exercise would show cell counts back to normal levels.

The major findings were the changes that caffeine elicited in the cytotoxic T cell subset. Cytotoxic or killer T cell counts rose significantly immediately post exercise. Both the tolerant and naïve groups had significant differences in each time (pre, post caffeine ingestion, and 1 hour post exercise) in the caffeinated run compared to immediately post exercise in their cell counts. When the groups were divided into caffeine naïve and caffeine tolerant the response could be attributed to the caffeine naïve group, in which significant differences were observed. Combined the exercise and caffeine together greatly increased the CD8 cell counts.

These findings are consistent and contradictory to the literature. Some studies have shown cytotoxic T cells to have greater modulatory effects than most other lymphocyte subsets with exercise (Simpson et al. 2007, Tanimura et al. 2009). Caffeine has been shown to increase epinephrine (Graham & Spriet 1995, Bishop et al. 2005), adrenaline (Graham 2001) catecholamine and cortisol levels (Hong et al. 2004, Bishop et
Bishop states that exercise-induced increases in catecholamine and cortisol levels are associated with transformations in immune cell function and movement of circulating leukocytes. CD8 cells express a greater density of beta-receptors than CD4 cells (2005). Perhaps the increase in CD8 cells may be due to the increases in circulating catecholamine and cytotoxic T cell’s sensitivity due to their greater receptor amounts. Exercise training has been shown to down-regulate beta-adrenergic receptors. As caffeine increases catecholamine levels perhaps the difference between the naïve and tolerant group’s responses were because of a down-regulation effect on beta-receptors. This same phenomenon has been seen with exercise training (Hong et al. 2004).

Bishop et al found an increase in CD4 and CD8 cell counts with caffeine ingestion and a significant decrease one hour post exercise with CD8 only (2004). We found a significant increase only in CD8 and only in immediately post exercise compared to all other time modules. We did not see a decrease one hour post exercise in any of the lymphocyte subsets. Bishop et al. did have a longer more intense exercise bout which may explain the difference in the cell counts an hour post exercise. Bishop also stated that caffeine has the ability to activate helper and cytotoxic T cells, and influences lymphocyte recruitment and activation which may help to explain the increase in cells with caffeine, although caffeine itself did not seem to be enough of a stimulant to produce significant effects without exercise. Hong et al. only had participants exercise at a moderate pace for twenty minutes and they saw an increase in CD4, CD8 and CD19 cell counts in response to exercise. Our subjects exercised for thirty minutes at a moderate pace but the only group with a significant increase in counts was the CD8 caffeinated run.
Caffeine has ergogenic effects and effects such as increased BP and HR (Stebbins et al. 2001) and anxiety. Our hypothesis stated that caffeine would affect the immune system as it does other bodily systems. Exercise reduces stress and anxiety. Exercise causes modulatory effects on circulating lymphocytes following intense exercise (Simpson et al. 2007, De Souza et al. 2008, Park et al. 2008) however, Hong et al. found lymphocytosis in Helper T, Cytotoxic T and B cells following a ‘moderate’ exercise bout that lasted for 20 min (2005). This is why we hypothesized that a moderate run would negate the effects of caffeine. Kantamala et al. found that caffeine exerted an inhibitory effect on both B and T proliferation. We saw no significant effect in cell counts for B or helper T cells; perhaps this is due to the inhibitory effect of caffeine even with exercise. Kantamala also hypothesized that chronic caffeine consumption could interfere with the level of intracellular calcium concentration of NK, and B cells, affecting their activities which may be why there was no significant increase or decrease in any of the cell populations with caffeine for the tolerant group.

Studies on caffeine have found its effects to alter homeostasis in different bodily systems (Liao, Graham and Spriet, Stebbins, Sale et al, Astorino et al, Haller et al, Bishop et al). In our study there was no effect to lymphocyte cell counts caused solely by caffeine as was seen by Bishop et al. It was noted that some participants were nervous about the finger prick and the ingestion of caffeine. Hong et al. found a significant increase in circulating cytotoxic and helper T cells after a psychological stressor, a speech (2004). Perhaps we did not see a significant difference between resting and after caffeine ingestion in the subjects because of anxiety. The resting sample may have already had elevated lymphocyte counts because of the stress of the unknown. Although thirty
minutes should be sufficient to allow blood saturation of caffeine (Roberts, 2003), Skinner et al. proposed that individuals metabolize caffeine differently which may alter the amount of time for caffeine to effect the immune system (2010).

