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The Effects of Repeated Anaerobic Bouts on Immune Parameters

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THE EFFECTS OF REPEATED ANAEROBIC BOUTS ON IMMUNE PARAMETERS

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The Effects of Repeated Anaerobic Bouts on Immune Parameters

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THE EFFECTS OF REPEATED ANAEROBIC BOUTS ON IMMUNE PARAMETERS

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To date, the exercise-induced immune response in has only been evaluated in subjects performing aerobic exercise. The primary purpose of this study is to determine if repeated bouts of anaerobic exercise will induce a similar immune response in human subjects as exhibited by aerobic exercise. Secondary to immune function, an analysis of performance from trial to trial will be made. Nine males between the ages of 18-25 were selected on a volunteer basis to participate in this study. Subjects performed Wingate tests set in 3 series with 120 seconds between trials within series and 405 seconds between series for an exercise duration of 30 minutes. Blood samples were taken again immediately post test, and 1 hour post test and analyzed for cytokine secretion, epinephrine, and Caspase-3. The present study found that repeated bouts of anaerobic exercise did not alter immune function.

Chapter I

Introduction

In the past studies on the human immune response to exercise have utilized an aerobic cycle or treadmill test at percentages of VO_{2max} to provide the stress needed to elicit a response. Variables such as intensity and duration are adjusted to determine thresholds and correlations to measured parameters of immune functioning. However, most sports are played in bouts of brief, high intensity activity. Also, shorter distance runs at higher intensities are being utilized in workouts for middle distance and marathon runners as well.

Oxidative stress that occurs with aerobic exercise is generally associated with the increase in oxygen consumption; however, when examining oxidative stress induced by anaerobic activity, it is believed that increased lipid and protein oxidation accompanied by the increase of catecholamine levels provides the same means for inducing an immune response which ultimately causes apoptotic cells (Groussard, 2003). This is supported by the data collected by Ježová et al. where only the cycling trial of 2 bouts for 4.5 minutes at a 5.0 W/kg workload (the trial representing the highest intensity level) consistently elicited a significant increase in catecholamine levels (1985).

When studying aerobic exercise, a comparison is usually drawn between intensities of 60% and 80% of max. Results from studies constructed by Mooren et al. (2004) and Wang et al. (2005) show higher intensity correlates with higher levels of apoptosis. Repeated bouts of anaerobic exercise at the appropriate workload would no doubt have an aerobic effect and would be on the higher end of intensity ($\geq 80\%$).

Anaerobic exercise has been associated with elevated levels of substances linked to

apoptosis such as catecholamines and glutathiones (Groussard, 2003.).

The primary purpose of this study is to determine if repeated bouts of anaerobic

exercise will induce a similar immune response in human subjects as exhibited by aerobic

exercise. Because all other studies have focused on stress applied by aerobic activity, it

is important to determine whether or not repeated anaerobic exercise is similar in immune

response. Secondary to immune function, an analysis of performance from trial to trial

will be made.

Statement of Purpose

To date, the exercise-induced immune response in has only been evaluated in

subjects performing aerobic exercise. Since many athletic events require a participant to

perform multiple bursts of anaerobic-type activity, it is important to assess the effect of

repeated intermittent high-intensity exercise on cells of the immune system. Anaerobic

exercise performed in repeated bouts for a prolonged period of time will exceed intensity

(which has been indicated as the primary factor in oxidative stress mediated levels of

apoptosis) of aerobic exercise. Thus, it is important that investigations are made into the

possibility that repeated bouts of anaerobic exercise meet or exceed the level of apoptosis

seen in aerobic exercise.

Hypotheses

- H_{01}: There will be no difference in [TH1/TH2] at Pre-, Post-, and 1HR Post-exercise.
- H_{A1}: [TH1/TH2] will be higher post-exercise than at Pre- and 1HR Post-exercise.

- H_{02}: There will be no difference in [Caspase-3] at Pre-, Post-, and 1HR Post-exercise.
- H_{A2}: [Caspase-3] will be higher post-exercise than at Pre- and 1HR Post-exercise.

- H_{03}: There will be no difference in power output from one Wingate test to the next.
- H_{A3}: Power output will decrease after the first Wingate.

- H_{04}: There will be no difference in performance when comparing Wingates of the same series position.
- H_{A4}: Power out put will decrease from series one to series three when analyzing Wingates of the same series position.

Assumptions

- Subjects did not participate in physical activity 48 hours before participating in trials.

- Subjects did not eat 8 hours before beginning a trial.

- Subjects did not consume alcohol or caffeine 24 hours before participating in trials.

- Subjects did get a full night of sleep before participating in trials.

- Subjects answered pre-participation forms honestly.

- Equipment measured accurately and reliably.

Delimitations

- Subjects performed a baseline Wingate power test.

- Subjects performed repeated Wingate power tests in three series with 120 s between each of the 3 tests in each series and 405 s between series.

- Workload was standardized for Wingate tests.

- Time of trials was uniform.

- Blood was taken by finger prick.

- Samples were taken at: pretest, posttest, 1 hr posttest.

Limitations

- May not be able to achieve statistical power.

- Could not catheterize subjects.

- Reliance upon subjects to adhere to guidelines.

- Sampling error

- Equipment/assay compatibility

- Equipment functioning correctly

Chapter II

Review of Literature

Training status is associated with immune function. Generally this association is that with relatively acute aerobic training, there is a favorable immune response such as maintaining healthy numbers of immune cells. However, upon entering a more chronic level of training, markers of decreased immune function begin to appear. What has yet to be investigated is performance of anaerobic activity in a repeated manner, and if the volume of training could reach a sufficient level as to produce an immune response. It has been proposed that as little as two weeks of sprint interval training (SIT) can markedly improve maximal activity of cytochrome c oxidase, citrate synthase activity, and endurance capacity in comparison to aerobic exercise (Gibala et al., 2006; Burgomaster et al., 2005). If this is the case, there must be a level of oxidative stress that comes from SIT.

Oxidative stress is the damage that results from a negative imbalance with "radical scavenging systems" and reactive oxygen species (ROS) (Wang and Huang, 2005). One negative effect from oxidative stress is the reduction of glutathione (GSH) levels. GSH is a cellular antioxidant meant to protect cellular organelles from oxidizing substances. Exercise has also been shown to alter intercellular calcium levels [Ca^{2+}] (Broadbent and Gass, 2006). When these and other markers exceed healthy levels for the cell, plans are made for the cell to die. This is called apoptosis.

