The Cytokine Response to Three Days of Intensive Training

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ABSTRACT

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This experiment determined the acute and cumulative responses of IL-6 and TNF-α to three consecutive days of intensive training (Session I, II and III). Results showed that exercise caused a similar increase in IL-6 from pre- to post-exercise for all sessions, but no changes in resting, baseline values. Results also showed an increase in TNF-α after Sessions II and III, but not Session I. The percent change in TNF-α was greatest for Session II, and resting TNF-α was significantly elevated by the end of the study. In conclusion, three days of intensive training separated by ~24 hours do not result in a significant widening of the "Open Window" period following exercise, as the environment created by exercise in predominantly anti-inflammatory, as evidenced by increased IL-6. This means that athletes who train intensively for three consecutive days do not appear to be at a greater risk of becoming ill.

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bpm	Beats per minute
CK	Creatine Kinase
dL	Deciliters
EDTA	Ethylenediaminetetraacetic Acid
H_b	Hemoglobin
Hct	Hematocrit
HR	Heart Rate
IL-6	Interleukin-6
kDa	Kilodalton
µg	Micro grams
mL	Milliliters
OTS	Overtraining Syndrome
pg	Picograms
RER	Respiratory Exchange Ratio
RPE	Rating of Perceived Exertion
TNF-α	Tumor Necrosis Factor-alpha
VO_{2max}	Maximal Oxygen Consumption
VO_{2peak}	Peak Oxygen Consumption
W	Watts

CHAPTER I

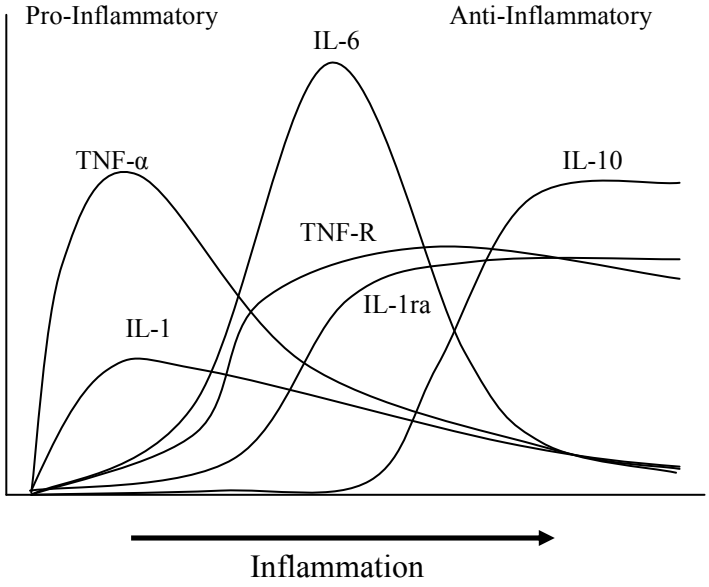
Introduction

Following an injury or an infection, there is a complex sequence of cellular and humoral responses aimed at limiting tissue damage and activating the repair process, collectively known as inflammation.[35] The inflammatory process begins with an initial phase of acute neutrophil accumulation, followed by mononuclear cell accumulation, and ends with a resolution phase, in which repair of injured tissue is completed.[5, 35] Inflammatory reactions that are acute in nature usually run a definite and limited time course; however, if the injurious stimulus is repeatedly applied, as may occur with sports training, chronic inflammation may develop.[35]

In both acute and chronic inflammation, there is an up-regulation of cytokines.[20, 25, 35] Cytokines are soluble glycoproteins, usually between 8-30 kilodaltons (kDa) in mass, which are produced by, and mediate communication between and within immune and non-immune cells, organs, and organ systems throughout the body.[10, 16, 17, 18, 21, 22] Cytokines can be functionally divided into two groups: pro-inflammatory cytokines and anti-inflammatory cytokines.[25] Pro-inflammatory cytokines are up-regulated by physical activity, trauma, autoimmune diseases, infection and certain types of cancer.[18, 20, 35] Pro-inflammatory cytokines are important in the initiation and progression of the inflammatory process.[21, 35] Two major pro-inflammatory cytokines are Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6).[20, 25, 28, 35] Conversely, anti-inflammatory cytokines serve to attenuate the

inflammatory network once the inflammatory process has begun.[19, 21, 35] Anti-inflammatory cytokines include Tumor Necrosis Factor-Receptor (TNF-R), Interleukin 1-receptor agonist (IL-1ra) and Interleukin10 (IL-10).[20, 21, 25, 28, 35] IL-6 has also been shown to have aspects of an anti-inflammatory cytokine. It may have immune regulatory effects by inhibiting TNF-α,[14, 26, 39] a major pro-inflammatory cytokine. It also induces upregulation of IL-1ra, a major anti-inflammatory cytokine.[20, 39] Figure 1 illustrates the proposed interrelationship of the major pro- and anti-inflammatory cytokines.

Figure 1. The cytokine cascade. Pro-inflammatory cytokines, TNF-α and IL-1 are released at the start of inflammation. Anti-inflammatory cytokines, TNF-R, IL1-ra and IL-10, attenuate the cascade once the inflammatory process has begun.[20, 21, 25, 28, 35] IL-6 has been shown to have aspects of both a pro- and anti-inflammatory cytokine.[16]

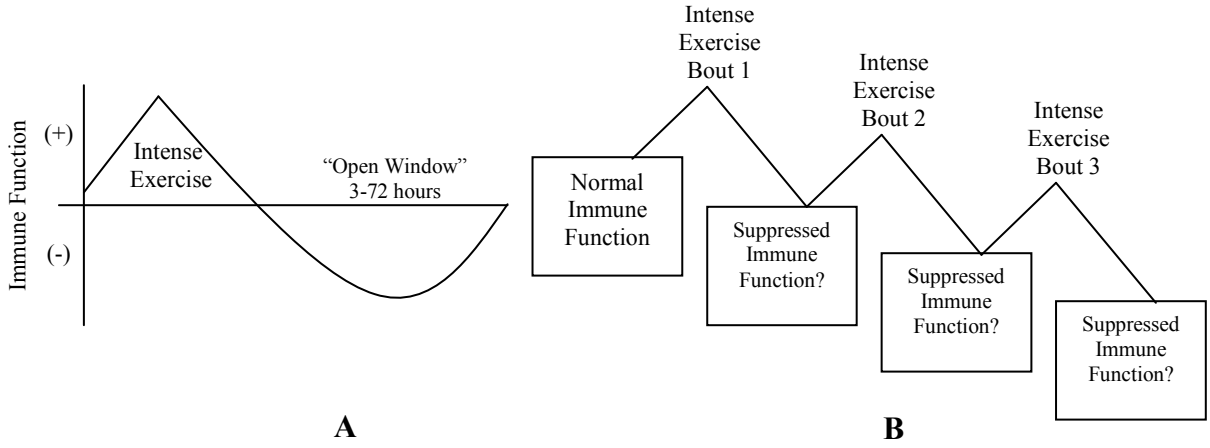


(Adapted from Pedersen, 2005)

Regular, moderate intensity exercise has been shown to offer protection against all-cause mortality [28, 32, 44, 47]; however, athletes engaged in intensive exercise have demonstrated an increased susceptibility to infectious illnesses, such as upper respiratory tract infections, as well as infections of the eye, ear and skin.[10, 12, 29, 30, 32, 35, 39, 44, 47] The increase in susceptibility

has been associated with the overtraining syndrome (OTS), or decrements, rather than improvements, in exercise performance that occur with frequent, intense training.[30,35, 36] The OTS is associated with suppressed immune function.[12] Pro-inflammatory cytokines are released into the circulatory system in response to intensive exercise, causing inflammation.[24] The immune system is then occupied with the task of responding to that inflammation. Researchers have suggested a cytokine "Open Window" (see Figure 2A) that occurs in the time period between three and 72 hours after exercise.[22, 35] During this time, pathogens, such as viruses and bacteria, may gain a "foothold" in the body, because the immune system cannot adequately respond to both the inflammation caused by the intensive exercise and the pathogen, thus increasing the risk of infection.[22, 35]

Figure 2. (A) The cytokine Open Window. This window occurs 3-72 hours post-exercise, and is a period of time in which athletes are more susceptible to illness. (B) Cumulative effects of the cytokine Open Window. These effects may result in chronically altered immune function.[22, 35]



Adapted from Smith (2000)

Several researchers have investigated the IL-6 response to exercise. Studies have shown an increase in IL-6 immediately after acute bouts of prolonged exercise ranging from one hour to six hours in duration (see Table 1).[5, 9, 10, 17, 20, 21, 24, 45] In the case of TNF-α,

participants who perform an acute bout of aerobic exercise, two hours or more in duration, demonstrate an increased circulation of TNF-α immediately post-exercise [5, 7, 9, 10, 17, 21, 45]; whereas participants who perform an acute bout of aerobic exercise less than two hours in duration do not demonstrate a change in circulating TNF-α immediately post-exercise (see Table 1).[10, 45]

Table 1. The IL-6 and TNF-α responses to exercise. These studies will be discussed in detail in the review of literature.

Cytokine	Author	Time (hr)	Intensity ($\%VO_{2max}$)	Circulating Concentration
IL-6	Ullum et al.[45]	1	75%	↑
	Haahr et al.[10]	1	75%	↑
	Brenner et al.[5]	2	60%	↑
	Nieman et al.[21]	2.5	74%	↑
	Nehlsen-Cannarella et al.[20]	2.5	77%	↑
	Moldoveanu et al.[17]	3	60-65%	↑
	Ostrowski et al.[24]	~3.5	"Competitive"	↑
	Gannon et. al.[9]	6	"Competitive"	↑
TNF-α	Ullum et al.[45]	1	75%	↔
	Haahr et al.[10]	1	75%	↔
	Brenner et al.[5]	2	60%	↑
	Dufaux & Order [7]	2.5	"Competitive"	↑
	Moldoveanu et al.[17]	3	60-65%	↑
	Ostrowski et al.[24]	~3.5	"Competitive"	↑
	Gannon et. al.[9]	6	"Competitive"	↑

The results of these investigations indicate that individuals who perform an acute bout of prolonged exercise may increase their susceptibility to infection in the post-exercise recovery period due to the up regulation of pro-inflammatory cytokines, and the subsequent inflammation. It is well known; however, that an athlete who is seriously training does not perform only a single, acute bout of exercise before taking a day to rest. It is more likely that an endurance athlete performs cycles consisting of several consecutive days of intensive training before a rest day, also known as a micro-cycle.[1] Elaborating on the Open Window idea presented earlier, researchers have proposed that an athlete who trains excessively

without adequate rest may show a cumulative effect of the Open Window period (see Figure 2B).[22, 35] In this case, the injurious stimulus is repeatedly applied, potentially leading to a more chronic type of inflammation. At this point, the cytokine response to consecutive intensive training days is unknown. Thus, it is not certain if changes in cytokine concentrations are cumulative over the course of an intensive micro-cycle. If pro-inflammatory cytokine concentrations are cumulative, an aerobic endurance athlete may be more susceptible to infection upon completion of a training micro-cycle due to the developing inflammation, meaning more rest time may be required for the athlete to appropriately recover before beginning another cycle of training. If the athlete does not have adequate time for rest, chronic inflammation may develop, along with an increased risk for development of the OTS.

Statement of the Problem

The purposes of this experiment were to determine the acute and cumulative responses of IL-6 and TNF-α to the three days of intensive training (i.e. micro-cycle), and to determine if there were differences in baseline values of IL-6 and TNF-α during three days of intensive training.

Research Questions

1. What is the acute effect of intensive training on IL-6?
2. What is the cumulative effect of intensive training on IL-6?
3. Is there a significant difference in resting levels of IL-6 during three days of intensive training exercise?
4. What is the acute effect of intensive training on TNF-α?
5. What is the cumulative effect of intensive training on TNF-α?

6. Is there a significant difference in resting levels of TNF-α during three days of intensive training?

Research Hypotheses

Hypothesis 1: Plasma IL-6 will be significantly increased from rest immediately post-exercise for each bout of intensive training.

Hypothesis 2: The change in IL-6 will be of a greater magnitude with each successive bout of intensive training.

Hypothesis 3: The resting concentrations of plasma IL-6 will be significantly elevated following each successive day of intensive training, and the following rest day.

Hypothesis 4: Plasma TNF-α will be significantly increased from rest immediately post-exercise for each bout of intensive training.

Hypothesis 5: The change in TNF-α will be of a greater magnitude with each successive bout of intensive training.

Hypothesis 6: The resting concentrations of plasma TNF-α will be significantly elevated for each successive day of intensive training, and the following rest day.