We hypothesized that caffeine would influence cell migration. Our results did not show change in migration for any of the cell populations for either group during any time period. There was no difference between placebo runs and caffeinated runs or even before and after exercise. There was a slight increase in each of the lymphocyte subsets, but nothing significant. Simpson et al. hypothesized that when no apoptosis was observed, perhaps the changes in cell counts were due to cells migrating in and out of the blood stream (2007). It is possible that the exercise intensity, the duration, or the combination of the two employed in the present study was insufficient to induce the significant changes in lymphocyte migration that has been observed in previous investigations.

If the cells are not migrating out of the blood stream perhaps they are dying, or undergoing apoptosis. Annexin-V is a ligand marker for early phase cell apoptosis which is what was utilized in the present study. Previous studies of intense or exercise of a long duration have found significant cell apoptosis perhaps causing the change in cell counts (Mars et al. 1998, Steensberg et al. 2002, Tanimura et al. 2009). Similar to cell migration, it is possible that this study did not have the participants exercise intensely or for a long duration which may be the reason why no significant apoptosis was observed.

This study found that cytotoxic T cells are affected the most when it comes to the combination of caffeine and exercise. Perhaps we did not see differences because the
exercise bout may not have been sufficient induce cell migration or apoptosis. One of the purposes was to see if caffeine had detrimental effects on the immune system through looking at cell counts and apoptosis and migration, also to see if the same dose of caffeine affected a naïve or tolerant person differently. The cytotoxic T cells were affected more in the naïve group showing consuming caffeine on a regular basis may reduce the effects caffeine has on your cytotoxic T cell circulating levels. One of the limitations observed was that many of the subjects were nervous for the blood draw which would increase their HR, BP and cause an immune response for the resting blood count levels. This may have caused the effects of caffeine to not show as well. Overall we found that caffeine does affect exercise and together they cause a greater change in CD8 cell counts. Chronically elevated lymphocyte counts are not healthy, but these were increased transiently in our study and by an hour after exercise those cell counts returned close to normal showing that caffeine does not have a detrimental effect on circulating lymphocyte cells in caffeine tolerant and naïve people.
Appendix A
APPLICATION FOR APPROVAL OF INVESTIGATIONS

IN Volving the use of human subjects

Submit to the Office of Sponsored Programs, 301 Potter Hall, by the first working Monday of the month for screening prior to the IRB meeting. Please add additional space between items as needed to describe your project.

The human subjects application must stand alone. Your informed consent document(s), survey instrument, and site approval letter(s) should be attached to the application and referred to in your write up of the appropriate sections so that reviewers may read them as they read your application. Thesis proposals or other documents that are meant to substitute for completing the sections of the application will not be read and should not be attached.

1. Principal Investigator's Name: Elizabeth Fedor
   Email Address: elizabeth.fedor889@wku.edu
   Mailing Address: 141 Coachman Court B Bowling Green, KY 42103
   Department: Kinesiology Recreation and Sport Phone: 270-313-8913
   Completion of the Citi Program Training? Yes X No
   Found at www.citiprogram.org Date 10/7/2009

2. If you are a student, provide the following information:

   Faculty Sponsor: James Navalta
   Department: Kinesiology Recreation and Sport Phone: 270-745-6037
   Faculty Mailing Address:

   Completion of the Citi Program Training? Yes ____ No_____
   Found at www.citiprogram.org Date _______________

   Student Permanent Address (where you can be reached 12 months from now):
   4631 Old Hartford Road Owensboro, KY 42303

   Is this your thesis or dissertation research? Yes X No
Policy of Research Responsibility. The Western Kentucky University Institutional Review Board defines the responsible party or parties of the research project as the Principal Investigator and Co-Principal Investigator. In those cases when a student holds the title of Principal Investigator, the Faculty Sponsor (Advisor, Supervisor, Administrator, or general managing Council) will conduct oversight of the research project and share in the accountability to assure the responsible conduct of research. Researchers outside of the Western Kentucky University campus system are required to provide proof of training to obtain approval for WKU Human Subjects protocols. This proof must be presented by the Compliance Official at the researcher’s institution to the WKU Compliance official. When no training requirement exists at the researcher’s host institution, training must be conducted through affiliation of Western Kentucky University CITI Program.org requirements. WKU faculty, staff, and students are required to complete the CITI Program Training modules outlined by the WKU HSRB.