Apoptosis, particularly lymphocyte apoptosis, is one of the primary means for evaluating the stress that exercise places on the immune system. Apoptosis is thought to

have two eliciting mechanisms: oxidative stress and receptor-mediated death. Inflammatory cytokines are main contributors to the latter mechanism. Both have been evaluated in aerobic exercise with results suggesting a positive correlation to apoptosis. Repeated anaerobic bouts at sufficient intensity have been shown to elicit similar training effects as aerobic exercise. If this is true, it would be expected that an increase in inflammatory cytokines and a decrease in anti-inflammatory cytokines would occur post-exercise. There is a particular group of cytokines that are known to initiate and mediate both chronic and acute inflammatory responses as well as cellular and humoral responses. These are known as Th1 and Th2 cytokines. Their name is related to their source. Th1 refers to the fact that they are secreted by Type I helper T cells, and Th2 refers to Type II helper T cells. To come to this conclusion, a review of the following is necessary: training status and apoptosis, exercise intensity and apoptosis, cytokine activity during exercise, and sprint interval training.

Training Status and Apoptosis

In addition to the previously mentioned variables, training status of subjects may influence apoptotic yields. The most obvious comparison to make is between trained and untrained individuals; however, researchers have extended this to compare highly trained subjects by setting an artificial dividing line to create two groups.

Mooren et al. composed a study to examine the effect training status has on lymphocyte apoptosis, and the possibility that it is ligand/receptor mediated (2004). 38 male subjects took part in VO_{2max} testing, after which they were either excluded from further testing or assigned to a highly or lower trained group. All the subjects ran the

2002 Munster marathon. Blood samples were taken from this group 2 days before and then immediately, 3 hours, and 24 hours after finishing the race. Ten healthy, male subjects were the sample for the treadmill run at 80% of max, of which 5 returned 10-14 days later to perform a run at 60% of max for an identical period of time. Blood samples were obtained before testing, immediately after testing, and 1 hour after completing the test. Lymphocytes were prepared by density gradient centrifugation and then measured by flow cytometry; pre- and post-exercise measures were tested by ANOVA. The main finding of this study was that while significant differences in lymphocytic apoptosis exists between highly trained runners and lesser trained runners, the only group that experienced significant increases in apoptosis was the lesser trained group. The authors concluded that training status does affect apoptotic levels in runners; however, no evidence can be provided that other mechanisms, besides ligands and receptors, are not responsible.

Steensberg et. al were aiming to investigate whether factors such as ROS and cortisol attribute to lymphocytopenia through apoptosis (2002). Eleven trained male runners between 24 and 50 years of age had their VO_{2max} determined one week before participation in the experimental trial. The experimental trial consisted of 2.5 hour run at 75% of maximal aerobic output with blood being taken at the following intervals: pretest, 0.5 hr into test, 1.5 hr into test, immediately posttest, 1 hr, 2 hr, 4 hr, and 8 hr posttest. Samples were taken to measure catecholamines, plasma cortisol, F2-isoprostanes, blood mononuclear cells, and apoptosis. Significant decreases were found in lymphocyte number and apoptotic lymphocytes post-exercise. However, from this study, the authors were not able to say that lymphocyte apoptosis contributed to lymphocytopenia.

Broadbent and Gass studied the effect endurance training has on intracellular calcium in helper T lymphocytes in both trained and untrained males (2006). Fourteen trained and nine untrained males between the ages of 23 and 26 years old took part in the 52 week study. During this time the trained group prepared for the Ironman triathlon, and the untrained group remained sedentary. VO_{2max} was determined pre- and post-participation. Resting blood samples were taken every 4 weeks and underwent treatment for extraction of CD4+ lymphocytes for flow cytometric analysis of calcium concentration. Significant findings include: increases in leukocytes in the untrained group between May and June and an increased number of leukocytes between March and April in the trained group. Only sequential changes within groups showed significance in other variables. From this study, the authors concluded that endurance training did not have a chronic immunosuppressive effect on calcium concentration, lymphocyte signaling, or leukocyte concentration.

Perhaps to a lesser extent than intensity, training status plays a role in accretion of both lymphocyte apoptosis and markers of apoptosis. Untrained individuals experience a greater apoptotic response to similar exercise intensities. This is likely due to unfamiliarity of the intensity or inability to handle the oxidative stress being placed on the immune system. Even trained individuals, when assigned as either highly or poorly trained, show differences in apoptotic response. This indicates that as training status increases, immune response decreases unless a stronger stimulus is applied.

Exercise Intensity and Apoptosis

When analyzing the role of exercise intensity on apoptosis, researchers use similar methods. One group will run or cycle for a long duration at roughly 60% of aerobic capacity, and another group will run or cycle for a shorter duration but at approximately 85% of aerobic capacity. It is established that with increasing intensity, increased apoptosis can be expected.

Peters et al. constructed this study to determine the effects that prolonged, high intensity aerobic exercise has on apoptosis in a controlled setting (2006). Eight male, endurance athletes who volunteered for the study, were selected through exclusion criteria to complete a 2.5 hour treadmill run at 75% of max oxygen uptake. Blood samples were taken pre-, post-, and 3 hours after exercise, allowed to clot at room temperature, frozen, and stored. Samples were then centrifuged to extract lymphocytes, which were then washed in PBS and centrifuged again. DNA size was determined by gel electrophoresis, and observed using a fluorescence microscope. Significant changes were found in neither DNA damage nor apoptosis.

Wang et al. conducted this study to observe the underlying mechanisms of apoptosis by inducing oxidative stress onto lymphocytes (2005). Eighteen healthy, sedentary men with an average age of 22.4 ± 1.5 years performed a cycle test in three minute stages which increased in resistance by 20 to 30 W each stage to determine VO_{2max}. Subjects then completed two protocols at approximately 60% and 80% of max for 40 minutes. Blood samples were obtained from the subjects at rest, immediately after, and 24 hours after exercise. Lymphocytes were isolated by density-gradient

centrifugation and treated to determine superoxide and GSH content. Other variables of interest include MTP, active caspases content, lymphocyte viability and DNA fragmentation. The only significant results were the increase in superoxide percent immediately after moderate exercise, GSH percent increase immediately and 24 hours after severe exercise, GSH decrease 24 hours after moderate exercise, lipid peroxidation 24 hours after moderate exercise, and MTP percent decrease both immediately and 24 hours after severe exercise. This study concludes that moderate intensity exercise improves anti-oxidative effects of oxidative stress, and that heavy exercise induces apoptosis through increased oxidative stress by decreasing GSH levels.