Definition of Terms

Inflammation – A complex sequence of cellular and humoral responses aimed at limiting tissue damage and activating the repair process.[21, 35] The inflammatory process begins with an initial phase of acute fluid and neutrophil accumulation to the site of injury, followed by mononuclear cell accumulation, and ends with a resolution phase, in which repair of injured tissue is completed.[21, 35]

Cytokine – A soluble glycoprotein, usually between 8-30 kDa in mass, which is produced by and mediates communication between and within immune and non-immune cells, organs and

organ systems throughout the body.[10, 16, 17, 18, 21, 22] Cytokines can be functionally divided into two groups: pro-inflammatory and anti-inflammatory.[18, 35]

Pro-Inflammatory Cytokine – Cytokines that are important in the initiation and progression of inflammation. They are up-regulated by physical activity, trauma, autoimmune diseases, infection and certain types of cancer.[18, 20, 35]. Two major pro-inflammatory cytokines are Tumor Necrosis Factor-alpha (TNF-α) and, in some cases, Interleukin-6 (IL-6).[20, 25, 28, 35]

Anti-Inflammatory Cytokine – Cytokines that are released with the main purpose of regulating the inflammatory network.[20, 21, 25, 28, 35] Major anti-inflammatory cytokines include Tumor Necrosis Factor- Receptor (TNF-R), Interleukin 1- receptor agonist (IL-1ra) and Interleukin -10 (IL-10), [20, 21, 25, 28] and in some cases IL-6.[14, 20, 26, 39]

Tumor Necrosis Factor-alpha (TNF-α) – A pro-inflammatory cytokine that is mainly a product of mononuclear phagocytes, but is also produced by T lymphocytes, Kupffer cells, neural cells, muscle cells and endothelial cells.[16, 18, 27] TNF-α increases vascular permeability and migration of leukocytes to sites of infection and inflammation.[16, 18] TNF-α also works in combination with IL-6 to induce the acute phase response and fever.[16]

Interleukin-6 (IL-6) – IL-6 is one of the most potent mediators of the acute phase immune response, and so it has been classified as both a pro- and an anti-inflammatory cytokine.[16] It is a glycoprotein produced by T lymphocytes, natural killer cells, monocytes and non-immune cells, such as smooth muscle, contracting skeletal muscle, chondrocytes, astrocytes and glial cells.[16, 27] IL-6 activates B cell growth and differentiation into antibody forming plasma cells.[16] It works with pro-inflammatory cytokines, such as TNF-α, to initiate the acute phase response and the synthesis of hepatic acute-phase proteins, such as C-reactive protein.[25, 40] IL-6 has also been shown to have aspects of an anti-inflammatory cytokine, in

that it may have immune regulatory effects by inhibiting TNF-α,[14, 26, 39] and inducing upregulation of IL-1ra, a major anti-inflammatory cytokine.[20, 39]

Cytokine Open Window - The time period between three and 72 hours after exercise in which pathogens, such as viruses and bacteria, may gain a "foothold" in the body.[22, 35] This may happen because the immune system cannot adequately respond to both the inflammation caused by the intensive exercise and the pathogen, thus increasing the risk of infection.[22, 35]

Intensive Training Trials – Three identical training trials, separated by ~24 hours, consisting of intensive prolonged exercise.

Intensive Prolonged Exercise - Exercise performed above 70% of maximal oxygen consumption for 60-120 minutes.[4, 11]

Assumptions

1. The participants adhered to all pre-experiment guidelines as instructed by the investigators.
2. The first resting, baseline blood sample that was taken before the first Intensive Training Trial was a valid, stable baseline.

Limitations

1. There was a small number of participants in this investigation, potentially leading to sampling error.
2. A typical athletic diet of 60% carbohydrate, 25% fat and 15% protein was recommended for all participants, and this diet was monitored; however, the participants were left to follow these guidelines without the provision of meals by the investigators.
3. The large degree of variability in the VO_2 measured during the Intensive Training Trials could have affected the results obtained.

Delimitations

1. Only healthy, non-smoking, moderately trained males aged 18-45 were recruited for the investigation.
2. Diet was not strictly controlled, but it was monitored. The standards were based on an athletic population, and may not be applicable to those who are part of a non-athletic population.
3. There was a 24 hour period between Intensive Training Trials in which post-exercise recovery blood samples were not obtained.
4. One hour of exercise at 75% of VO_{2peak} was chosen as the exercise duration and intensity of the study.

CHAPTER II

Review of Literature

This review of literature will be presented in the following format: It will begin with a brief overview of basic cytokine physiology related to exercise. Following the overview, the IL-6 and TNF-α responses to exercise will be discussed in detail. For both the discussion of IL-6 and the discussion of TNF-α, the review will be organized based on duration of the exercise protocol. Studies utilizing the shortest duration of exercise (60 minutes) will appear first, followed by studies of increasing exercise duration, and ending with studies utilizing the longest exercise duration (6 hours). The review of literature concludes with a summary statement.

Cytokine Physiology

Exercise causes many alterations within the immune system. Some of these changes include release of inflammatory mediators, activation of white blood cells and induction of acute phase proteins, which enhance the immune response to a pathogen.[16, 22] Cytokines, as described earlier, are the inflammatory mediators. They are molecules involved in communication between cells involved in the immune response and non-immune cells.[9, 16, 17, 20, 21,22] If cytokine production is either excessive or insufficient, infectious, immunological and inflammatory diseases may result.[22]

Exercise bouts that are intensive and prolonged or that have a strong eccentric component, which cause muscle damage, can cause the release of cytokines.[22] The first

cytokines released are those involved with inflammation, or pro-inflammatory cytokines such as IL-1, TNF-α and IL-6.[18, 24, 25, 28] It is thought that pro-inflammatory cytokines are released in response to mechanical stress, local ischemia, and/or the production of free radicals in the active skeletal muscles; however, the exact mechanisms underlying increased cytokine production after exercise remain unknown.[21] The release of pro-inflammatory cytokines induces the secretion of several molecules from the liver called acute phase proteins.[22, 28] These proteins have several functions, some of which serve to enhance the body's immune response to a pathogen.[22] They are also released as a first step towards beginning the process of tissue repair.[21, 22]

The release of pro-inflammatory cytokines is followed closely by the release of anti-inflammatory cytokines, such as IL-10 and IL-1ra, which attenuate the inflammatory process once it has begun.[18, 20, 24, 25, 28] As previously noted, IL-6 has been shown to have aspects of both a pro-inflammatory and an anti-inflammatory cytokine.[16] It is anti-inflammatory in the respect that it may have immune regulatory effects by inhibiting TNF-α,[14, 26, 39] and inducing upregulation of IL-1ra.[20, 38] A balance of pro- and anti-inflammatory cytokines is of the utmost importance for the maintenance of a reliable immune system.[22] It should be noted that, in contrast to intensive prolonged exercise, moderate exercise, such as brisk walking or low intensity cycling, does not cause a significant increase in the cytokine levels within the blood.[22]

Results of many studies, some of which will be discussed at length in the proceeding sections, show significant elevations in cytokine levels after exercise; however, some do not. Changes in plasma levels of cytokines are not always seen post-exercise.[22] One explanation for the lack of observed cytokine response is that they may be produced and act locally only

within a very small area of the tissue.[22] Accessing these sites after exercise may not be possible. A second explanation is that cytokines are rapidly cleared from the blood by receptors on both immune cells and non-immune cells.[22] This does not mean that cytokines are not being produced. Rather, it means that blood levels may not necessarily reflect changes in production, secretion, and activity of a certain cytokine.[22]

IL-6 Responses to Exercise

The IL-6 response to exercise of various durations has been studied at length as well. Ullum et al.[45] investigated the effect of one hour of cycling exercise at 75% of VO_{2max} on plasma levels of IL-6 and pre-mRNA for IL-6 in blood mononuclear `cells in seventeen moderately trained men.[45] Blood samples were taken before exercise, during the last few minutes of exercise, and for several hours into recovery. Results indicated that plasma IL-6 was significantly increased from during the last few minutes of exercise and levels returned to baseline at one hour post-exercise. Pre-exercise IL-6 was 51 pg/mL. IL-6 increased to 83 pg/mL post-exercise, corresponding to an increase of 62.7%. In addition, pre-mRNA for IL-6 could be detected in blood mononuclear cells, but, once again, the amounts of mononuclear cells did not change in relation to exercise, leading the researchers to conclude that the absolute amount of monocytes increases with exercise; however, the increase in plasma levels of IL-6 during exercise is not likely to be a result of activated monocytes in peripheral blood.

Haahr et al.[10] examined the effect of one hour of cycling exercise at 75% VO_{2max} on the production of IL-6. Ten young, healthy volunteers completed the protocol. Blood samples were taken before exercise, during exercise, and several hours into recovery in this investigation as well. Results of this study showed that IL-6 significantly increased to ~25

pg/mL two hours after exercise, followed by a decrease to baseline values, ~15 pg/mL, at 24 hours post-exercise. These results indicate that one hour of cycling exercise at 75% VO_{2max} significantly influences plasma levels of IL-6 at two hours post-exercise.

Brenner et al.[5] compared the impact of three different types of exercise and seated rest on the production of IL-6. Eight healthy male subjects completed five minutes of cycle ergometer exercise at 90% of VO_{2max}, a circuit training routine involving biceps curl, knee extension, hamstring curl, bench press, and leg press, two hours of cycling at 60% of VO_{2max} and five hours of seated rest. Results of this study indicated significant increases in plasma IL-6 from pre-exercise only for the group that completed two hours of cycling at 60% of VO_{2max}. Peak values of IL-6 were obtained after three hours of recovery, followed by a return to near-baseline at 72 hours post-exercise. Pre-exercise IL-6 was 1.14 ± 0.31 pg/mL whereas the peak IL-6 value was 6.06 ± 1.95 pg/mL. These results led the researchers to conclude that two hours of cycling exercise at 60% of VO_{2max} increased plasma levels of IL-6, showing an activation of components of the inflammatory response. It is interesting to note that in the study by Ullum et al.[45], which used one hour of exercise, IL-6 was increased in the last few minutes of exercise and reached a baseline only one hour after exercise. In the present study [5], which utilized two hours of exercise, peak values were obtained three hours into exercise, and a near-baseline value was not obtained until approximately 24 hours post-exercise.

Nieman et al.[21] studied the effect of carbohydrate ingestion and mode of exercise on cytokine responses to exercise. Ten experienced triathletes (eight men and two women) were broken into carbohydrate or placebo groups, and served as their own controls. The subjects completed four sessions of running or cycling for 2.5 hours at 75% of VO_{2max} under the

following conditions: running/placebo, running/carbohydrate, cycling/placebo, cycling/carbohydrate. Results showed a post-exercise increase in plasma IL-6 above baseline in all four conditions. The lowest immediate post-exercise plasma concentration was 10.7 pg/mL, and was obtained in the group that consumed carbohydrate and cycled for 2.5 hours. In contrast, the highest immediate post-exercise plasma concentration was obtained in the placebo group that ran for 2.5 hours, which was 51.6 pg/mL. These results led the researchers to conclude that IL-6 increases in both cycling and running exercise at 75% of VO_{2max} and that carbohydrate ingestion attenuates the cytokine response. These results are in agreement with those of Ullum et al.[45] and Brenner et al.[5] discussed earlier.

Nelson-Cannarella[20] also studied the cytokine response to 2.5 hours of high intensity running (76.7 ± 0.4% VO_{2max}) in 30 experienced marathon runners (24 men, six women) who were split into two groups. One group consumed a 6% carbohydrate solution during the 2.5 hours of running, while the other group consumed a placebo solution. Results of this study indicated that plasma IL-6 was significantly increased immediately post-run and remained elevated for 1.5 hours post-run for both groups. The increase; however, was of a lesser magnitude for the group that consumed carbohydrate during the run. The post-exercise increases in IL-6 for the carbohydrate and placebo groups were 421 and 753%, respectively. Similar to the results of Nieman et al.[21], these results indicate that 2.5 hours of high intensity exercise increases plasma IL-6; however, carbohydrate attenuates some of the rise in plasma IL-6.

Moldoveanu et al.[17] studied the effect of three hours of cycling and inclined walking at 60-65% VO_{2peak} on plasma levels of IL-6. Ten healthy, but untrained men were recruited for this study; however, cytokines were only analyzed on six of the ten subjects who

completed the protocol. Results indicated a significant increase in plasma IL-6 from pre-exercise to one hour into exercise and three hours into exercise. Pre-exercise IL-6 was 1.2 pg/mL. Post-exercise IL-6 was 4.8 pg/mL, which corresponds to an increase of 300% from pre-exercise. Plasma concentrations of IL-6 were still significantly elevated at two hours post-exercise, but then decreased to baseline levels at 24 hours post-exercise, indicating that prolonged exercise at 60-65% VO_{2peak} elevates plasma levels of IL-6. These results are similar to those of Haahr et al.[10] and Brenner et al.[5], in that levels of IL-6 were still elevated above baseline several hours into recovery from exercise.

Ostrowski et al.[24] ivestigated the IL-6 response to a long distance run in ten male highly trained subjects. All subjects completed the Copenhagen Marathon (time range 2:40 – 4:20) followed by a four hour rest period. Mean plasma IL-6 peaked at ~75 pg/mL immediately after the race and then declined during the four hours of the resting period. The recovery levels of IL-6 were declining, but remained significantly different from baseline (~0 pg/mL) until four hours post-run. Once again, these results agree with the majority of those presented thus far, in that IL-6 stays elevated for several hours into the recovery period after exercise. These results led the researchers to conclude that strenuous exercise induces an increase in pro-inflammatory cytokines, specifically IL-6. The researchers also looked at several anti-inflammatory cytokines and found that they had increased during exercise as well, indicating that pro-inflammatory cytokines are balanced by an increase in anti-inflammatory cytokines that restrict the duration of the inflammatory response.