3. Title of project: Effects of caffeine and exercise on lymphocytes

4. Project Period: Start [ ] upon HSRB approval [ ] End May 10, 2011

   month, day, year

   Note: Your project period may not start until after the HSRB has given final approval.

5. Has this project previously been considered by the HSRB? Yes [X] No [ ]

   If yes, give approximate date of review:

6. Do you or any other person responsible for the design, conduct, or reporting of this research have an economic interest in, or act as an officer or a director of, any outside entity whose financial interests would reasonably appear to be affected by the research?

   Yes [ ] No [X]

   If "yes," please include a statement below that may be considered by the Institutional Conflict of Interest Committee:

7. Is a proposal for external support being submitted? Yes [ ] No [X]
If yes, you must submit (as a separate attachment) one complete copy of that proposal as soon as it is available and complete the following:

a. Is notification of Human Subject approval required?  Yes _____ No _____

b. Is this a renewal application?  Yes _____ No _____

c. Sponsor's Name:

d. Project Period:               From:                         To:

8. You must include copies of all pertinent information such as, a copy of the questionnaire you will be using or other survey instruments, informed consent documents, letters of approval from cooperating institutions (e.g., schools, hospitals or other medical facilities and/or clinics, human services agencies, individuals such as physicians or other specialists in different fields, etc.), copy of external support proposals, etc.

9. Does this project SOLELY involve analysis of an existing database?  Yes _____ No  _X_

If yes, please provide the complete URLs for all databases that are relevant to this application, then complete Section A and the signature portion of the application and forward the application to Sponsored Programs:

If the database is not available in an electronic format readily available on the internet, please provide evidence that the data were collected using procedures that were reviewed and approved by an Institutional Review Board, then complete Section A and the signature portion of the application and forward the application to Sponsored Programs.

In the space below, please provide complete answers to the following questions. Add additional space between items as needed.
I. PROPOSED RESEARCH PROJECT

A. Provide a brief summary of the proposed research. Include major hypotheses and research design.

The proposed purpose of this study is to determine the effects of caffeine, and exercise on caffeine, in tolerant and caffeine naïve lymphocyte apoptosis and migration in specific cell subsets.

In other words I would like to test people who consume caffeine regularly (≥300mg caffeine a day) and those who do not (≤50mg caffeine a day) with caffeine ingestion and exercise. I will test their lymphocyte counts before and after ingesting caffeine (6mg/kg body weight) and after exercise. My hypothesis are that there will be an increase in blood lymphocyte counts with ingestion of caffeine. And caffeine will cause a general increase in lymphocyte apoptosis and migration. Also caffeine will cause an increase in lymphocyte apoptosis in caffeine naïve group but not in caffeine tolerant group. Finally I think that exercise will cause apoptotic levels to move back to resting, pre-caffeine levels in both groups.

Participants will also complete a daily caffeine intake survey to determine which group they belong to, caffeine naïve or tolerant (Caffeine tolerant ≥300mg or more per day, caffeine naïve ≤50mg or less per day). All participants will complete a VO2 max test a week prior to the study to determine 60% of HHR. Blood samples will be taken through a finger stick where 10µl of blood (about ¼ teaspoon) will be collected into tubes to then be analyzed. Resting blood will be taken, next all participants will complete two exercise bouts. Before the exercise each subject will be given a drink, randomly one trial will be a placebo; the other trial will contain 6mg/kg of caffeine, this maximizes blood caffeine levels (Graham, Spriet. 1995). 30min after ingestion of the drink, blood samples will be taken again to allow for caffeine blood saturation. The participant then will perform moderate intensity treadmill exercise according to the ACSM guidelines of 60% HHR for 30min. Immediately post exercise blood will be taken then again 60min post exercise.