Hsu et al. constructed this study to investigate the effects mitochondrial depolarization of leukocytes may have on apoptosis. Twelve trained, male runners (age 21.1±1.8 years, weight 61.4±7.3 kg) volunteered for the study. Subjects performed a VO_{2max} test and ran at 35% of max for subsequent trials on days 1, 3, 5, and 7 (D1, D3, D5, and D7) with blood being taken either pre or post exercise. Subjects were randomly assigned to groups of higher (85%) and lower (60%) intensities for D2. Blood samples were treated for 10 minutes then washed for 5 minutes. Fluorescence was used for MTP and DNA fragmentation then evaluated by flow cytometry. Significant decrease in MTP was seen after day 3 with 60% of max. When intensity was increased to 85%, decreases were seen immediately after exercise. MTP and PMN increased significantly only when aerobic activity was performed above 60% of max. This study concludes that leukocyte mitochondria are affected by exercise in an intensity dependent manner.

Navalta et al. designed this study to determine the exact time of onset of apoptosis, and the point after exercise when apoptosis begins to decrease (2007). Fourteen males

and females (ages 19-32) were chosen on the basis of certain exclusion criteria. They completed a discontinuous treadmill VO_{2max} test with incremental increases in speed (13.4 m/min) until a desired speed was met at which time increases where made by increasing the incline by 2% each stage. Bouts were separated by 2 minute rest periods to collect blood samples. Blood samples of 7mL were taken before and immediately after exercise as well. Whole blood samples were observed under a light microscope; cells were considered apoptotic if they exhibited decreased cell volume, membrane blebbing, or apoptotic bodies. Significant increase in apoptosis occurred at 60% of max reaching peak levels immediately after exercise. Apoptotic index did not significantly decrease until 20 minutes after exercise. The main finding of this study is that a threshold intensity for apoptosis does exist between 40 and 60 percent of max.

Exercise intensity is the primary factor that influences apoptotic yields. There is a positive correlation that exists between these variables in that as intensity increases so does apoptotic yields. While threshold for apoptosis has been set between 40% and 60% (generally accepted more toward 60%), the immune response and characteristics of apoptosis are much more visible as exercise intensifies. Having said this, repeated anaerobic exercise should be much higher intensity exercise, thus creating an even greater apoptotic response than even prolonged endurance exercise.

Cytokine Activity During Exercise

Cytokines are important molecules in mediating a variety of immune responses. Most cytokines are produced by one the various leukocytes with intentions to act upon another leukocyte. There primary functions include but are not limited to cell

proliferation, cell activation, enhanced function of the target cell, and, more systemically, inflammation. During exercise a major concern is the interaction of IL-8 with neutrophils and muscle damage. Many of the important actions exhibited by cytokines relate to their ability to impose positive or negative feedback on other cytokines causing cascade effects thus greatly enhancing a process with little concentration change in individual cytokines.

The purpose of this study, Endurance Exercise Causes Interaction Among Stress Hormones, Cytokines, Neutrophil Dynamics, and Muscle Damage, by Suzuki et al. was to determine if a communication exists between nervous, endocrine, cytokine, and neutrophil behavior, and if so, is it a possible mechanism for exercise-induced muscle damage (1999). Subjects consisted of eight apparently healthy males between the ages of 19 and 21. Subjects performed 90 minute cycling sessions on 3 consecutive days at a workload of 90W. Blood samples (12 mL) were obtained through venipuncture catheterization pre- and post-exercise on all 3 days. Leukocytes were stained then counted by microcell counter with a minimum of 200 cells/slide. Neutrophils were isolated and treated for the purposes of evaluating responsiveness and analyzed through luminescence. Plasma samples were separated for cytokine and hormonal evaluation and frozen until analysis was to be conducted. Findings of this study include: 1) significant increase in plasma growth hormone and IL-6 post-exercise; both were positively correlated to neutrophil response, 2) creatine kinase and myoglobin increased post exercise showing a "delayed onset" in muscle damage-also strongly correlated to neutrophil response, and 3) a negative correlation between catecholamine levels and neutrophil response, thus suggesting high [catecholamine] is a protective measure to muscle damage.

The purpose of the study TNF-α-induced macrophage death via caspase-dependent and independent pathways by Tran et al. was to determine if macrophages inhibited by nuclear factor kappa-light-chain-enhancer of activated B cells, NF-κB, would result in TNF-α-induced lysosomal membrane permeability (LMP) (2009). Buffy coats form healthy donors were isolated by Histopaque. Cells were then either infected with adenovirus (followed by introduction of TNF-α to the culture) or trasnsfected with cathespin B siRNA after cell differentiation. Cells were analyzed for viability, subdiploid DNA, lysosomal integrity, caspase-8 activity, cytosolic enzyme activity, and cytosolic cyotochrome c. Primary results of this study are: 1) loss of lysosomal integrity precedes both DNA fragmentation and cell viability, 2) cathespin B is a contributing factor in TNF-α-induced macrophage death, 3) [caspase-8] and lysosomal integrity are independent of each other, and 4) TNF-α-induced NF-κB suppresses A20, a protein known to prevent TNFα-mediated macrophage death). The author concludes that TNF-α induces macrophage death through both caspase-dependent and –independent pathways.

The purpose of the study Carbohydrate intake during endurance exercise increases natural killer cell responsiveness to IL-2 by McFarlin et al. was to determine the effect carbohydrate supplementation (CHO) during exercise has on NK cell responsiveness (NKCR) to IL-2 (2003). Thirteen male subjects participated in 1 of 2 trials in which either CHO or placebo was administered to them. Exercise consisted of 1 hour of cycling during which time HR and VO_2 were monitored and both CHO and placebo were given every 15 minutes of exercise. One hundred microliter venous blood samples were obtained pre-, post-, 2 hours post-, and 4 hours post-exercise. Whole blood analysis of NKCR to IL-2 was determined by a gamma counter. The main finding of this study is at

post-exercise and 4 hours post-exercise, IL-2 stimulated NKCR was significantly greater in the CHO group than it was in the placebo group.

Timmons et al. designed this study to determine the effectiveness of cytometric bead assay (CBA) in the analysis of cytokine secretion compared to that of ELISA method (2009). Twenty-three subjects (13 females and 10 males) underwent dietary and exercise control. Venous blood samples were obtained both at rest and immediately following 90 minutes of cycling at 65% maximal aerobic capacity. Blood samples were tested for IL-1β, -6, -8, -10, -12, and TNF-α for CBA, but ELISA was only utilized for the analysis of IL-6. While both methods did find a significant increase in IL-6 concentration post-exercise (CBA = 3.75 pg/mL and ELISA = 7.0), correlations of 0.56 show that CBA did not accurately determine the magnitude of IL-6 change.