Gannon et al.[9] studied the effect of prolonged and strenuous cycling in six amateur cyclists on the IL-6 response. All of the subjects completed a 250-km road race (race time: 404.0 ± 3.5 minutes). Plasma IL-6 concentrations were approximately doubled at 10-25

minutes after completion of the road race, and, again were still elevated 150 minutes into recovery from the race. These results led the investigators to suggest that performance in a road race lasting approximately 6.5 hours increases plasma levels of IL-6 and induces a pro-inflammatory cytokine response.

The studies discussed in this section agree that exercise is a stimulus that increases circulating plasma levels of IL-6; however they do not include the source of the IL-6. Steensberg et al. investigated the IL-6 response to five hours of one-legged dynamic knee extensor exercise.[40] One leg performed the exercise, which represented 40% of peak power output, while the other leg remained stationary. Results of this study showed that the net IL-6 measured in the exercising leg increased dramatically from 0.74 ng/L to 14.13 ng/L after five hours, whereas the resting leg had an unchanged net IL-6 release, leading these researchers to conclude that IL-6 is released exclusively from the contracting muscle during exercise.

TNF-α Responses to Exercise

The TNF-α response to exercise of various durations has been studied at length. Ullum et al.[45] investigated the effects of cycling exercise on plasma levels of cytokines and pre-mRNA for cytokines in blood mononuclear cells. Seventeen healthy moderately trained men completed one hour of cycle ergometer exercise at 75% of VO_{2max}. Results indicated that post-exercise plasma TNF-α was below the detection limit in most subjects. In addition, pre-mRNA for TNF-α could be detected in blood mononuclear cells, but the amounts did not change in relation to exercise, leading these researchers to conclude that one hour of cycling exercise performed at 75% of VO_{2max} does not increase plasma TNF-α.

Haahr et al.[10] performed an experiment very similar to that of Ullum et al.[45], and observed very similar results. In this experiment, ten young, healthy volunteers completed one hour of cycling exercise at 75% VO_{2max}. Results of this investigation showed that blood levels of TNF-α did not change from pre- to post-exercise. There were also no changes at two hours post-exercise and 24 hours post-exercise, indicating, once again, that one hour of cycling exercise at 75% VO_{2max} does not influence blood levels of TNF-α; however, Zack [48] has shown that 30 minutes of submaximal exercise has been a sufficient stimulus to cause an increase in the amount of circulating plasma TNF-α.

Brenner et al.[5] compared the impact of strenuous all-out exercise, circuit training, and two hours of exercise on TNF-α production. Eight healthy male subjects completed exercise under four conditions: five minutes of cycle ergometer exercise at 90% of $VO_{2max,}$ a circuit training routine involving five different exercises (biceps curl, knee extension, hamstring curl, bench press, and leg press), two hours of cycling at 60% of VO_{2max} and five hours of seated rest. For purposes of this review, the focus is only on the two hours of cycle ergometer exercise performed at 60% of VO_{2max}. Results of this study indicated significant increases from pre-exercise plasma TNF-α for two hours of cycling at 60% of VO_{2max}. Pre-exercise TNF-α was 1.05 ± 0.13 pg/mL. Peak values of 1.87 ± 0.27 pg/mL were obtained after 72 hours of recovery. These results led the researchers to conclude that two hours of prolonged cycling exercise at 60% of VO_{2max} increases plasma levels of TNF-α; thus, activating components of the inflammatory response. These results are contrary to those of Ullum et al.[45] and Haahr et al.[10], which utilized only one hour of exercise.

Dufaux and Order [7] investigated the TNF-α response to 2.5 hours of running (marathon) in eight healthy male subjects. Results indicated a significant increase in plasma

TNF-α one hour after the cessation of exercise (~27 pg/mL vs. ~17 pg/mL pre-exercise), but not immediately post-exercise (~21 pg/mL). Plasma concentrations of TNF-α were still elevated at both three hours post-exercise and at 24 hour post-exercise. These results indicate that 2.5 hours of running exercise is sufficient to evoke a TNF-α response and that there is involvement and activation of parameters of the inflammatory response after prolonged exercise. The results obtained in this experiment differ from those obtained by Brenner et al.[5] in that there was no significant increase in plasma TNF-α immediately post-exercise.

Moldoveanu et al.[17] studied the effect of three hours of cycling and inclined walking at 60-65% VO_{2peak} in ten healthy, but untrained men. Plasma cytokine level were only analyzed in six of the ten subjects who completed the protocol. Results of this study indicated a significant increase from pre-exercise TNF-α at one hour into exercise and at the completion of exercise. Pre-exercise TNF-α was 1.0 pg/mL, which increased to 1.2 and 1.9 pg/mL after 60 and 180 minutes, respectively. These concentrations decreased at two hours and 24 hours post- exercise; however, they were still significantly higher than the initial baseline value, indicating that prolonged exercise at 60-65% VO_{2peak} elevates plasma levels of TNF-α. The results of this study both agree with and contradict the results of other investigations. These results are similar in that TNF-α was significantly elevated above baseline immediately post- exercise[5], and several hours into recovery.[5,7] They are contradictory; however, because significant differences in plasma TNF-α were observed after only one hour of exercise at 60-65% in this study, whereas in the studies discussed by Ullum et al.[45] and Haahr et al.[10], no significant differences were found for one hour of exercise performed at 75% of VO_{2max}.

Ostrowski et al.[24] studied the TNF-α response to strenuous exercise in ten highly trained male subjects. All of the subjects completed the Copenhagen Marathon (time range 2:40 – 4:20) followed by a four hour rest period. Similar to the results of both Brenner et al.[5] and Moldoveanu et al.[17], mean plasma TNF-α peaked immediately after the race (2-3 fold increase) and then declined during the four hours of the resting period. Despite the fact that the plasma levels of TNF-α were declining, they remained significantly elevated from baseline until 2.5 hours after the completion of the marathon. These results led the researchers to conclude that strenuous exercise induces an increase in plasma TNF-α.

Finally, Gannon et al.[9] studied the effect of prolonged and strenuous cycling on TNF-α as well. Six amateur cyclists completed a 250-km road race (race time: 404.0 ± 3.5 minutes). Plasma TNF-α concentrations were approximately doubled at 10-25 minutes and 150 minutes after completion of the competition. These results led these investigators to suggest that performance in a road race lasting approximately 6.5 hours increases plasma levels of TNF-α and induces a pro-inflammatory cytokine response.

Timmons[43] recently reviewed immunological changes in children that occur with exercise and suggested that IL-6 produced during exercise may function as an antagonist to TNF-α. He notes that TNF-α is expressed in, but not released from skeletal muscle during contraction, and so the ratio of IL-6 to TNF-α may be a useful marker of the inflammatory environment during and following exercise.

Summary

Cytokines are molecules which are produced by and mediate communication between and within immune and non-immune cells, organs, and organ systems throughout the body.[10, 16, 17,18, 22] Cytokines are classified as either pro-inflammatory or anti-inflammatory, and a

balance between the two types is of the utmost importance for the maintenance of a reliable immune system.[22] Following an intensive prolonged bout of exercise, pro-inflammatory cytokines such as IL-1, TNF-α and IL-6 are released.[16, 18, 22] These cytokines are responsible for inducing the secretion of acute phase proteins which are involved with augmenting the body's immune response to a pathogen and beginning the process of tissue repair.[22]

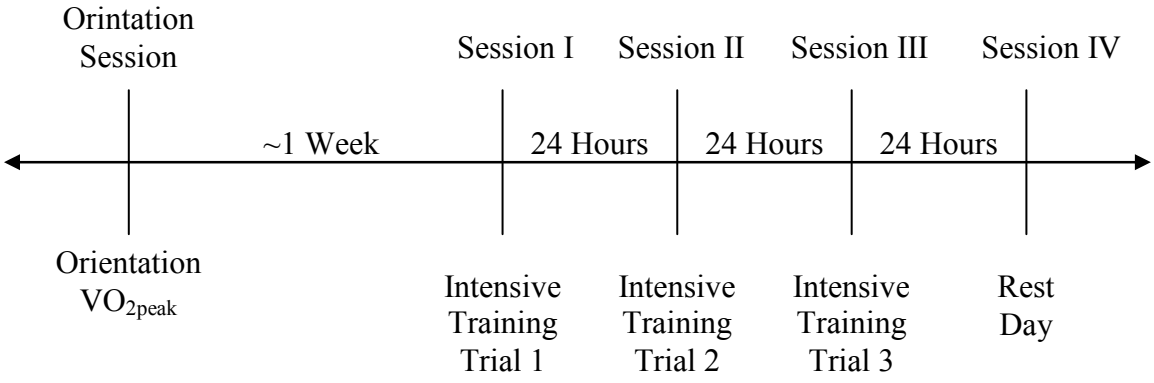
Anti-inflammatory cytokines, such as IL-10 and IL-1ra, are released shortly after pro-inflammatory cytokines. As previously noted, the IL-6 released shortly after the onset of exercise can also be considered an anti-inflammatory cytokine in that it may have immune regulatory effects by inhibiting TNF-α,[14, 26, 39] and inducing upregulation of IL-1ra.[20, 39] These anti-inflammatory cytokines are involved in attenuating the inflammatory process once it has begun.[16, 22] The literature on single sessions of exercise (some of great duration) show that major pro-inflammatory cytokines, TNF-α and IL-6, increase following exercise, and, in most cases, remain elevated for several hours, even days, into the recovery period. However, the responses of TNF-α and IL-6 to multiple sessions separated by 24 hours of recovery are unknown.

CHAPTER III

Methodology

This study consisted of five total visitation sessions for each participant (see Figure 3). The first session was an orientation session, which was followed by four consecutive days of testing each separated by 24 hours. In the orientation session, the protocols were explained, participant characteristics were obtained, and peak oxygen consumption, VO_{2peak}, was determined. Sessions I, II and III were Intensive Training Trials in which participants performed 60 minutes of cycling at 75%VO_{2peak}. Baseline and immediate post-exercise blood samples were taken to determine IL-6 and TNF-α concentrations. In Session IV, the participant had a resting, baseline blood sample taken to determine IL-6 and TNF-α concentrations. No exercise was performed in this final session.

Figure 3. General outline of the experimental protocol used in the study.



Participants

Highly trained male endurance athletes, 18 to 45 years of age, were recruited for this study. In order to be included in this study, participants had to be training for a minimum of five days per week for a minimum of 60 minutes each day and had to be current participants in competitive sporting events such as marathon, triathlon or cycling competition. In addition, all participants were willing to consume a glucose polymer supplement, Polycose®, on the day preceding the Intensive Training Trials, as well as on the days of the Intensive Training Trials. If any of the following were present, participants were excluded: history of any chronic medical conditions or orthopedic injury, history of infection in the six weeks period prior to the study, and use of any medications, including all non-steroidal anti-inflammatory drugs such as Aleve®, Advil®, Bayer®, Celebrex®, etc. Each participant was informed of the experimental protocol, made aware of the possible risks and signed an informed consent prior to participation. All participants refrained from strenuous physical activity for 24 hours prior to VO_{2peak} testing and 24 hours prior to the start of the Intensive Training Trials. In addition, participants were asked to reduce training to an "easy" level two days prior to the start of the Intensive Training Trials in order to mimic a typical "micro-cycle"[1]. Participants did not perform any physical activity on the days of the Intensive Training Trials. Finally, participants did not eat, smoke or consume alcohol and caffeine eight hours prior to Sessions I-IV.

Instrumentation

Height was determined to the nearest 0.01 cm using a portable standiometer (Perspective Enterprises, Portage, MI). Body mass was determined to the nearest 0.01 kg using a calibrated mechanical scale (Detecto, Webb City, MO). Skinfolds were measured to

the nearest) 0.5 mm using a Lange skinfold caliper (Model 68902, Cambridge Scientific Industries, Inc., Cambridge, MA). Peak oxygen consumption was determined during a continuous, incremental cycling test on a Monark Ergomedic 894 E cycle ergometer (Monark Exercise, Sweden). Respiratory gases were obtained for the orientation session, as well as all Intensive Training Trials using the Parvo Medics TrueMax 2400 Metabolic System (Parvo Medics, Salt Lake City, UT). Heart rate was monitored continuously using the Polar telemetry system (Polar Electro Inc., Lake Success, NY). Ratings of perceived exertion were determined using Borg's original 6-20 scale.[3] Hematocrit was determined using an Adams MHCT II microhematocrit centrifuge (Becton Dickinson, Frankin Lakes, NJ) and an International Microcapillary Reader (International Equipment Company, Needham Heights, MA). Hemoglobin was determined from absorbance values obtained using a Milton Roy Spectronic 1201 flow through cell spectrophotometer (Milton Roy, Ivyland, PA). Whole blood samples were placed in an IEC Centra-8R refrigerated centrifuge (International Equipment Company, Needham Heights, MA) and the resultant plasma was stored. Plasma TNF-α and IL-6 were measured using high-sensitivity enzyme-linked immunosorbent assay kits (ELISA; Cell Sciences, Canton, MA). Cytokine assays were read with a Finstruments Microplate reader (Model 310, MTX Lab Systems Inc., Vienna, VA) with Spectra software analysis (MTX Lab System Inc., Vienna, VA). Lactate and total CK were measured using the Vitros DT60 II (Ortho-Clinical Diagnostics, Rochester, NY).