B. Describe the source(s) of subjects and the selection criteria. Specifically, how will you obtain potential subjects, and how will you contact them?

Are the human subjects – under 18 years of age, pregnant women, prisoners, or fetus/neonates?

☐ Yes  ☒ No

Students at WKU will be recruited from Exercise Science graduate and undergraduate courses, The Church of Jesus Christ of Latter day Saints student institute classes and through word of mouth. Participation is completely voluntary. Prospective participants will complete a PAR-Q to determine health status. Only participants with ‘low risk’ will be allowed to participate
in the study. Full-time athletes will be excluded. Participants will also complete a daily caffeine intake survey to determine which group they belong to, caffeine naïve or tolerant (Caffeine tolerant ≥300mg or more per day, caffeine naïve ≤50mg or less per day). **All subjects for this study will be between the ages of 20–30 years of age and will be classified as “low risk” according to American College of Sports Medicine (ACSM) guidelines. “Low Risk” means that they are either females <55 years of age or males <45 years of age, that they are asymptomatic, and meet no more than one risk factor threshold for coronary artery disease.** Contacting subjects will involve a) announcements in Exercise Science courses, and LDS institute classes, and word of mouth recruitment.

C. Informed consent: Describe the consent process and attach all consent documents.

All subjects will complete a written informed consent (see attachment) prior to admittance indicating requirements for participation, risks involved, and benefits. The form also will indicate that participation is completely voluntary and that a subject may choose to drop out at any point. They will also complete an ACSM PAR-Q (2006) indicating their risk level. These guidelines classify individuals as “low”, “moderate”, or “high” risk for exercise participation. ONLY subjects classified as “low” risk will be allowed to participate in the current study.

D. Procedures: Provide a step-by-step description of each procedure, including the frequency, duration, and location of each procedure.

All procedures will be located in the Western Kentucky Exercise Science Lab, with a CPR certified and blood borne pathogen trained supervisor present. All participants will complete a VO2 max treadmill test a week prior to the study to determine 60% HHR. Participants will refrain from caffeine intake for 8 hours previous to study (ie. no coffee, tea, nicotine, chocolate, soft drinks, energy drinks, etc.) before each of the two trials. Before, and after each trial blood samples will be taken through a finger stick and 10 µl of blood will be collected into tubes to then be analyzed. After resting blood is taken, all participants will complete two exercise bouts 48 hours apart. Before the exercise each subject will be given a drink, counter balanced and double blinded, one trial will be a placebo; the other trial will contain 6mg/kg of caffeine, this maximizes blood caffeine levels (Graham, Spriet. 1995). 30min after ingestion of the drink, which will allow for blood saturation, blood samples will be taken again. The participant then will warm up for 3min walking on the treadmill, perform 30min of 60%HHR treadmill exercise. Immediately post exercise blood will be taken and again 60min post exercise. Participants will be allowed to cool down for 5min post exercise. Participants will be asked about their RPE at the end of each session.

Blood will be analyzed using flow cytometry for lymphocyte (Helper T, Cytotoxic T and B cell) counts, apoptosis and migration. Blood will be stained with Annexin-V, 7-AAD and CD-95 antigens to measure cell apoptosis. 10µl blood will be added to 250µl standing buffer with antibody cocktail. This will incubate in the dark at room temperature for 30min. After 30min it will centrifuge for 10min, decant and stir using the vortex. Add 300µl Lysis buffer, vortex, let sit for 15min. Add 300µl PBS, centrifuge for 10min. Decant, vortex and analyze using the flow cytometer to determine cell counts, apoptosis, and migration percentages.
E. How will confidentiality of the data be maintained? (Note: Data must be securely kept for a minimum of three years on campus.)

Data will be numerically coded and stored on computer disks, which will be locked in the primary investigators office (Smith Stadium 1058).

F. Describe all known and anticipated risks to the subject including side effects, risks of placebo, risks of normal treatment delay, etc.