Inflammatory cytokines do correlate well to the occurrence of apoptosis. TNF-α is perhaps the most potent of these cytokines in directly influencing lymphocyte apoptosis; however, others are involved in activating NK cells, controlling inflammation due to muscle damage, and indirectly controlling immune responses by controlling caspase-dependent pathways and mitochondrial membrane potential.

Sprint Interval Training

In order to make a claim for studying apoptosis in repeated anaerobic bouts, first it must be established that repeated anaerobic bouts utilizes oxidative pathways to some degree. The following studies have examined the effect that training in this manner has on aerobic/endurance performance. In doing so, the researchers have looked at change in aerobic performance and changes in enzyme activity associated with aerobic capacity.

The purpose of the study by Gibala et al. was to determine if low volume, sprint interval training (SIT) solicits similar training adaptations as high volume, endurance training (ET) (2006). Sixteen males of no specific training were randomly assigned to two groups: 8 in SIT group and 8 in ET group. Each subject performed a cycle VO_{2peak} test and time trials at 50 and 750 kJ. SIT group also performed a Wingate test, while the ET group performed a submax test to determine a workload corresponding to 65%. Subjects also had a pre-training muscle biopsy taken. Both training procedures lasted 2 weeks with training days being Monday, Wednesday, and Friday. SIT group performed 30 s repeats at ~700 W with four minutes of rest between bouts, and performed four repeats during trials 1-2, 5 during 3-4, and 6 during 5-6. ET group cycled continuously at 65% of VO_{2peak} for 90 min during sessions 1-2, 105 min during 3-4, and 120 during 5-6. Post-training assessments were conducted in the same manner as pre-training. Cytochrome c was determined with a spectrophotometer. Western blots were used to determine COX II and IV content after separation and buffering of proteins. Absorbency was used to determine RNA concentration and purity. Muscle buffering capacity and resting glycogen storage capacity were all increased in the SIT group over the ET group; however, the difference were not significant. The authors concluded from this study that the two training methods produced very similar muscle adaptations in regards to increased exercise tolerance.

Burgomaster et al. constructed this study to determine the effects of six sessions of cycle SIT on oxidative potential, VO_{2peak}, and time to fatigue (2005). Sixteen subjects (14 males and 2 females) volunteered for the study. Six males and both females were assigned to the 2 week SIT protocol. The other 8 males were assigned to a control group

which took the performance tests two weeks apart. Each subject performed a VO_{2peak} test, a "practice ride" to determine a workload of 80% of peak, and an endurance capacity test at 80% of peak until volitional fatigue. Subjects performed repeated Wingate tests (D1:4; D2:5; D3: 6; D4: 6; D5: 7; D6:4) with four minutes of rest between bouts three times per week with 1-2 days of rest. Subjects were instructed to maintain a similar diet during all stages of testing. Biopsies were used to determine citrate synthase (CS) and metabolite concentrations. Both CS and muscle glycogen content increased significantly over the control group, and cycle endurance capacity doubled in the experimental group. From this study, the authors concluded that repeated bouts of very intense exercise can rapidly increase oxidative capacity.

Billaunt et al. constructed this study to examine the performance changes in repeated sprints with various recovery periods and if gender plays a role. Thirty-three subjects (20 males and 13 females) volunteered to participate. Each subject performed a Force-velocity (F-v) test after a 4 minute warm-up period to determine power output and optimal force (F_{opt}). During the experimental trials, subjects completed a series of 2-8 s sprints against F_{opt}. Each series was separated by 240 s with each sprint being separated by 15, 30, 60, or 120 s. Peak and ending power were recorded along with total work performed. No significant results were found at any rest interval or between gender and rest interval. The authors concluded that power output is not dependant upon rest intervals during short bouts of anaerobic exercise.

Groussard et al. looked to test their hypothesis that short-term, sub-maximal, anaerobic exercise may be a source of oxidative stress (2002). Eight healthy, college age males were recruited on the basis of the physical activity over the last year and visited the

lab on 2 days separated by 8-15 days. On day 1, anthropometric measures, VO_2 estimates, and load corresponding to maximal power output were established. On day 2, subjects were given a standardized breakfast 2 hours before placement of the antecubital catheter, warm-up, and subsequent Wingate test. Blood samples were obtained at rest, immediately after exercise, and 5, 10, 20, and 40 minutes after exercise to analyze plasma TBARS, erythrocyte GSH and SOD, GPx activity, free radical production, and hemoglobin and hematocrit concentration (plasma chemical measures were corrected for change in plasma volume associated with exercise). Results show a significant increase in free radical production from lipids and decreases in plasma TBARS and erythrocyte SOD. Groussard concludes that the increase seen in lipid radical production indicates that anaerobic exercise induces oxidative injury.

As far as the study by Billaunt is concerned, it actually does make sense. Peak power output and minimum could be approximately the same (close enough to say no difference). However, we know nothing of what happened in the middle. It is likely subjects had a much sharper drop-off and spent the last few seconds, especially of longer bouts, at the lower end of their minimum power output. As for the rest of the studies, much evidence is provided to say that similar adaptations occur from training. While one study provides that this happened without increasing VO_{2max}, perhaps after training in this manner perceived exertion has an effect where performing traditional aerobic exercise seems easier.

Conclusion

It is well documented that aerobic exercise causes lymphocyte apoptosis. There is

also support that exercise intensity changes lymphocyte apoptotic levels with a greater response coming from exercise at a higher intensity. Also, the increase in inflammatory cytokines as well as the decrease in anti-inflammatory cytokines correlates well with lymphocyte apoptosis, markers of apoptosis, macrophage death, and activity of pathways that regulate these occurrences. From the literature, sprint interval training solicits a response similar to aerobic training which means there is a level of oxidative stress being placed on the body. This stress should be extended onto the immune system. Thus, it is worth investigating whether repeated anaerobic exercise can induce lymphocyte apoptosis in groups that vary in training status.

Chapter III

Methods

Subjects

Nine males between the ages of 18-25 were selected on a volunteer basis to participate in this study (see Table 1). All subjects agreed to and signed HSRB informed consent documents. All subjects came to the laboratory initially for the purposes of familiarization with the protocol and collection of descriptive data (see table 1). During this time, subjects completed a preparticipation health screening and were classified as "low risk" as established by ACSM guidelines (Whaley, 2006).