Protocol

Orientation Session

The participant arrived at the Applied Physiology Laboratory and was be informed of the experimental protocol, made aware of the possible risks associated with the protocol, and

signed an informed consent. After consenting to participate in the experiment, the participant underwent a medical screening, including a 12 lead electrocardiogram, and a physical screening. After the participant was deemed fit to participate in the experiment, physical characteristics, including height, weight and age were obtained. Percent body fat was determined from the sum of skinfolds using the method of Jackson and Pollock.[13]

Following collection of participant characteristics, VO_{2peak} was determined during a continuous, incremental cycling test on a cycle ergometer. The cycle ergometer was fitted to the participant to ensure that he was comfortable and could perform the VO_{2peak} protocol without injury. The participant completed a 10 minute warm up which consisted of five minutes of cycling at a resistance of 50 watts (W) followed by five minutes of stretching focused on the lower body musculature. After warm up, resting oxygen consumption (VO_2) was recorded for four minutes. The participant then began cycling at 50W. Resistance was increased by 50W every three minutes for 12 minutes. Resistance was then increased by 25 W every minute thereafter until volitional fatigue (see Figure 4).[15, 38] Heart rate (HR) and respiratory gases were monitored continuously for the entire duration of the VO_{2peak} determination. HR and rating of perceived exertion (RPE) were recorded at the end of each stage of the protocol. The participant was provided with strong verbal encouragement during the entirety of the test. At the conclusion of the test, the participant was allowed to recover actively or passively. Participants were deemed safe to leave the laboratory once HR less than or equal to 100 beats per minute (bpm). For the VO_{2peak} test to be considered valid, the participant had to achieve a respiratory exchange ratio (RER) equal to or greater than 1.1, a HR equal to or greater than the age predicted maximal heart rate (220-age ± 5%), and had to report an RPE equal to or greater than 18.[15] A power output estimated to require 75% of

VO_{2peak} was determined from a linear regression equation which plotted steady state oxygen consumption (VO_2) values against the corresponding workloads.[15] Prior to the conclusion of the orientation session, participants were given a canister of Polycose® glucose polymer (Ross Products, Columbus, OH) and were instructed to consume 25% of the canister (~90g) on the day prior to the start of Session I, and an additional 25% of the canister on the days of Sessions I, II and III. Upon completion of the exercise protocol, participants were asked to return the empty canister to the investigators to ensure compliance with the participant instructions. Polycose® glucose polymer, a carbohydrate supplement, was given to the participants due to the fact that research has shown that decreased carbohydrate intake can potentially increase the cytokine response to exercise.[21]

Sessions I-III: Intensive Training Trials.

At least one week after determination of VO_{2peak}, participants reported to the Applied Physiology Laboratory for three consecutive days of Intensive Training Trials. Two days prior to the first Intensive Training Trial, participants were instructed to complete an "easy" day of training. The day before the first Intensive Training Trial, participants were instructed to rest. No exercise of any kind was permitted on the day before the first Intensive Training Trial. In addition, participants completed a food diary for the day preceding Session I, as well as on the days of Sessions I, II, and III. Participants arrived after an overnight fast (minimum of 8 hours) to complete each Intensive Training Trial, which was performed in the morning, between the hours of 6:30 A.M. and 11:00 A.M. on each day. The time at which each participant arrived for Session I was replicated for Sessions II, III and IV. This was done in order to minimize differences in hormonal concentrations across the Intensive Training Trials for each of the participants.

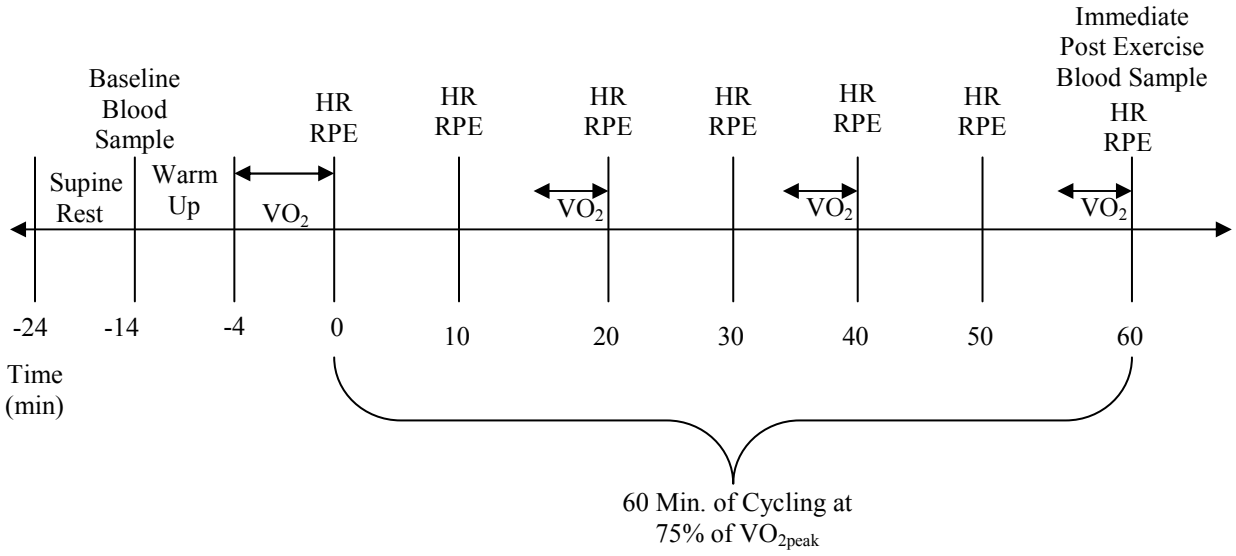
Upon arrival, participants were asked if they complied with the guidelines set by the investigators, and verbal responses were recorded. In addition, dietary logs were collected prior to the start of each session and examined to ensure that participants were consuming adequate calorie and carbohydrate to complete the exercise tasks. After compliance was confirmed, participants rested in the supine position for ten minutes. After ten minutes of rest, three mL of venous blood were obtained using the standard Venipuncture technique. The blood sample was placed into a sterile K_2 - EDTA (purple top) Vacutainer® tube and immediately put on ice. After resting blood sampling, participants performed a ten minute warm up consisting of five minutes of cycling followed by five minutes of stretching focused on the lower body musculature. After warm up, resting oxygen consumption was recorded for four minutes (see Figure 4). The participants then cycled for 60 minutes at the previously determined workload that elicits 75% of VO_{2peak}. Sixty minutes of cycling exercise was chosen in order to mimic a typical athletic training regimen. HR and RPE were monitored continuously and recorded at minutes 0, 10, 20, 30, 40, 50 and 60. Oxygen consumption was measured from minutes 16-20, 36-40 and 56-60. Water was ingested ad libidum by the participants for all of the Intensive Training Trials. Upon completion of the 60 minutes of cycling exercise, a three mL blood sample was obtained and immediately put on ice. After blood sampling, participants were deemed safe to leave the laboratory once HR was less than or equal to100 bpm.

Session IV: Rest Day

Twenty four hours after completion of the third Intensive Training Trial, participants returned to the Applied Physiology Laboratory between the hours of 6:30 A.M. and 11:00 A.M. for a final, resting blood sample. Upon arrival, participants rested in the supine position

for ten minutes. After the rest period, a three mL blood sample was obtained using the standard Venipuncture technique, placed in a purple top Vacutainer® tube and immediately put on ice.

Figure 4. Outline of the three identical Intensive Training Trials (Sessions I, II, and III) used in the study.



Blood Procedures

Hematocrit

Resting and post-exercise Hematocrit (Hct) values for each of the three Intensive Training Trials were determined in triplicate from blood samples. Whole blood was drawn into 75 mm microhematocrit capillary tubes (Fisher Scientific International Inc., Hampton, NH) and sealed using Critoseal (Krakeler Scientific, Inc., Albany, NY). Capillary tubes were then be spun in a microhematocrit centrifuge for three minutes at 10,000 RPM. After three minutes, the capillary tubes were removed from the centrifuge and placed on a hematocrit wheel to determine the ratio of formed elements to plasma in each sample. A mean of the three hematocrit determinations from each whole blood sample was calculated and used in data analysis.

Hemoglobin

Resting and immediate post-exercise Hemoglobin (H_b) values were determined in triplicate from the blood samples for each of the three Intensive Training Trials by using the cyanmethemoglobin technique. Twenty µL of whole blood were placed into five mL of cyanmethemoglobin for each sample. Samples were then drawn into a flow through cell spectrophotometer and an absorbance value was obtained for each. The amount of H_b present in each sample was determined mathematically via regression analysis using Beers Law, and a mean was calculated for use in data analysis.

Plasma Volume Shift

Exercise-induced changes in plasma volume were calculated from Hct and H_b values according to the method described by Dill and Costill.[6] Plasma volume shift was used as a potential explanatory variable for changes in cytokine concentrations, and is not a primary outcome measure.

Cytokines

Whole blood was centrifuged at 3,000 *g* for 10 min at 4°C. The resultant plasma portion was transferred to Eppendorf tubes (Eppendorf North America, New York, NY) and stored at −80°C until cytokine analyses were conducted. Plasma levels of TNF-α and IL-6 were measured using high-sensitivity enzyme-linked immunosorbent assay kits (ELISA; Cell Sciences, Canton, MA). Sensitivity of the TNF-α assay was <2.0 pg/mL. Sensitivity of the IL-6 assay was < 0.8 pg/mL. All cytokine assays were performed in triplicate and intra-assay coefficients of variation were calculated (For details, see Appendix D).

Additional Blood Measures

To assess the participants level of physical stress during the Intensive Training Trials, lactate and total creatine kinase (CK) were measured.[11] Lactate and total CK were measured using the Vitros DT60 II (Ortho-Clinical Diagnostics, Rochester, NY). All assays were performed with duplicate determinations.

Data Analysis

Data was analyzed using SPSS statistical software (Version 14.0, Chicago, IL). Statistical significance for all analyses was set at $\alpha < 0.05$. Descriptive statistics were presented as means ± standard deviations (SD).

To examine the cardiovascular and respiratory responses (VO_2, heart rate, RPE and plasma volume shift) to the Intensive Training Trials (Sessions I, II and III), a repeated measures Analyses of Variance (ANOVA) with Bonferroni adjusted t-tests was performed. These analyses were used to investigate whether or not participants were exercising at a physiological steady state for the 60 minutes of exercise in each of the Intensive Training Trails, and to determine if there were any significant differences in mean cardiovascular and respiratory responses between exercise in Sessions I, II and III. Additionally, to examine the participants' level of physical stress during the Intensive Training Trials, a repeated measures ANOVA with Bonferroni adjusted t-tests was performed with the creatine kinase (CK) and lactate data obtained.

To determine the acute effect of exercise on cytokine concentrations (*hypotheses 1 and 4*), a 3 X 2 (3 intensive training trials X 2 blood sampling times) completely repeated measures ANOVA analysis with Bonferroni adjusted t-tests was performed. To determine the cumulative effect of exercise on cytokine concentrations *(hypotheses 2* and *4*), three

percent change (%Δ) scores were computed for both IL-6 and TNF-α, for a total of six %Δ scores. The first set of %Δ scores assessed changes in IL-6 and TNF-α from rest to immediate post-exercise in Session I. The second set of %Δ scores assessed changes in IL-6 and TNF-α from rest to immediate post-exercise in Session II. The third %Δ score assessed changes in IL-6 and TNF-α from rest to immediate post-exercise in Session III. All %Δ scores were computed using the formula:

$$\%\Delta = \{(\text{Post-exercise} - \text{Pre-exercise})/ \text{Pre-exercise}\} X\ 100$$

A one way (1 change score X 3 intensive training trials) repeated measures ANOVA analysis with Bonferroni t-tests was then performed. To determine if there were significant differences in the mean resting cytokine concentrations over the the three Intensive Training Trials and the rest day (*hypotheses 3* and *6*), a one way (1 blood sampling time X 4 days of measurement) repeated measures ANOVA with Bonferroni adjusted t-tests was performed.

Finally, the pre-exercise and post-exercise IL-6:TNF-α ratios were computed for each participant by dividing the IL-6 values obtained by the TNF- α values obtained. The resultant number was then multiplied by 100 to make the ratio into a percentage. The formula used in computing the IL-6:TNF-α ratio can be seen below:

$$\text{IL-6:TNF-}\alpha\text{ ratio} = (\text{IL-6/TNF-}\alpha) * 100$$

A 3 X 2 (3 intensive training trials X 2 blood sampling times) repeated measures ANOVA with Bonferroni adjusted t-tests was performed in order to determine the acute effect of exercise on the IL-6:TNF-α ratio. To determine if there were significant differences in the mean resting IL-6:TNF-α ratio over the course of the three Intensive Training Trials and the following rest day, a one way (1 blood sampling time X 4 days of measurement) repeated measures ANOVA analysis with Bonferroni adjusted t-tests was performed.