Risks associated with this study are running on the treadmill which could result in muscle soreness, cramping or fatigue; and consuming caffeine. Persons may fall off a treadmill, pass out, or in worst case die. The treadmill V02 max test stresses the body’s cardiovascular system to its max. However, we are only using healthy people with low risk of any cardio-pulmonary issues, thus lessening the chances of causing harm to anyone. Another risk may be through the finger stick; it might bruise, swell or cause soreness. I have completed the blood borne pathogen training and so will anyone else who may be taking blood. I am CPR certified and will be present during all testing, if I cannot be there, there will be someone who is CPR certified present. Lastly caffeine is a stimulant that may increase heart rate, blood pressure and cause headaches; however the dose we propose to give has been tested to have a stimulatory effect on the body without causing over-dose effects. The informed consent states all of these concerns.

G. Describe the anticipated benefits to subjects, and the importance of the knowledge that may reasonably be expected to result.

Benefits to the subject include knowing one’s aerobic capacity through the V02 max test. Caffeine has been shown to increase white blood cell counts and high white blood cell counts have been linked to risks of many diseases such as CHD, CAD, hypertension, and diabetes (Jee et al. 2005). Chronic intake of caffeine causes white blood cell counts to rise, but exercise causes white blood cell levels to fall to normal. If we find that exercise can reverse the effects of caffeine it shows that exercise is extremely necessary for habitual caffeine users to buffer the white blood cell count increase caused by caffeine. Also we may confirm the effects of caffeine on blood leukocyte levels, and obtain knowledge of whether caffeine naive and caffeine tolerant individuals react differently to caffeine immunologically.

H. List of references (if applicable):

Additions to or changes in procedures involving human subjects, as well as any problems connected with the use of human subjects once the project has begun, must be brought to the attention of the IRB as they occur.
II. SIGNATURES

A. I certify that to the best of my knowledge the information presented herein is an accurate reflection of the proposed research project.

___________________________________   _____________
Principal Investigator      Date

___________________________________   _____________
Co-Investigator       Date

B. Approval by faculty sponsor (required for all students):

I affirm the accuracy of this application, and I accept the responsibility for the conduct of this research, the supervision of human subjects, and maintenance of informed consent documentation as required by the IRB.

___________________________________   _____________
Faculty Sponsor       Date

C. Approval by Department Head is not required (Some departments require approval by the Department Head. Please verify with your department head if their signature is required). If PI is a director or department head, then the PI's immediate superior should sign.

I confirm the accuracy of the information stated in this application. I am familiar with, and approve of the procedures that involve human subjects.

___________________________________   _____________
D. Advising Physician*:

I certify that I am a duly licensed physician in the State of Kentucky and that, acting as advising physician, I accept the procedures prescribed herein.

___________________________________   _____________
Physician’s Name and Signature    Date

*Physician signature is needed only if the project involves medical procedures and the investigator is not a licensed physician.
Project Title: Effects of caffeine and exercise on white blood cells

Investigator: Elizabeth Fedor, KRS, 270-313-8913

(This portion is for IRB use only.)

HSRB Determination:

Exempt from Full Review ( ) Expedited Review ( ) Full HSRB Review ( )

( ) Disapproval ( ) Approval

( ) Above minimal risk ( ) Minimal risk

a. approval, subject to minor changes

b. approval in general but requiring major alterations, clarifications or assurances

c. restricted approval

Date of review: _____________________
If you have questions regarding review procedures or completion of this IRB application, contact the Office of Sponsored Programs:

Director -- Dr. Steve Haggbloom, Human Protections Administrator, (270) 745-4652

E-mail: Steven.Haggbloom@wku.edu

Compliance Coordinator -- Mr. Paul Mooney, Human Protections Administrator, (270) 745-2129

E-mail: Paul.Mooney@wku.edu
Appendix B
In future correspondence, please refer to HS11-030, September 2, 2010

Elizabeth Fedor  
c/o Dr. James Navalta  
Kinesiology  
WKU

Elizabeth Fedor:

Your research project, *Effects of Caffeine and Exercise on Lymphocytes*, was reviewed by the HSRB and it has been determined that risks to subjects are: (1) minimized and reasonable; and that (2) research procedures are consistent with a sound research design and do not expose the subjects to unnecessary risk. Reviewers determined that: (1) benefits to subjects are considered along with the importance of the topic and that outcomes are reasonable; (2) selection of subjects is equitable; and (3) the purposes of the research and the research setting is amenable to subjects’ welfare and producing desired outcomes; that indications of coercion or prejudice are absent, and that participation is clearly voluntary.