Methodology

Day 1: Descriptive Data

On the first visit to the lab, subjects had height and weight measured on the Health o meter 402KL (Sunbeam Products, Inc., Purvis, MS). Body composition was determined by 7-site skinfold assessment using a Lange caliper (Beta Technology, Santa Cruz, CA). Subjects performed Wingate pretest on a Monark 824E (Monark Exercise, Vansboro, Sweden). This is a test to of power output in which subjects pedaled against 7.5% of their body weight, which was then applied to the flywheel of the cycle. They were given 5 seconds to pedal as fast as possible before the resistance was applied. Once the resistance was applied, subjects pedaled as fast as possible for 30 seconds. Power output was calculated as a function of rotations per minute (rpm) which were counted by the SMI Optosensor-and resistance (kg) through SMI Power 5.2.20 software (Sports Medicine Industries, St. Cloud, MN).

Table 1. Descriptive data (N=9)

Table 1	Height (cm)	Weight (kg)	body comp	peak power (W)	peak power (W/kg)	mean power (W)	mean power (W/kg)	Fatigue Index (%)
001-2	180.6	61.4	3.43	646	10.52	522	8.51	35.2
001-4	173.4	70.0	4.94	658	9.42	574	8.21	32.7
001-6	178.0	78.8	7.90	839	10.65	605	7.68	47.9
001-7	182.6	90.6	16.42	855	9.47	699	7.74	41.3
001-8	167.6	81.5	8.00	815	10.27	530	6.68	78.6
002-1	175.3	83.6	24.90	880	10.55	523	6.27	69.0
002-2	185.6	105.5	20.20	941	8.92	717	6.80	57.0
002-3	180.3	88.6	5.70	819	9.26	712	8.05	32.2
002-4	182.9	74.5	8.16	776	10.44	597	8.02	49.6
Mean	178.5	81.6	11.07	803.22	9.94	608.78	7.55	49.3
S.D.	5.6	12.8	7.55	97.32	0.67	81.37	0.78	16.35

Day 2: Experimental Trial

Upon arrival subjects gave a resting blood sample. All blood samples were taken by finger stick, collected in heparinized capillary tubes (Chase Scientific Glass, Inc., Rockwood, TN), and transferred to microcentrifuge tubes for separation of plasma. Subjects then performed Wingate tests set in 3 series. Wingates (Wg) of the same series were separated by 120 seconds. Between series subjects rested for 405 seconds for a total exercise duration of 30 minutes as follows: Wg1-120s-Wg2-120s-Wg3-405s- Wg4-120s-Wg5-120s-Wg6-405s-Wg7-120s-Wg8-120s-Wg9. Blood samples were taken again immediately post test, and 1 hour post test. All blood samples were stored at -40°C in the So-Low Ultra-low freezer (Environmental Equipment, Cincinnati, OH) until used and analyzed for cytokine secretion, epinephrine, and Caspase-3.

Whole Blood Analysis of Cytokines, Caspase-3, and Epinephrine

Whole blood was centrifuged with Histopaque for the separation of plasma. Plasma was then separated into the needed volumes as determined for Th1/Th2 CBA and caspase-3 ELISA (Bender MedSystems GmbH, Vienna, Austria) (25 and 10 μL respectively). Detection of cytokines (IL-1β,-2,-4,-5,-6,-8,-10,-12, TNF-α,-β,-r, and INF-γ) was run as previously described by Chan et al. (2005). Twenty-five microliters of sample was transferred to designated sample tubes, followed by addition of bead mixture and diluted biotin-conjugate for specific cytokine binding. The contents of each tube were mixed and incubated at room temperature for 2 hours. The resulting immuno-complexes were then introduced to a diluted buffer of streptavidin-PE which binds the biotin-conjugate and emit fluorescent signals. After a 1 hour incubation at room temperature the immuno-complexes were suspended in 500μL of the provided assay buffer. The bead suspensions were analyzed by flow cytometry (C6, Accuri Cytometers, Inc., Ann Arbor, MI). Cytokine standards and blanks were handled in the same manner as the samples.

Caspase-3 was analyzed according to the procedure described by Bender MedSystems. One hundred and forty microliters of deionized water was added to the sample wells. Ten microliters of each sample was added to each well and mixed. The plate was then covered and incubated at room temperature (18ºC to 25ºC) for 3 hours. Plates were then uncovered, emptied, and washed 6 times with approximately 400 μL of wash buffer per well. Then, 100 μl of TMB substrate solution was added to all wells, including the blank wells. The microwell strips were again incubated at room temperature (18° to 25°C) for about 10 min. The enzyme reaction was stopped by quickly

pipetting 100 μl of Stop Solution into each well. Absorbance of each microwell was read on a microplate reader (ELx800, Bio-Tek Instruments, Inc., Winooski, VT) at 450 nm.

The epinephrine ELISA was performed according to the instructions provided by the manufacturer (IBL International, Hamburg, Germany). Twenty microliters of each Standard and Control and 500 μL of each plasma sample were pipetted into wells of the extraction plate. Five hundred microliters of DI water was added to the needed wells to correct for differences in volume. Extraction Buffer was pipetted into each well (1000 μL). Extraction took place for 30 min at RT (18-25°C) on an orbital shaker. The plate was immediately emptied. DI water was pipetted into each well (2 mL). The plate was covered with a new adhesive foil and shaken for 5 min at RT on an orbital shaker. The plate was immediately emptied. Extraction Buffer and Acylation Reagent was pipetted into each well (50 μL). The plate was immediately mixed. Extraction took place for 20 min at RT on an orbital shaker. The plate was emptied and residual fluid was removed. DI water was pipetted into each well (2 mL). The plate was covered with new adhesive foil and shaken for 5 min at RT on an orbital shaker. The plate was emptied and residual fluid was removed. Release Buffer was pipetted into each well (300 μL) and shaken for 30 min at RT on an orbital shaker. COMT Enzyme Solution was pipetted into each well of the Microtiter Plate (75 μL). Extracted Standards, Controls and samples were pipetted into the respective wells of the microtiter plate (100 μL). Adrenalin Antiserum was pipetted into each well (50 μL). The plate was covered with adhesive foil and incubated for 120 min at RT on an orbital shaker. The plate was washed 4 times with approximately 250 - 300 μL of diluted Wash Buffer. Excess solution was removed by tapping the inverted plate on a paper towel. Enzyme Conjugate was pipetted into each well (100 μL).

The plate was covered with a new adhesive foil and incubated for 60 min at RT on an orbital shaker. The plate was washed 4 times with 250 - 300 µL of diluted Wash Buffer. Substrate Solution was pipetted into each well (200 µL) and incubated for 40 min at RT on an orbital shaker. The substrate reaction was stopped by adding 50 µL of PNPP Stop Solution into each well. Optical density was measured with a microplate reader at 405 nm (Reference-wavelength: 620-650 nm) within 60 min after pipetting of the Stop Solution.

Statistical Analysis

Data was analyzed by a 1 (subjects) x 3 (pre-, post-, 1 hr post-exercise) repeated measures ANOVA. Statistical significance was established at the $P < 0.05$ level.