CHAPTER IV

Results

Participant Characteristics

Twenty highly trained male endurance athletes were recruited for participation in this study. Seven of the 20 participants completed all aspects of the protocol (Table 2). Prior to participation in this study, all participants were briefed on the nature of the study, made aware of the possible risks associated with the protocol, and gave written informed consent to act as human subjects.

Table 2. Physical characteristics of the participants (n=7). Values are Means (± SD).

Measure	Mean ± SD	Range
Age (yr)	29.1 ± 7.0	22.0 – 42.0
Height (cm)	180.7 ± 7.4	173.0 – 191.5
Mass (kg)	78.8 ± 4.7	72.0 – 86.3
Body Fat (%)	13.8 ± 2.6	11.3 – 17.5

All participants met the inclusion criteria; i.e., they were currently training for a minimum of five days per week for a minimum of 60 minutes per day, and were participating in or training for competitive sporting events such as triathlons, marathons, and cycle competitions, etc. All participants complied with the experimental guidelines set by the investigators (see Methods chapter).

VO_{2peak} Testing

Four of the seven participants met the criteria for a valid VO_{2peak} test (see Methods chapter). For these participants, oxygen consumption peaked at the end of the test and then decreased, indicating that peak oxygen consumption had been reached, leading the researchers to conclude that the test was valid.

The mean absolute VO_{2peak} obtained was 4.7 ± 0.8 L/min (range of 3.3 – 5.9 L/min) and relative VO_{2peak} obtained was 59.7 ± 7.7 ml/kg/min^{-1} (range of 45.8 – 68.3 ml/kg/min^{-1}). Mean VO_{2peak} test time was 19.0 ± 2.8 minutes in duration (range of 14.0 to 23.0 minutes). The mean maximal workload completed by the participants was 360.7 ± 55.6W (range of 250.0 – 425.0W).

Intensive Training Trials

Each participant completed three submaximal Intensive Training Trials separated by approximately 24 hours with a subsequent resting only trial (Session IV), 24 hours after the third Intensive Training Trial.

Basic Physiological Responses

Each Intensive Training Trial was to consist of 60 minutes of cycling at 75% of VO_{2peak}. The actual mean trial workload was 218.6 ± 42.3W, which elicited a mean VO_2 of 3.4 ± 0.6 L/min, corresponding to 71.3 ± 8.0 % of VO_{2peak}.

The VO_2 responses of the participants indicate that they were working at a physiological steady state for the duration of each of the 60 minute Intensive Training Trials (see Table 3). ANOVA results revealed no significant differences from the start of exercise (20 minutes) through the completion of 60 minutes of exercise for all of the Intensive

Training Trials, indicating a steady state VO_2 for the duration of the Intensive Training Trials ($p > 0.05$).

The HR results of the participants; however, indicate that they were working at a physiological steady state for some, but not all of the Intensive Training Trials (see Table 3). ANOVA results showed a significant session X time interaction for HR. *Post Hoc* analyses indicate no significant differences in the resting HR of the participants for Sessions I, II and III ($p > 0.05$). *Post Hoc* analyses; however, revealed a significantly higher HR during minutes 30 and 60 of Session II than minutes 30 and 60 of Session III ($p = 0.038$ and $p = 0.020$, respectively). Finally, analyses revealed a significantly higher HR at minute 60 of Session II than at minute 10 of Session II ($p = 0.001$).

No significant interaction was found for RPE; however, ANOVA results indicated a significant main effect for time ($p = 0.001$). *Post Hoc* analyses indicated significantly higher mean RPE at minutes 30, 40, 50 and 60 than at minute 10 of exercise ($p = 0.001$). In addition, *Post Hoc* analyses revealed a higher mean RPE at minutes 40, 50, and 60 of exercise than at minute 20 of exercise ($p = 0.015$, $p = 0.009$ and $p = 0.030$, respectively).

Finally, plasma volume declined during Sessions I, II and III. Mean plasma volume loss for Sessions I, II and III were -17.2 ± 5.6%, -18.8 ± 3.8% and -18.3 ± 6.6% over the 60 minutes of exercise, respectively. There were, however, no significant differences in mean plasma volume loss between each of the training Sessions ($p > 0.05$).

Pre-exercise, post-exercise and percent change values for CK can be found in Table 5. ANOVA results indicate a significant session X time interaction for CK ($p = 0.04$). *Post Hoc* analyses reveal significant increases in mean CK from pre-to post-exercise for Session I, Session II and Session III ($p = 0.005$, $p = 0.001$ and $p = 0.002$, respectively). Mean CK

concentrations can be seen in Table 5. ANOVA results; however, indicate no significant differences in the percent change in CK for sessions I, II and III ($p > 0.05$). ANOVA results also indicate significant differences in mean baseline concentrations of CK ($p = 0.04$). *Post Hoc* analyses indicate a significantly higher baseline CK concentration for Sessions III and IV than for Session I ($p = 0.008$ and $p = 0.006$, respectively). Baseline values of CK can be found in Table 5.

ANOVA analysis was performed in order to assess changes in post-exercise lactate concentration. Pre-exercise lactate was not examined because it is widely accepted that blood lactate concentrations are approximately 1.0 mmol/L.[34] ANOVA results reveal no significant differences in mean post-exercise blood lactate concentration ($p > 0.05$). Assuming the pre-exercise values were approximately 1.0 mmol/L, this means that blood lactate increased from pre- to post-exercise for Sessions I, II and III; however, the increases were all substantially elevated and were of the same magnitude, which confirms the intensive level of the exercise. Post-exercise lactate concentrations can be seen in Table 5.

Primary Outcome Blood Responses

IL-6. The intra-assay coefficient of variation for the IL-6 assay was 6.99 %. Quality control standards were within 10% of determined values, suggesting the assay displayed a high level of accuracy.

Pre-exercise and post-exercise values for IL-6 can be found in Table 4. ANOVA results indicated a significant main effect of time for IL-6 ($p = 0.002$). Mean pre-exercise IL-6 was measured as 4.43 ± 2.67 pg/mL. After 60 minutes of exercise, IL-6 rose to 8.23 ± 3.67 pg/mL, which represents an increase of 85.78%. There was no session X time interaction ($p > 0.05$).

Percent change and baseline values of IL-6 can be found in Table 4. ANOVA results show no significant differences in the percent change of IL-6 across the three days of intensive training ($p > 0.05$). In addition, ANOVA results indicated that there were also no significant differences in the baseline values of IL-6 over the course of Sessions I-IV ($p > 0.05$).

TNF-α. The intra-assay coefficient of variation for the TNF-α assay was 7.15%. Quality control standards were within 10% of determined values, suggesting the assay displayed a high level of accuracy.

ANOVA results indicated no significant main effects or session X time interaction for TNF-α ($p > 0.05$). Mean pre-exercise TNF-α was 217.38 ± 529.16 pg/mL. Post-exercise TNF-α was 238.13 ± 578.63 pg/mL, which corresponds to an increase of only 9.55. In order to decrease the variability within the data, a log transformation of the TNF-α concentrations was performed, and the ANOVA analysis repeated. Results of the ANOVA using the log transformation data indicated a significant session X time interaction ($n = 7$, $p = 0.001$). *Post Hoc* analyses show that TNF-α increased from pre- to post-exercise in both Session II and Session III ($p = 0.001$ and $p = 0.017$, respectively), but not Session I.

Pre-exercise and post-exercise TNF-α concentrations can be found in Table 4, and are depicted in Figure 6. The ANOVA analysis was repeated with the outlier subject data completely removed. Once again, results show a significant session X time interaction ($n = 6$, $p = 0.001$). *Post Hoc* analyses reveal a significant increase in mean TNF-α from pre- to post-exercise for both Sessions II and III ($p = 0.001$ and $p = 0.010$, respectively).

Percent change for TNF-α can be found in Table 4. ANOVA results show significant differences in the percent change of TNF-α across the three Intensive Training Trials ($n = 7$,

p = 0.001). *Post Hoc* analyses indicate that the percent change was significantly higher for Session II than for Session I (p = 0.001). Once again, the outlier subject data for TNF-α were removed and the ANOVA repeated. Analysis without the outlier subject data also indicates a significant difference in the percent change in TNF-α across the three Intensive Training Trials (n = 6, p = 0.001). *Post Hoc* analyses show that the change in mean TNF-α was significantly higher for Session II than for both Session I and Session III (p = 0.001 and 0.006, respectively), indicating that the magnitude of change in TNF-α was the greatest for Session II, which supports the data from the ANOVA analysis with seven subjects.

Baseline values for TNF-α can be found in Table 4. The ANOVA analysis also indicated that there were no significant changes in baseline TNF-α over Sessions I-IV (n = 7, p > 0.05). As with previous TNF-α analyses, the outlier subject data were removed, and the ANOVA analysis for baseline TNF-α repeated. Results of the analysis without the outlier data for TNF-α showed a significant main effect for session, indicating a significant difference in baseline concentration of TNF-α over the course of Sessions I-IV (n = 6, p = 0.003). *Post Hoc* analyses revealed significantly higher baseline concentrations of TNF-α for Session IV than for Session II (p = 0.002).

IL-6:TNF-α Ratio. ANOVA results show a significant main effect for time of the IL-6:TNF-α Ratio percentage (n = 7, p = 0.005). The mean pre-exercise IL-6:TNF-α ratio was 21.22 ± 15.98%. Post-exercise, this ratio increased to 34.14 ± 20.18%, a 1.6 fold increase. As with all analyses involving TNF-α, the outlier subject data were removed, and the ANOVA repeated. ANOVA results with the outlier data removed also indicate a main effect for time of the IL-6:TNF-α Ratio (n = 6, p = 0.002). The mean pre-exercise IL-6:TNF-α ratio with the

outlier subject data removed was 24.69 ± 15.47%. Post-exercise, this ratio increased to 39.69 ± 16.36%, a 1.6 fold increase. Values of the IL-6:TNF-α ratio can be found in Table 4.

Table 3. Mean (± SD) VO_2, HR, and RPE for Sessions I, II and III. Dashed lines (---) indicate that measurements were not recorded during that time period. * Indicates a significantly lower VO_2 than minutes 20, 40 and 60 ($p < 0.001$). § Indicates a significantly higher HR than for the corresponding time in Session III ($p < 0.03$)‡ Indicates a significant difference in RPE from minute 10 within the session ($p < 0.001$). † Indicates a significant difference in RPE from minutes 20 within the session ($p < 0.03$).

		Time (minutes)						
	Measure	Rest	10	20	30	40	50	60
Session I	VO_2 (L/min)	* 0.4 ± 0.1	---	3.4 ± 0.7	---	3.4 ± 0.6	---	3.4 ± 0.6
	HR (bpm)	58.6 ± 9.3	154.9 ± 2.3	158.3 ± 20.8	159.6 ± 17.1	160.0 ± 14.5	161.1 ± 16.0	159.1 ± 14.0
	RPE	---	13.6 ± 1.3	14.2 ± 1.5	‡ 14.9 ± 1.6	‡† 15.6 ± 2.0	‡† 15. 9± 2.0	‡† 15.6 ± 1.8
Session II	VO_2 (L/min)	* 0.4 ± 0.1	---	3.4 ± 0.7	---	3.4 ± 0.6	---	3.4 ± 0.6
	HR (bpm)	61.3 ± 11.1	151.0 ± 20.8	155.7 ± 17.9	§ 160.1 ± 16.4	160.4 ± 13.0	160.3 ± 14.9	§ 160.1 ± 14.2
	RPE	---	13.7 ± 1.6	14.6 ± 1.6	‡ 15.1 ± 1.6	‡† 15.6 ± 1.8	‡† 15.6 ± 1.3	‡† 15.8 ± 1.7
Session III	VO_2 (L/min)	* 0.4 ± 0.1	---	3.3 ± 0.7	---	3.4 ± 0.6	---	3.3 ± 0.6
	HR (bpm)	61.7 ± 11.9	150.0 ± 20.9	154.6 ± 19.4	154.0 ± 16.7	156.1 ± 15.9	155.4 ± 18.5	153.7 ±16.0
	RPE	---	13.4 ± 2.2	13.9 ± 1.7	‡ 14.2 ± 1.3	‡† 14.4 ± 1.2	‡† 14.3 ± 1.0	‡† 14.0 ± 0.8