1. In addition, the IRB found that you need to orient participants as follows: (1) signed informed consent is required; (2) Provision is made for collecting, using and storing data in a manner that protects the safety and privacy of the subjects and the confidentiality of the data. (3) Appropriate safeguards are included to protect the rights and welfare of the subjects.

   This project is therefore approved at the Expedited Review Level until May 10, 2011.

2. Please note that the institution is not responsible for any actions regarding this protocol before approval. If you expand the project at a later date to use other instruments please re-apply. Copies of your request for human subjects review, your application, and this approval, are maintained in the Office of Sponsored Programs at the above address. Please report any changes to this approved protocol to this office. A Continuing Review protocol will be sent to you in the future to determine the status of the project. Also, please use the stamped approval forms to assure participants of compliance with The Office of Human Research Protections regulations.

Sincerely,

[Signature]

Paul J. Mooney, M.S.T.M.  
Compliance Coordinator  
Office of Sponsored Programs  
Western Kentucky University

cc: HS file number Fedor HS11-030

The Spirit Makes the Master

Office of Sponsored Programs | Western Kentucky University | 1906 College Heights Blvd. #11026 | Bowling Green, KY 42101-1026

Equal Education and Employment Opportunities • Printing paid from state funds, KRS 57.375 • Hearing Impaired Only: 270.745.3189
INFORMED CONSENT DOCUMENT

Project Title: Effects of caffeine and exercise on white blood cells

Investigator: Elizabeth Fedor, KRS, 270-313-8913
(include name, department and phone of contact person)

You are being asked to participate in a project conducted through Western Kentucky University. The University requires that you give your signed agreement to participate in this project.

The investigator will explain to you in detail the purpose of the project, the procedures to be used, and the potential benefits and possible risks of participation. You may ask him/her any questions you have to help you understand the project. A basic explanation of the project is written below. Please read this explanation and discuss with the researcher any questions you may have.

If you then decide to participate in the project, please sign on the last page of this form in the presence of the person who explained the project to you. You should be given a copy of this form to keep.

1. **Nature and Purpose of the Project:** The purpose of this project is to test the effects of caffeine on white blood cells. I would like to see if consuming caffeine causes your blood white blood cell levels to increase/decrease or not change at all. I also want to measure what effects exercise has on your caffeinated white blood cells.

2. **Explanation of Procedures:** You will fill out this PARQ and if you qualify you will also fill out this habitual caffeine form. Next you will be put into either a caffeine naïve or tolerant group (Caffeine tolerant ≥300mg or more per day, caffeine naïve ≤50mg or less per day). The next time we meet you will perform a V02 max treadmill test that will take about 15min where you will run until exhaustion. From this we will measure what intensity is right for you. A week later you will meet, I will sample your blood through a simple finger stick where we will take a little, about ¼ of a teaspoon, of your blood. Then you will be given a drink, then wait for 30min, after which I will take another blood sample. Finally you will warm up for 3min, run for 30min on a treadmill at an assigned pace (60% or your HRR). I will take another blood sample immediately following your exercise then again after 60min rest.

3. **Discomfort and Risks:** Finger stick may cause soreness and or bruising. Maximal treadmill test requires you to run to exhaustion. 30min of treadmill running. Possible injury if you fall off the treadmill. Shortness of breath, fatigue, and/or soreness. Ingestion of caffeine may increase your heart rate, blood pressure, or cause headaches and loss of focus.

4. **Benefits:** Knowledge of your aerobic capacity, which means the health of your heart and lungs, through the max V02 test. Two exercise sessions and flavored drink. By the end of the study I hope to find out if exercise can reverse some of the harmful effects of caffeine. Knowledge that you will help to further the study of exercise and stimulants on your immune system.
system.