Chapter IV

Results

Power Output

Absolute Peak

When comparing power output within a series, only the third series of Wingates did not exhibit a statistical drop-off from first to last (see Table 2). In the first series, Wg1 and Wg2 were significantly greater than Wg3 ($P = 0.035$ and 0.047); in series 2, the first (Wg4) was significantly greater than the third (Wg6) ($P = 0.018$). When comparing Wingates in terms group position (Wg1 is the first Wingate in Series 1, Wg4 is the first Wingate in Series 2, Wg7 is the first in Series 3, etc.), Wg1 was significantly greater than Wg7 ($P = 0.002$) but not Wg4. When looking at Wg2, Wg5, and Wg8, power output decreased from Wg2 to Wg5 and Wg8, but Wg5 was not different from Wg8 (Wg2:Wg5 $P = 0.004$; Wg2:Wg8 $P = 0.00$). Finally, Wg3, Wg6, and Wg9 were all similar in power output (see Figure 1).

Relative Peak

A line of division appears in this variable at Wg5. Wg1, Wg2, and Wg4 were not statistically different. Wg3 was greater but not statistically different from Wg5 or Wg7. Wingates 6-9 were all statistically lower than Wg1, Wg2, and W4 but did not differ from each other (see Figure 2). P-values for Wg1 vs Wg6-9 were: $P = 0.001$, 0.003, 0.000, and 0.001. P-values for Wg2 vs. Wg6-9 were: $P = 0.003$, 0.026, 0.000, and 0.001.

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Finally, P-values for Wg4 vs. Wg6-9 were calculated at the following levels: P = 0.014, 0.038, 0.001, and 0.011.

Absolute Mean

In series 1, power output decreased significantly from Wg1 to Wg3 (Wg1:Wg2 P = 0.006; Wg1:Wg3 P = 0.002; Wg2:Wg3 P = 0.001). Series 2, consisting of Wg4-Wg6, exhibited the same trend (Wg4:Wg5 P = 0.020; Wg4:Wg6 P = 0.007; Wg5:Wg6 P = 0.010). In series 3, Wg7-Wg9, Wg7 was significantly greater than 8 and 9 (Wg7:Wg8 P = 0.011; Wg7:Wg9 P = 0.010). When comparing Wg1, Wg4, and Wg7 (the first trial in each series), 1 and 4 were significantly greater than 7 (Wg1:Wg7 P = 0.000; Wg4:Wg7 P = 0.028). Power output of Wg2, Wg5, and Wg8 decreased significantly from one to the next (Wg2:Wg5 P = 0.000; Wg2:Wg8 P = 0.000; Wg5:Wg8 P = 0.021). Upon comparison of Wg3, Wg6, and Wg9, each was significantly lower than its predecessor (Wg3:Wg6 P = 0.000; Wg3:Wg9 P = 0.001; Wg6:Wg9 P = 0.045) (see Figure 1).

Relative Mean

Relative mean power output on Wg1 was significantly greater than each subsequent Wingate performed (Wg1:Wg2 P = 0.003; Wg1:Wg3 P = 0.001; Wg1:Wg4 P = 0.003; Wg1:Wg5 P = 0.000; Wg1:Wg6 P = 0.000; Wg1:Wg7 P = 0.000; Wg1:Wg8 P = 0.000; Wg1:Wg9 P = 0.000). Series 1 and 2 (Wg1-3 and Wg4-6 respectively) both exhibited the trend of decreasing power output from one to the next (Wg2:Wg3 P = 0.001) (Wg4:Wg5 P = 0.000; Wg4:Wg6 P = 0.000; Wg5:Wg6 P = 0.010). In Series 3 only Wg7 was significantly greater than Wg8 and Wg9 (Wg7:Wg8 P = 0.005; Wg7:Wg9 P = 0.003).

When comparing Wingates of the same series position, power output decreased in all from series to series (see Figure 2).

Fatigue Index

Fatigue index for Wg1 was significantly lower than each subsequent Wingate (Wg1:Wg2 P = 0.009; Wg1:Wg3 P = 0.001; Wg1:Wg4 P = 0.005; Wg1:Wg5 P = 0.016; Wg1:Wg6 P = 0.002; Wg1:Wg7 P = 0.003; Wg1:Wg8 P = 0.016; Wg1:Wg9 P = 0.020). The only series that exhibited a trend in fatigue index was Series 1 (Wg1<Wg2<Wg3) (Wg2:Wg3 P = 0.026) (see Figure 3).

Table 2. Power output

Table 2	Abs Peak	Rel Peak	Abs Mean	Rel Mean	FI
Wg1	799.00±141.04	9.8±0.51	612.11±70.20	7.62±0.85	43.94±16.59
Wg2	763.00±108.13	9.41±.69	536.33±63.38	6.73±1.34	49.76±16.75
Wg3	717.44±114.40	8.84±0.84	486.89±85.48	6.13±1.52	55.16±14.54
Wg4	731.44±133.33	9.01±1.05	556.33±88.42	6.55±1.31	52.36±17.8
Wg5	662.22±99.82	8.19±0.97	456.78±76.65	5.77±1.50	51.07±19.92
Wg6	650.89±88.74	8.05±0.84	440.22±68.46	5.55±1.37	52.97±17.56
Wg7	688.67±166.17	8.39±1.06	471.33±71.14	5.88±1.06	55.03±20.93
Wg8	626.78±105.84	7.73±0.89	432.67±63.10	5.43±1.19	54.2±17.02
Wg9	625.56±100.93	7.74±1.04	413.11±60.36	5.21±1.23	53.44±23.17