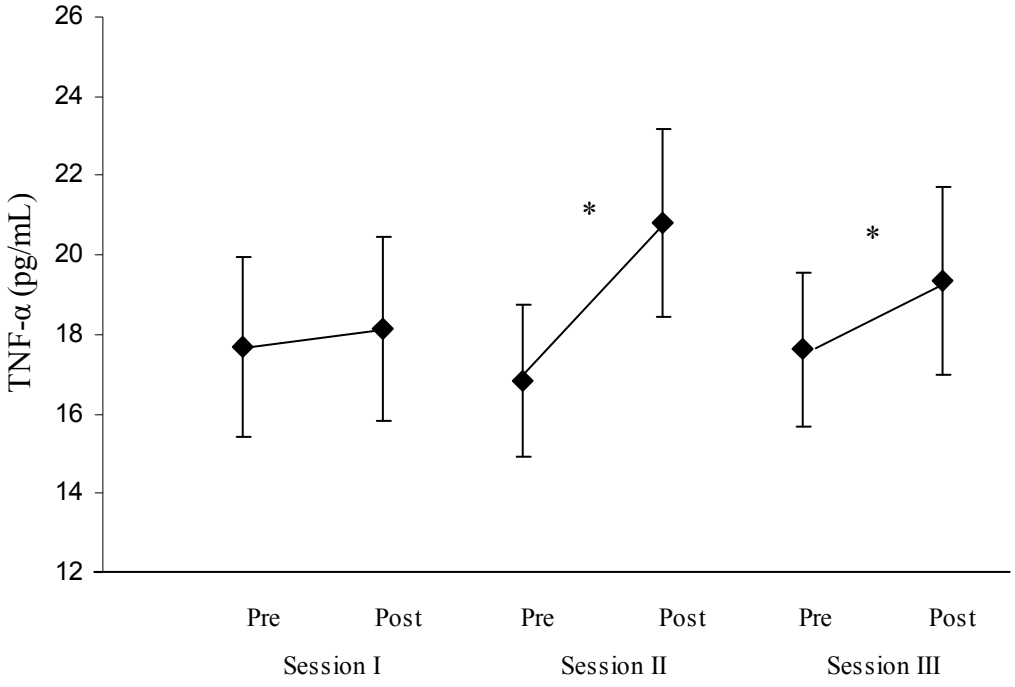
Table 4. Mean (± SD) pre-exercise (baseline) values, post-exercise values, and calculated percent change (%Δ) for IL-6, TNF-α and the IL-6:TNF-α ratio. Dashed lines (---) indicate that measurements were not calculated. Note: The values presented for TNF-α are means ± SD for the ANOVA with the outlier subject data removed (n = 6). * Indicates a significantly higher post-exercise value ($p < 0.01$). § Indicates a significantly higher %Δ than Session I and Session III ($p < 0.006$). ‡ Indicates a significantly higher baseline than Session II ($p < 0.002$).

Session	I			II			III			IV
Measure	Pre	Post	Change (%Δ)	Pre	Post	Change (%Δ)	Pre	Post	Change (%Δ)	Pre
IL-6 (pg/mL)	4.57 ± 1.93	* 7.86 ± 3.20	80.77 ± 53.40	4.87 ± 4.59	* 9.56 ± 6.19	137.28 ± 108.65	3.85 ± 1.87	* 7.28 ± 2.87	97.07 ± 38.18	5.56 ± 4.35
TNF-α (pg/mL)	17.69 ± 5.78	18.16 ± 5.65	3.21 ± 9.32	16.87 ± 4.69	* 20.82 ± 5.78	§ 25.10 ± 16.19	17.62 ± 4.72	* 19.34± 5.80	9.71 ± 10.28	‡ 18.78 ± 4.38
IL-6:TNF-α ratio (%)	24.5 ± 8.7	* 46.1 ± 17.1	---	29.2 ± 26.2	* 38.6 ± 22.5	---	20.4 ± 8.8	* 34.5± 10.2	---	30.4 ± 23.7

Table 5. Mean (± SD) pre-exercise (baseline) values, post-exercise value and percent change (%Δ) for CK and Lactate. Dashed lines (---) indicate that measurements were not recorded during that time period. * Indicates a significant increase from baseline values within the session ($p < 0.004$). § Indicates a significantly higher baseline than Session I ($p < 0.008$).

Session	I			II			III			IV
Measure	Pre	Post	Change (%Δ)	Pre	Post	Change (%Δ)	Pre	Post	Change (%Δ)	Pre
CK (u/L)	114.1 ± 72.6	* 221.8 ± 79.1	64.93 ± 56.47	234.7 ± 116.4	* 369.0 ± 153.2	65.11 ± 36.76	§ 261.8 ± 82.6	* 347.0 ± 125.4	29.67 ± 18.48	§ 266.2 ± 108.1
Lactate (mmol/L)	---	5.56 ± 1.70	---	---	5.41 ± 3.25	---	---	5.19 ± 3.15	---	---

Figure 6. Mean (± SE) TNF-α response to exercise. * Indicates a significant increase in TNF-α from pre to post-exercise (n = 6, $p < 0.01$).



CHAPTER V

Discussion

The purposes of this experiment were to determine the acute and cumulative responses of IL-6 and TNF-α to three days of intensive training (i.e. training micro-cycle), and to determine if there were differences in baseline values of IL-6 and TNF-α during the three days of intensive training. The investigator hypothesized that each cytokine would increase from pre-exercise to post-exercise, that the change in each cytokine would be of greater magnitude with each successive bout of intensive training, and that resting concentrations of each cytokine would be significantly elevated following each Intensive Training Trial. In order to test these hypotheses, seven highly trained male athletes completed a VO_{2peak} test, followed by three days of steady state cycling at ~75% VO_{2peak} separated by approximately 24 hours, and a subsequent rest day assessment.

Exercise Responses

The workloads at which the participants cycled elicited a mean VO_2 of 71.3 ± 8.0 % of VO_{2peak} across the three days, which is slightly lower than the goal of 75% of VO_{2peak}. The workloads to elicit the 75% were determined from regression analysis of VO_{2peak} results. This variability between desired intensity and actual elicited responses, while not ideal, are within the error of the regression technique. Any workload adjustments made during Session I were repeated for Session II and Session III to ensure that the workloads were identical for all of the Intensive Training Trials. Despite the fact that the trial workloads were held constant,

some measures varied within each session and from day to day. Lactate and CK responses suggest that all of the Intensive Training Trials were equally difficult. Similarly, VO_2 results indicated the three Intensive Training Trials were of a comparable difficulty, and that the participants were working at steady-stateVO_2 for each; however, HR and RPE responses did not show steady state responses.

Results showed that mean HR measured at minutes 30 and 60 of Session II were significantly higher than minutes 30 and 60 of Session III, indicating that two time points in Session II were more physiologically taxing than the same time points in Session III. In addition, the HR results also indicated that the end of exercise (minute 60) was significantly more physiologically taxing than the beginning of Session II (minute 10), meaning HR was not at physiological steady-state for the duration of Session II. It is likely that this increase in HR at the end of Session II was due to cardiovascular drift and plasma volume loss. Interestingly, all subjects verbally indicated that they perceived Session II to be the most difficult of all of the Intensive Training Trials, and noted that Session III was substantially easier than Session II, which is supported by the HR data.

Additionally, RPE results suggest that the second half of each of the Intensive Training Trials was perceived as more difficult than the first half of each of the trials. These results show the workloads of three trials were the same for each of the three days, in that the responses for all three of the Intensive Training Trials were similar. Despite the fact that the workloads elicited similar RPE responses for all three trials, the responses were not steady-state because RPE increased at the end of the exercise trials. It is likely that general fatigue development over the course of the exercise contributed to the higher RPE values recorded at the end of each trial.

IL-6

Plasma IL-6 significantly increased from pre-exercise to post-exercise for each of the Intensive Training Trials. The pre-exercise and post-exercise values obtained in the present investigation are in accordance with those found in other studies.[5, 10, 17, 20, 21, 42] The post-exercise values obtained in this study; however, were lower than those found in some studies.[24, 45] The participants in both the Ostrowski et al.[24] study and the Ullum et al.[45] study were of a similar age and fitness status of those utilized in the present study, so the differences in IL-6 increase are likely due to other factors. One possible factor is that the protocol utilized by Ostrowski et al.[24] involved completion of the Copenhagen marathon (42.2 km). This type of competitive exercise involves running at an intensity that is not necessarily steady-state for several hours continuously. It has been noted that IL-6 release is dependent on both exercise intensity and duration.[14, 28, 37, 40] It may be that the degree of muscle damage and glycogen depletion that occurred during exercise that is several hours in duration would be greater than that observed during the 60 minutes of exercise utilized in the present investigation. Research has shown that muscle damage elicits a repair process, including the entry of macrophages and neutrophils into the cell, which causes an increase in IL-6 production, thus greater muscle damage could result in increased IL-6 concentrations.[14, 17, 25, 37] This is supported by the results of Rogers et al.[31] who found the CK response to a marathon to be ~10 times greater than that presently observed.

It has also been demonstrated that IL-6 acts directly on liver cells to increase glucose release during prolonged exercise if prior carbohydrate ingestion is inadequate.[8, 26, 28, 40, 41] In a situation such as a marathon, the IL-6 produced in both the muscle cells and the liver acts on the liver cells in order to increase glucose output to support the demands of the exercise.[8,

[26, 28, 40, 41] These examples could explain the considerably larger increase in IL-6 found in the Ostrowski et al. study.[24]

In the case of the study by Ullum et al.[45], the participants completed 60 minutes of cycling exercise at 75% of VO_{2max}, which is a comparable to the present investigation. The substantially greater post-exercise increase in IL-6 may be due, in some part, to differences in assay technology, as the Ullum et al.[45] study was conducted approximately 13 years ago.[17, 25] Early cytokine assays exhibited greater levels of cross-reactivity between the various cytokines due to lack of monoclonal antibody specificity. Interestingly; though, the magnitude of change post-exercise between the studies was similar (~1.6 fold vs.1.8 fold).

Percent change in IL-6 was not of a greater magnitude with each successive Intensive Training Trial. It was thought that the percent change for Session II would be of a greater magnitude than Session I, and that the percent change for Session III would be of a greater magnitude than Session II, indicating an accumulation of this cytokine in the plasma over the course of the Intensive Training Trials. This was not observed in the present investigation. Despite the lack of statistically significant findings regarding percent change in IL-6, the percent change values observed are supported by other research literature. Suzuki et al.[42] had participants complete a comparable protocol to the one utilized in the present investigation. Percent change calculated from their data suggests that the percent change in IL-6 from pre- to post-exercise are very similar to those obtained in the present investigation (Session I = 74% vs. 80%).

It is unclear why there was a lack of significant findings regarding percent change over the course of the Intensive Training Trials. Perhaps this was due to the fact that the participants were given a carbohydrate supplement throughout the study, monitored for

caloric intake, and were trained enough that 24 hours of recovery was adequate time to recover and clear the IL-6 produced during the exercise. Obviously, if full recovery is achieved and the IL-6 cleared from the blood, there is no possibility for accumulation of the cytokine across trials.

Finally, the baseline concentrations of IL-6 were not significantly elevated following each successive Intensive Training Trial, and the following rest day. Several studies have shown that IL-6 increases in response to exercise of various intensities and durations; however, it then decreases to baseline values after 24 hours post-exercise.[5, 10, 17, 20, 21, 45] In the context of this study, 24 hours post-exercise from Session I corresponds to the pre-exercise (baseline) of Session II, and so on. That being said, the results of the aforementioned studies support the non-significant baseline results of this investigation. It appears that within the context of the current design, 24 hours is adequate recovery between exercise trials.

TNF-α

Upon initial analysis, there were no significant increases in TNF-α from pre-exercise to post-exercise for each bout of intensive prolonged exercise. However, there is some disagreement between the TNF-α values measured in this experiment and TNF-α reported in the research literature. While the values obtained in the present investigation agree with the findings of Dufaux and Order.[7] and Suzuki et al.[42], they are remarkably higher (4-10X) than those found in several other studies.[5, 17, 24, 45]

It was noted; however, that the TNF-α data for one participant was considerably out of the range of values found in these studies noted above. Thorough scrutiny of the data for this participant led the investigators to conclude that the participant may have simply been a very high responder, as a resting blood sample taken from this participant several weeks after

completion of the protocol also showed TNF-α values that were significantly out of the typical range. Initially, a log transformation of the TNF-α was performed in order to decrease the variability in the data set (n = 7). After analyzing the standard data values, the subject was finally deemed an outlier [19], and removed from the data set for TNF-α. Once removed from the data set, the mean values for TNF-α were much more representative of the values found in the above noted studies.

There were significant increases in TNF-α after both the log transformation of the data was performed, and after the outlier subject data were removed from the analysis. Both of theses analyses showed a significant increase in TNF-α for Sessions II and III pre- to post-exercise. It was thought that TNF-α would increase from pre-exercise for all three Intensive Training Trials; however, this was not the case. Interestingly, the data obtained in this experiment indicated that some of the highest CK values were measured after Sessions II and III, which suggests that a greater amount of muscle damage existed during these two sessions than in Session I. As discussed earlier, this muscle damage and resulting tissue inflammation elicits a repair process, thus greater muscle damage, as evidenced by higher CK values, which may promote increased plasma TNF-α.[25, 37] It has been demonstrated that CK shows a delayed response in most individuals, peaking anywhere from 24 to 96 hours post-exercise.[2, 23, 35] While the CK values obtained in this study show that the highest values were obtained after Session II and Session III, it is a possibility that the increases seen after these sessions are the result of CK accumulation from Session I.