5. **Confidentiality:** You will be assigned a numerically coded identification number. Your data will be stored on computer disks, which will be locked in the primary investigators office (Smith Stadium 1058).

6. **Refusal/Withdrawal:** Refusal to participate in this study will have no effect on any future services you may be entitled to from the University. Anyone who agrees to participate in this study is free to withdraw from the study at any time with no penalty.

   *You understand also that it is not possible to identify all potential risks in an experimental procedure, and you believe that reasonable safeguards have been taken to minimize both the known and potential but unknown risks.*

   Signature of Participant ___________________________  Date __________

   Witness ___________________________  Date __________

THE DATED APPROVAL ON THIS CONSENT FORM INDICATES THAT
THIS PROJECT HAS BEEN REVIEWED AND APPROVED BY
THE WESTERN KENTUCKY UNIVERSITY INSTITUTIONAL REVIEW BOARD
Paul Mooney, Human Protections Administrator
TELEPHONE: (270) 745-4652

[Stamp: HUMAN SUBJECTS REVIEW BOARD
APPROVED]

[Stamp: HSRB APPLICATION # 11-0330
APPROVED 9/12/10]

[Stamp: EXEMPT EXPEDITED FULL BOARD
DATE APPROVED 9/12/10]
Appendix C

Caffeine Questionnaire
Caffeine Survey

Please list each type of beverage or foods, how many days a week you consume said item and how many ounces, cups, bars etc.

<table>
<thead>
<tr>
<th>Type of:</th>
<th>How many days of the week:</th>
<th>How much per day:</th>
</tr>
</thead>
<tbody>
<tr>
<td>soda</td>
<td></td>
<td>cans</td>
</tr>
<tr>
<td>tea</td>
<td></td>
<td>cups</td>
</tr>
<tr>
<td>chocolate</td>
<td></td>
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<tr>
<td>Energy bar</td>
<td></td>
<td>bars</td>
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<tr>
<td>Energy drink</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>other</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix D
Modified AHA/ACSM Health/Fitness Facility Preparticipation Screening Questionnaire
Assess your health status by marking all true statements

History
You have had:

- a heart attack
- heart surgery
- cardiac catheterization
- coronary angioplasty (PTCA)
- pacemaker/implantable cardiac device
- defibrillator/rhythm disturbance
- heart valve disease
- heart failure
- heart transplantation
- congenital heart disease

If you marked any of these statements in this section, consult your physician or other appropriate health care provider before engaging in exercise. You may need to use a facility with a medically qualified staff.

Symptoms

- You experience chest discomfort with exertion.
- You experience unreasonable breathlessness.
- You experience dizziness, fainting, or blackouts.
- You take heart medications.

Other health issues

- You have diabetes.
- You have asthma or other lung disease.
- You have burning or cramping sensation in your lower legs when walking short distances.
- You have musculoskeletal problems that limit your physical activity.
- You have concerns about the safety of exercise.
- You take prescription medication(s).
- You are pregnant.

Cardiovascular risk factors

- You are a man older than 45 years.
- You are a woman older than 55 years, have had a hysterectomy, or are postmenopausal.
- You smoke, or quit smoking within the previous 6 months.
- Your blood pressure is >140/90 mm Hg.
- You do not know you blood pressure.
- You take blood pressure medication.
- Your blood cholesterol level is >200 mg/dL.
- You do not know your cholesterol level.
- You have a close blood relative who had a heart attack or heart surgery before age 55 (father or brother) or age 65 (mother or sister).
- You are physically inactive (i.e., you get <30 minutes of physical activity on at least 3 days per week)
- You are > 20 pounds overweight.

If you marked two or more of the statements in this section consult your physician or other appropriate health care provider before engaging in exercise. You might benefit from using a facility with a professionally qualified exercise staff to guide your exercise program.

- You participate in aerobic exercise 1-2h per session, at least 3-4 days per week
- None of the above

You should be able to exercise safely without consulting your physician or other appropriate health care provider in a self-guided program or almost any facility that meets your exercise program needs.
Bibliography