Values are expressed as means ± standard deviation. Values labeled as absolute (Abs) are in watts (W) and those labeled relative (Rel) are in watts per kilogram (W/kg). FI is expressed as a percentage.
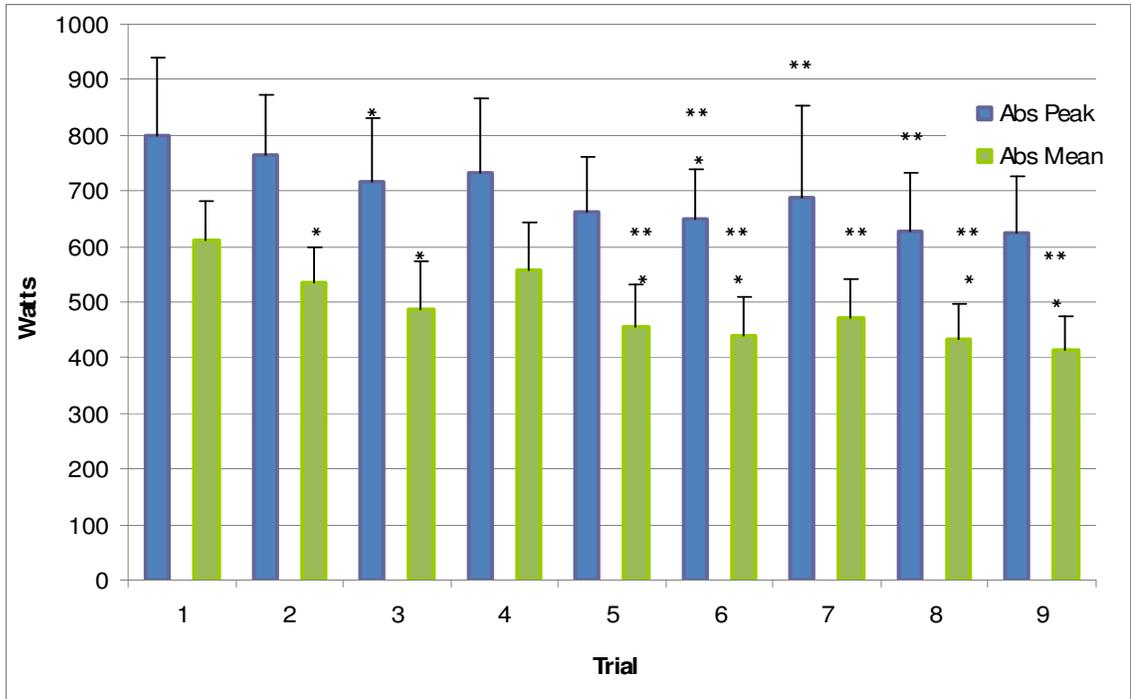
Figure 1. Absolute peak and mean power outputs. An * denotes within series decreases in power output. ** denotes decreases in power output in series position.
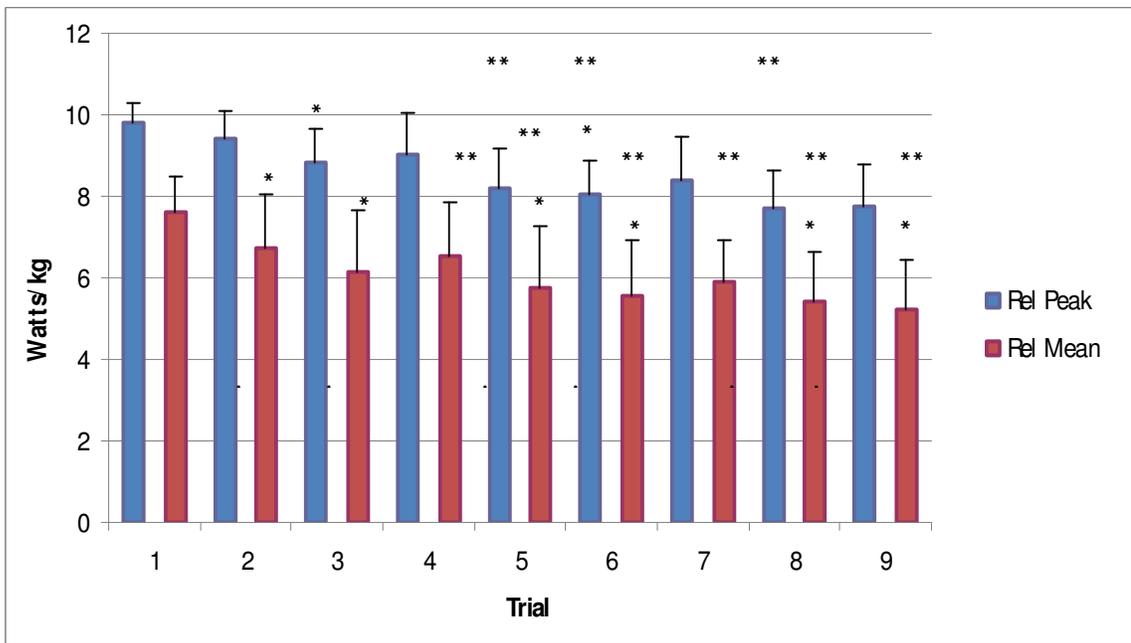

Figure 2. Relative peak and mean power outputs. * denotes within series decreases in power output. ** denotes decreases in power output in series position.
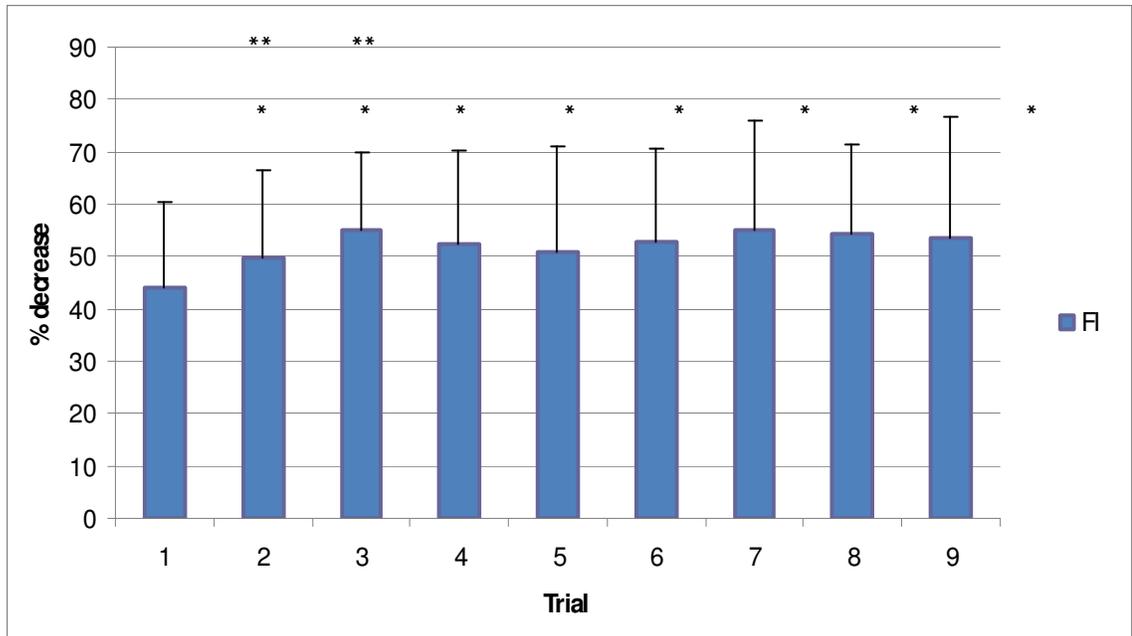
Figure 3. Fatigue index. * denotes an increase in FI from the previous trial. ** denotes increases within a series.