In addition, the percent change in TNF-α with exercise was not of a greater magnitude with each successive Intensive Training Trial as had been hypothesized. The greatest percent change in TNF-α was achieved during Session II, which, as mentioned, was

perceived as the most difficult day for all participants in this study. Despite the fact that VO_2 was steady-state and lactate increased in a similar fashion in the Intensive Training Trials, HR results for Session II indicated that HR at several points, including the end of exercise (60 minutes), was greater than the same time point in Session III, which suggests that Session II was more stressful than Session III. The CK data also showed that the highest CK values were measured during Session II. Taken together, these points suggest that Session II was the most stressful of the Intensive Training Trials, which could explain the greater percent change in TNF-α for Session II than for Sessions I and III.[37] Once again, it must be noted that CK shows a delayed response, and the increase in CK during Session II may be the result of CK accumulation in the blood.[2, 23, 33] Muscle damage likely occurred during Session I, as evidenced by increased post-exercise CK. The CK then began to accumulate in the blood, as more muscle damage was caused during Session II. The increasing amount of muscle damage likely induced a local inflammatory response which caused an upregulation in TNF-α, as evidenced by the increase in the percent change for Session II. Due to the fact that the inflammation was not due to an invading pathogen, TNF-α began to be cleared, as evidenced by the lack of significant percent change for Session III, while CK continued to increase.

Finally, the resting concentrations of plasma TNF-α were not significantly elevated on each successive day of intensive prolonged exercise and the final rest day. It was suspected that baseline values of TNF-α would increase each day, and that the highest baseline value of TNF-α would be found for Session IV. The highest baseline value of TNF-α was, in fact, found for Session IV; however, baseline concentrations did not increase over the course of Sessions I, II and III, as was originally thought. Baseline TNF-α significantly increased only from Session II to Session IV.

Some of the existing research literature indicates that exercise has no effect on baseline values of TNF-α.[7, 24, 42] These studies showed that TNF-α concentrations were not significantly elevated over the pre-exercise baseline values when taken one to three and a half hours post-exercise.[6, 22, 38] Post-exercise recovery blood samples were not taken in the present experiment, so it is not possible to know if TNF-α returned to baseline in this one to three and a half hour time period, but we know that it happened 24 hours post-exercise.

In contrast, other studies demonstrate that TNF-α remained elevated 24 hours after exercise, and in some cases 72 hours after the initial pre-exercise value.[5, 17] This was not found in the present experiment. The differences in baseline TNF-α between the studies could be due to several factors. Brenner et al.[5] utilized a protocol that involved moderately trained athletes completing two hours of exercise at 60% of VO_{2max}. As noted earlier, cytokine production and release is directly related to exercise intensity and duration.[14, 28, 40] The fact that the Brenner et al.[5] exercise protocol was twice the length of the present protocol could account for the elevated TNF-α found 72 hours post-exercise. The elevated baseline TNF-α for several days after the completion of exercise could also be due to the fact that the athletes that completed these two hours of exercise were not as fit as those who participated in the present investigation.[46] Viru and Viru [46] have noted that with many hormones, an increase in the blood occurs if the activity is above a certain threshold; however, with exercise training, this threshold increases. This means that at an identical absolute intensity, the trained athlete will have a lower response, or the response will disappear entirely. These trained athletes may release more TNF-α, but they are better at clearing it than the moderately trained athlete. The same principles can be applied to the Moldoveanu et al.[17]

study, which found that TNF-α was still elevated 24 hours post-exercise, as healthy, but untrained men completed three hours of exercise in their investigation.

IL-6: TNF-α ratio

Results showed a significant main effect of time for the IL-6:TNF-α ratio, indicating that the ratio increased from pre-exercise to post-exercise during the Intensive Training Trials. Timmons suggests that the IL-6:TNF-α ratio could be a useful tool in order to look at the inflammatory environment of an individual in response to exercise.[43] That is, the ratio could be used to decipher if exercising is putting the individual in a position that would make them more susceptible to infection. An increase in the ratio indicates a greater predominance of IL-6, which would suggest a more anti-inflammatory environment. In contrast, a decrease in the ratio, due to TNF- α predominance, would suggest a more pro-inflammatory environment. Our IL-6:TNF-α ratio data suggest that exercise is not making these particular athletes more susceptible to infection. It is clear that the ratios increase slightly with exercise, due to the increase in circulating IL-6. The increase in ratio is not detrimental, as it suggests that that the environment at the time of exercise was one that was essentially anti-inflammatory. In addition, since both cytokines, IL-6 and TNF- α, appeared to be cleared within 24 hours post-exercise there was a lack of an additive effect. It may be, though, that three days of intensive prolonged exercise is the upper limit, as the baseline value of TNF-α was higher than all other baselines for Session IV. It is possible that intensive prolonged exercise for a fourth day may cause an even greater increase in TNF-α, which would result in a lower IL-6:TNF-α ratio. This lowering of the ratio would indicate a greater pro-inflammatory environment, which could mean that the athlete is more susceptible to

infection. However, this was not examined in the present investigation, and is speculative, thus further research is warranted on this matter.

Conclusion

In conclusion, three consecutive days on intensive training does not appear to increase an athletes susceptibility to infectious illnesses as previously reported in the research literature.[10, 12, 29, 30, 32, 35, 39, 44, 47] It was originally suggested that the increase in susceptibility to infection was associated with OTS, in which frequent, intense training caused a decrease in performance, rather than an increase in performance.[30, 35, 36] Researchers have suggested a cytokine Open Window that occurs in the time period between three and 72 hours after exercise, in which an increase in pro-inflammatory cytokines begin the inflammatory process. During this time period, pathogens may gain a "foothold" in the body, because the immune system cannot adequately respond to both the inflammation caused by the intensive exercise and the pathogen, thus increasing the risk of infection.[22, 35] The data from the present investigation involving three consecutive days of cycling exercise at 75% of VO_{2peak} suggest that while IL-6 concentration in the blood is generally increased with exercise (this response was mirrored by TNF-α, but only over the last two days of exercise), it stayed elevated for less than 24 hours and does not appear to have an additive effect, indicating a clearance of the cytokine.

CHAPTER VI

Summary, Conclusions and Recommendations

Summary

Athletes engaged in intensive exercise have demonstrated an increased susceptibility to infectious illnesses.[10, 12, 30, 32, 35, 39, 44, 47] Pro-inflammatory cytokines are released into the circulatory system in response to intensive exercise, causing inflammation.[24] The immune system is then occupied with the task of responding to that inflammation for up to several days following the exercise.[18] The cytokine Open Window model, in which several consecutive days of intensive training decrease function of the immune system, has been suggested to explain this increased susceptibility to illness that occurs in the time period between three and 72 hours after exercise.[22, 35]

The purposes of this experiment were to determine the acute and cumulative responses of IL-6 and TNF-α to the three days of intensive training, and to determine if there were differences in baseline values of IL-6 and TNF-α during three days of intensive training. Seven highly trained males (Mean ± SD: age = 29.1 ± 7.0 yr, height = 180.7 ± 7.4 cm, mass = 78.8 ± 4.7 kg, body fat % = 13.8 ± 2.6, VO_{2peak} = 59.7 ± 7.7 ml/kg/min^{-1}) completed three Intensive Training Trials (Sessions I-III) which consisted of 60 minutes of cycling at ~75%VO_{2peak}. Resting, baseline and post-exercise blood samples were taken to determine IL-6 and TNF-α concentrations for Sessions I-III. Session IV consisted of only a resting, baseline blood sample.

Results indicated a significant main effect of exercise for IL-6 ($p = 0.002$). Mean pre-exercise IL-6 was 4.43 ± 2.67 pg/mL and mean post-exercise IL-6 was 8.23 ± 3.67 pg/mL; an increase of 85.78%. There were no significant differences in the percent change or baseline IL-6 over the course of the Intensive Training Trials ($p > 0.05$). Results also indicated a significant session X time interaction ($n = 6$, $p = 0.001$) for TNF-α. Analyses revealed a significant increase in mean TNF-α from pre- to post-exercise for both Sessions II and III. Results also showed a significantly higher percent change in TNF-α for Session II than for both Session I and Session III, as well as a significantly higher baseline concentration of TNF-α for Session IV than for Session II. Finally, there was a significant main effect for the ratio of IL-6:TNF-α. The mean pre-exercise IL-6:TNF-α was 24.69 ± 15.47. Post-exercise, this ratio increased 60.76% to 39.69 ± 16.36, suggesting the existence of anti-inflammatory environment.

These results indicate that three days of Intensive Training Trials consisting of 60 minutes of cycling exercise at ~75% of VO_{2peak} do not result in a significant widening of the Open Window period, meaning that athletes who train intensively for three consecutive days do not appear to be at a greater risk of becoming ill or developing the OTS. It is clear that IL-6 increases with exercise (TNF- α partially); however, it stays elevated for only a short period of time, as evidenced by both the clearance of this cytokine within 24 hours post-exercise and the lack of an additive effect.

Conclusions

Hypothesis 1: Plasma IL-6 will be significantly increased from rest immediately post-exercise for each bout of intensive training. This hypothesis was *accepted*. IL-6 was

significantly increased from pre-exercise to post-exercise for all three of the Intensive Training Trials.

Hypothesis 2: The percent change in IL-6 will be of a greater magnitude with each successive bout of intensive training. This hypothesis was *rejected*. The percent change in IL-6 did not change over the course of the three Intensive Training Trials.

Hypothesis 3: The resting, baseline concentrations of plasma IL-6 will be significantly elevated following each successive day of intensive training, and the following rest day. This hypothesis was *rejected*. There were no significant increases in resting, baseline IL-6. This baseline remained stable throughout the duration of the three days of Intensive Training Trials.

Hypothesis 4: Plasma TNF-α will be significantly increased from rest immediately post-exercise for each bout of intensive training. This hypothesis was *rejected*. TNF-α was significantly elevated immediately post-exercise for Sessions II and Session III, but not for Session I.

Hypothesis 5: The percent change in TNF-α will be of a greater magnitude with each successive bout of intensive training. This hypothesis was *rejected*. The percent change obtained for Session II was of a significantly greater magnitude than both Sessions I and III.

Hypothesis 6: The resting, baseline concentrations of plasma TNF-α will be significantly elevated for each successive day of intensive training, and the following rest day. This hypothesis was *rejected*. Resting, baseline TNF-α was expected to significantly increase with each successive training trial; however, it was only significantly higher for Session IV than for Session II.

Recommendations for Future Research

In order to gain more insight into the cytokine response to several of intensive training, the investigator recommends that future researchers:

- Repeat the experiment with an increased number of participants
- Repeat the experiment with a fourth Intensive Training Trial
- Repeat the experiment using a different mode of exercise, such as running
- Repeat the experiment using different intensities and durations of exercise
- Repeat the experiment with sedentary individuals, as well as moderately trained athletes
- Repeat the experiment and include other cytokines in addition to IL-6 and TNF-α
- Continue to take blood samples for several hours into recovery
- Have participants complete two exercise sessions within one day
- Strictly control the caloric intake of the participants in the study, instead of asking them to follow general guidelines and consume a carbohydrate supplement
- Control VO_2 more tightly during exercise

APPENDICES

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APPENDIX A:

Medical Screening Forms

* These materials are not included in the copyright of Melissa B. Behr

Department of Exericse and Sport Science
Medical History

Subject ID:_____ Telephone: _____

Address:_____

Occupation: _____ Age: _____

YES NO

Patient History

1. How would you describe your general health at present? _____ _____
   Excellent _____ Good _____ Fair _____ Poor _____
2. Do you have any health problems at the present time? _____ _____
3. If yes, please describe: _____

4. Have you ever been told you have heart trouble? _____ _____
5. If yes, please describe: _____

6. Do you ever get pain in your chest? _____ _____
7. Do you ever feel lightheaded or have you ever fainted? _____ _____
8. If yes, please describe: _____

9. Have you ever been told that your blood pressure has been elevated? _____ _____
10. If yes, please describe:_____

11. Have you ever had difficulty breathing either at rest or with exertion? _____ _____
12. If yes, please describe: _____

13. Are you now, or have you been in the past 5 years, under a doctor's care for any reason? _____ _____
14. If yes for what reason? _____

15. Have you been in the hospital in the past 5 years? _____ _____
16. If yes for what reason? _____

17. Have you ever experienced an epileptic seizure or been informed that you have epilepsy? _____ _____
18. Have you ever been treated for infectious mononucleosis, hepatitis, pneumonia, or another infectious disease during the past year? _____ _____
19. If yes, name the disease. _____
20. Have you been treated for or told you might have diabetes? _____ _____
21. Have you been treated for or told you might have low blood sugar? _____ _____
22. Do you have any known allergies to drugs? _____ _____
23. If so, what? _____

24. Have you ever been "knocked out" or experienced a concussion? _____ _____
25. If yes, have you been "knocked out" more than once? _____ _____
26. Have you ever experienced heat stroke or heat exhaustion? _____ _____
27. If yes, when? _____

_____

28. Have you ever had any additional illnesses or operations? (Other than childhood diseases) _____ _____
29. If yes, please indicate specific illness or operations: _____

_____

30. Are you now taking any pills or medications? _____ _____
31. If yes, please list: _____

_____

32. Have you had any recent (within 1 year) difficulties with your:
    a. Feet _____ _____
    b. Legs _____ _____
    c. Back _____ _____

Family History

33. Has anyone in your family (grandparent, father, mother, and/or sibling) experienced any of the following?
    a. Sudden death _____ _____
    b. Cardiac disease _____ _____
    c. Marfan's syndrome _____ _____

Mental History

34. Have you ever experienced depression? _____ _____
35. If yes, did you seek the advice of a doctor? _____ _____
36. Have you ever been told you have or has a doctor diagnosed you with panic disorder, obsessive-compulsive disorder, clinical depression, bipolar disorder, or any other psychological disease? _____ _____
37. If yes, please list condition and if you are currently taking any medication.