Caspase-3

No significant changes occurred in [Caspase-3] between resting (14.49±6.7ng/mL) and post-exercise values (20.3±11.3ng/mL) or between post-exercise to 1 hr post-exercise values (15.7±10ng/mL).

Cytokines

While select cytokines (IL-5, IL-8, and TNF-beta) did exhibit the trend we expected to see (higher post values than rest and 1hr post values returning to resting levels), none of them experienced enough change from rest to post and from post to 1 hr post to reach significance. IL-12 was the closest to achieving significance between resting and post-exercise values at the 0.112 level. An interesting note is that IL-1β and TNF- beta continued to rise up to 1 hour post-exercise.

Table 3. Concentration of Cytokines

Th1

Cytokine	Pre	Post	1-h Post
IL-2	3510.111±2040.578	5026.667±5549.411	3551.556±1364.223
IL-12	330.2222±29.83613	353.5556±47.48713	352.4444±64.11535
INF-γ	216.3494±26.88542	224.5519±29.37552	221.1627±30.82857
TNF-β	222.2937±221.0077	237.1275±224.8676	239.39166±216.2856

Th2

Cytokine	Pre	Post	1-h Post
IL-4	87319.11±550.7371	87579.11±1195.367	87306.67±1013.032
IL-5	23355.13±42659.76	24837.64±41872.66	23308.87±42679.98
IL-6	321.1111±90.6731	310.8889±45.79696	335.3333±87.13208
IL-10	9382.769±10716.27	9375.446±10722.31	9384.652±10712.66

Cytokine	Pre	Post	1-h Post
IL-1β	3071.778±708.1643	3177.333±820.7937	3221.667±888.5222
IL-8	16606.67±20512.62	16766.75±20378.35	16602.83±20513.32
TNF-α	481.6667±47.44997	496.7778±50.68969	488.3333±52.67352
TNF-r	243.1045±178.9916	252.3656±183.8467	249.171±179.8026

Cytokines are grouped as those that are secreted by Type I helper T cells, secreted by Type II helper T cells, and cytokines that have origins other than T helper cells. Values express the concentrations of the given cytokine in pg/mL. .

Chapter V

Discussion

The purpose of this study was to analyze the effects of repeated anaerobic bouts on immune function as determined by the concentrations of secreted cytokines and caspase-3. Also, due to the use of a novel protocol, an investigation into performance during each of the trials was made. We hypothesized that analysis of cytokines and caspase-3 would yield higher concentrations of both post-exercise than at rest or 1 hour post-exercise. We also hypothesized that performance on the Wingate trials would decrease after Wg1 and that performance would change when analyzing trials of the same series position.

From our findings, the hypothesis that the concentration of cytokines would increase post-exercise above that of resting and 1 hour post-exercise values must be rejected. A study by Timmons et al. did find that cytokines increased post-exercise; however, their results also showed that cytometric bead assays (CBA) do not correlate well with ELISA, and the correlation is worst at the post-exercise interval(approximately 30% lower readings with CBA) (2009). Studies in which cytokines were found to increase post-exercise relied upon other means of evaluation such as ELISA and gamma counter (Suzuki, 1999; Tran, 2009; McFarlin, 2004).

The hypothesis that caspase-3 would increase post-exercise above resting and 1 hour post-exercise values also must be rejected as there were no differences between the three sample times. Caspase-3 is a marker of apoptotic cells as it is a catalyst for the cleaving of cellular proteins (Porter, 1999). That caspase-3 did not increase post-exercise

is in line with the findings of Steensberg et al. where circulating lymphocytes decreased following a 2.5 hour treadmill run; however, the percentage of early apoptotic cells did not change post-exercise. Also, Peters et al. found that circulating lymphocytes increased, but apoptotic yields were not significantly different post-exercise. Both studies utilized flow cytometry for the determination of apoptotic lymphocytes.

As for the hypothesis that performance would decrease from the first bout to the last, we can accept this hypothesis for Fatigue Index and relative mean power output. This is supported by the results from the study by Billaut et al. in which 8 maximal effort cycling trials were performed, and performance did decrease from trial 1 to trial 2 (2003). Other studies of this type have controlled power output such that there should be no or little difference in performance (Gibala, 2006). No other study has separated Wingate tests into series to analyze performance on the basis of series position. We can accept the hypothesis that performance would change depending on which series the Wingate occurred as this was the case in relative mean power, absolute mean power, and absolute peak to an extent.

Concentrations of epinephrine were not obtained because the standards did not fall in the appropriate ranges and could not be used to create a standard curve. However, had we been able to obtain values, it is likely that since we do not have increases in cytokines, we would have seen an increase in epinephrine. It has been proposed that increased catecholamine secretion is a protective measure against the inflammatory response mediated by cytokines (Suzuki, 1999). It is also possible they would have increased from the exercise itself as was reported by French et al. (2007) and Ehsani et al. (1984).

Part of the lack in significant differences may be due in part to the exercise itself. While performance of the exercise was difficult, the fact is that cycling requires much more concentric effort than eccentric. This is supported by the study Quadriceps femoris electromyogram during concentric, isometric and eccentric phases of fatiguing dynamic knee extensions by Pincivero et al. in which EMG muscle activation was analyzed during a 5 minute bout of cycling (2006). Results showed that muscles of the quadriceps are most active during the concentric phase and least active during the eccentric phase of cycling. The eccentric phase of a movement is known to cause greater muscle damage than concentric. Thus, it is possible cytokines responsible for the recruitment of neutrophils and macrophages to the site of injury were not needed due to a lack of exercise induced muscle damage.

It is possible that the difference in performance from Wg1-Wg5 to Wg6-Wg9 could have altered the results. As a consideration for furthers studies, perhaps the workload should be altered such that power output is more consistent throughout the trials. Although intensity is generally credited as the primary contributing factor in inducing an immune response in aerobic exercise, it is possible that the opposite is true of anaerobic exercise (Peters, 2006; Hsu, 2002; Wang, 2005). Having said that, future studies should consider the possibility of increasing the duration of the exercise protocol to 45 minutes or one hour.

The present study found that repeated bouts of anaerobic exercise did not alter immune function. As previously stated cytokines did not reach significant differences to cause a receptor-mediated immune response. It is also likely that the duration of exercise was not long enough to cause an immune response due to oxidative stress. New research

even suggests that transient levels of exposure to oxidative stress improve immune

function (Hurst, 2009).

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