Condition Medication

_____

_____

_____

Bone and Joint History

34. Have you ever been treated for Osgood-Schlatter's disease? _____ _____
35. Have you ever had any injury to your neck involving nerves or vertebrae? _____ _____
36. Have you ever had a shoulder dislocation, separation, or other injury of the shoulder that incapacitated you for a week or longer? _____ _____
37. Have you ever been advised to or have you had surgery to correct a shoulder condition? _____ _____
38. Have you ever experienced any injury to your arms, elbows, or wrists? _____ _____
39. If yes, indicate location and type of injury: _____

40. Do you experience pain in your back? _____ _____
41. Have you ever had an injury to your back? _____ _____
42. If yes, did you seek the advice of a doctor? _____ _____
43. Have you ever been told that you injured the ligaments or cartilage of either knee joint? _____ _____
44. Do you think you have a trick knee? _____ _____
45. Do you have a pin, screw, or plate somewhere in your body as the result of bone or joint sugery that presently limits your physical capacity? _____ _____
46. If yes, indicate where: _____
_____
47. Have you ever had a bone graft or spinal fusion? _____ _____

Activity History

48. During yoru early childhood (to age 12) would you say you were:
Very active _____ Quite active _____ Moderately active _____ Seldom active _____
49. During your adolescent years (age 13-18) would you say you were:
Very active _____ Quite active _____ Moderately active _____ Seldom active _____
50. Did you participate in:
a. Intramural school sports? _____ _____
b. Community sponsored sports? _____ _____
c. Varsity school sports? _____ _____
d. Active family recreation? _____ _____
51. Since leaving high school, how active have you been?
Very active _____ Quite active _____ Moderately active _____ Seldom active _____
52. Do you participate in any vigorous activity at present? _____ _____
53. If yes, please list:

Activity	Frequency	Duration	Intensity

54. How would you describe your present state of fitness?
Excellent _____ Good _____ Fair _____ Poor _____
55. Please list the type(s) of work you have been doing for the previous ten years:

Year	Work	Indoor/Outdoor	Location (city/state)

56. Whom shall we notify in case of emergency?
Name: _____
Phone: (Home) _____ (Work) _____
Address: _____

57. Name and address of personal physician: _____

_____

_____

All of the above questions have been answered completely and truthfully to the best of my knowledge.

Signature: _____ Date: _____

APPENDIX B:

Physical Screening Forms

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Examination status: Approved Disapproved

Department of Exercise & Sport Science
Physical Examination Screening

Name: _____ Age: _____ Gender: _____

Please respond to each of the following in writing.

Pulse rate and regularity: _____ ECG Interpretation: _____

Blood Pressure:

Supine: _____ Sitting: _____ Standing (Left side): _____

Squat: _____ Standing (Right side): _____

Marfan Syndrome evaluation: (Δ BP, Physical Char.) _____

Palpation of Pulses: Carotid: _____ Radial: _____ Pedal: _____

Auscultation of the Lungs:

Back: Lower: _____ Middle: _____ Upper: _____

Front: Middle: _____ Upper: _____

Auscultation of Heart Sounds:(Supine, Standing, Squatting)

Non-Specific HS: _____/_____/_____

Murmur: _____ Gallop: _____ Click: _____ Rub: _____ Click w/ Murmur: _____

Bruits: Carotid: _____ Abdominal: _____

Edema: Abdominal: _____ Calf: _____ Pedal: _____

Tenderness: Abdominal: _____ Other: _____

Xanthoma or xanthelasm: _____

Medical / Family History:

High Blood Pressure: _____ Diabetes: _____ CHD/CAD: _____

Last examination w/ physician: _____

Medications (prescription / counter): _____

Examiner: _____ Date: _____

APPENDIX C:

Data Collection Forms

Subject_____ **Orientation Session**
Date_____

Informed Consent
1. Inform participant of the experimental protocol
2. Make participant aware of the possible risks
3. Sign informed consent

Participant Compliance Questions
1. Did the participant perform strenuous physical activity for 24 hours prior to VO_{2peak} testing:
   Y N
2. Did the participant eat, smoke or consume alcohol or caffeine 2 hours prior to testing:
   Y N

Examinations _____
1. Medical Examination
2. 12 Lead EKG
3. Physical Examination

Physical Characteristics
1. Sex _____
2. Age _____ yrs
3. Height _____ cm
4. Weight _____ kg
5. Percent Body Fat _____%; Skinfolds:
   a. Chest _____mm _____ mm _____ mm; Mean = _____mm
   b. Ilium _____mm _____mm _____mm; Mean = _____mm
   c. Abdomen _____mm _____mm _____mm; Mean = _____mm
   d. Axilla _____mm _____ mm _____ mm; Mean = _____mm
   e. Sum of Skinfolds _____mm

Before VO_{2peak} Protocol
1. Set up metabolic system (calibrate, mouthpiece, etc)
2. Fit cycle ergometer to the participant - record seat position using flexible tape (line tape up from black cross bar on back of seat to the clear piece of tape on the cycle)
   Seat height: _____ cm
3. Fit polar heart rate (HR) monitor to participant
4. Make sure polar heart rate monitor picks up signal
5. Place RPE scale near cycle ergometer/explain RPE to participant

Warm Up
1. 5 minutes of cycling at 50W
2. 5 minutes of stretching focused on the lower body
3. Record resting oxygen consumption for 4 minutes (have participant sit on bike) – headpiece (NOT mouthpiece) is mandatory for all MAX tests

VO_{2peak} Protocol
1. Stage 1: 50W for 3 minutes →HR _____; RPE _____
2. Stage 2: 100W for 3 minutes →HR _____; RPE _____
3. Stage 3: 150W for 3 minutes →HR _____; RPE _____
4. Stage 4: 200W for 3 minutes →HR _____; RPE _____
5. Stage 5: 225W for 1 minute →HR _____; RPE _____
6. Stage 6: 250W for 1 minute →HR _____; RPE _____
7. Stage 7: 275W for 1 minute →HR _____; RPE _____
8. Stage 8: 300W for 1 minute →HR _____; RPE _____
9. Stage 9: 325W for 1 minute →HR _____; RPE _____

10. Stage 10: 350W for 1 minute →HR _____; RPE _____
11. Stage 11: 375W for 1 minute →HR _____; RPE _____
12. Stage 12: 400W for 1 minute →HR _____; RPE _____
13. Increase workload until volitional fatigue → add more stages if necessary (record to right)
14. Recovery - reduce resistance and have participant continue pedaling
15. Participant rests (supine) until HR is less than or equal to 100 bpm

Prior to Participant Exiting – Give canister of polycose supplement. Instruct the participant as follows:

1. Consume 25% of the canister throughout the day before Session II
2. Consume 25% of the canister throughout the day of Session II
3. Consume 25% of the canister throughout the day of Session III
4. Consume 25% of the canister throughout the day of Session IV
5. Instruct the participant to bring the empty canister with them for Session V
6. Instruct the participant to write down everything they eat and drink on the day preceding Session II, as well as on the days of Session II, III and IV

Criteria for valid VO_{2peak} Test

1. Did the participant have a maximal RER equal to or greater than 1.1? RER = _____
2. Did the participant reach age predicted maximal HR (220-age ± 5%)? HR_{max} = _____
3. Did the participant have a RPE equal to or greater than 18? RPE = _____
4. Was the total test time equal to or greater than 12 minutes? Test time = _____

Power Output Estimation for Intensive Training Trials

1. VO_{2peak} = _____
2. Peak Workload = _____
3. Workload that corresponds to 75% VO_{2peak} = _____

Subject_____ **SESSION I II III**
Date_____ (circle one)
Time_____

Special Notes

1. Session I must start at least one week after determination of VO_{2peak}
2. Sessions I-III must be spaced 24 hours apart, at or about the same time each day

Participant Compliance Questions (some only apply to Session II)

1. Did the participant perform an easy day of training 2 days prior to the start of session I?Y N N/A
2. Did the participant perform activity the day prior to Session I? Y N N/A
3. Did the participant consume 25% of the polycose supplement the day prior to the Session? Y N
4. Has the participant been sick in the 6 week period prior to testing? Y N N/A
5. Did the participant eat, smoke or consume alcohol or caffeine 8 hours prior to testing? Y N
6. Has the participant taken any NSAIDS in the past 24 hours? Y N

Before Starting Exercise Protocols for Sessions II-IV

1. Collect the Food Diary from the participant
2. Set up metabolic system (calibrate, mouthpiece, etc) NOTE: use only the mouthpiece for the submaximal exercise sessions.
3. Set up blood supplies
4. Set up cycle ergometer to previously recorded seat height: _____ cm
5. Fit polar heart rate (HR) monitor to participant
6. Make sure polar heart rate monitor picks up signal
7. Place RPE scale near cycle ergometer

Exercise Protocol for Sessions II-IV

1. The participant will rest in the supine position for 10 minutes
2. Obtain 3-mL of venous blood using the standard Venipuncture technique
3. Placed blood into a sterile K_2 - EDTA (purple top) Vacutainer® tube
4. Place tube on ice immediately
5. 10 minute warm up
    a. 5 minutes of cycling at 50W
    b. 5 minutes of stretching focused on the lower body
6. Record resting oxygen consumption for 4 minutes with participant seated on bike (remove mouthpiece after sampling)
7. Cycle for 60 minutes at the previously determined workload that elicits 75% of VO_{2peak}
    a. Min 0 → HR _____; RPE _____
    b. Minute 10 → HR _____; RPE _____
    c. Minutes 16-20 → VO_2 measurement (remove mouthpiece after sampling)
    d. Minute 20 → HR _____; RPE _____
    e. Minute 30 → HR _____; RPE _____
    f. Minutes 36-40 → VO_2 measurement (remove mouthpiece after sampling)
    g. Minute 40 → HR _____; RPE _____
    h. Minute 50 → HR _____; RPE _____
    i. Minutes 56-60 → VO_2 measurement (remove mouthpiece after sampling)
    j. Minute 60 → HR _____; RPE _____
8. Recovery - assist participant off bike, and have them rest (supine)
9. Obtain 3-mL of venous blood using the standard Venipuncture technique
10. Placed blood into a sterile K_2 - EDTA (purple top) Vacutainer® tube
11. Place tube on ice immediately
12. Participant rests (supine) until HR is less than or equal to 100 bpm

Subject_____ **SESSION IV**
Date_____
Time_____

Special Notes

- Sessions IV must occur ~24 hours after session IV

Participant Compliance Questions

1. Did the participant eat, smoke or consume alcohol or caffeine 8 hours prior to testing? Y N
2. Has the participant taken any NSAIDS in the past 24 hours? Y N
3. Did the participant consume 25% of the polycose supplement the day prior to the Session? Y N

Before Starting Protocols for Sessions V

1. Collect the Food Diary from the participant
2. Set up blood supplies

Session V Protocol

1. No exercise will be performed on this day
2. The participant will rest in the supine position for 10 minutes
3. Obtain 3-mL of venous blood using the standard Venipuncture technique
4. Placed blood into a sterile K_2 - EDTA (purple top) Vacutainer® tube
5. Place tube on ice immediately

APPENDIX D:

Assay Information

IL-6 Assay

ASSAY METHOD

a) Before use, mix all reagents thoroughly without making foam.

b) Determine the number of microwell strips required to test the desired number of samples, plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control samples should be assayed **in duplicate**. Remove sufficient microwell strips from the pouch.

c) Add 100 µl of appropriate standard diluent to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, F2. Reconstitute standard vial with the appropriate volume as described in the chapter reagents preparation. Pipet 200 µl of standard into wells A1 and A2 (see Plate Scheme below). Transfer 100 µl from A1 and A2 to B1 and B2 wells. Mix the contents by repeated aspirations and ejections. Take care not to scratch the inner surface of microwells. Repeat this procedure from the wells B1, B2 to wells C1, C2 and from wells C1, C2 to D1, D2 and so on creating two parallel rows of IL-6 standard dilutions ranging from 200 to 6.25 pg/ml. Discard 100 µl from the content of the last microwells used (F1, F2).

d) Add 100 µl of appropriate standard diluent to the blank wells (G1-G2).

e) Add 100 µl of samples to sample wells and 100 µl of control to the control wells (H1, H2).

f) Preparation of biotinylated anti-IL-6 : see reagents preparation.

g) Add 50 µl of diluted biotinylated anti-IL-6 to all wells.

h) Cover with a plate cover and incubate for 1 hour at room temperature (18°C - 25°C).

i) Remove the cover plate and wash the plate as follows:
   1) aspirate the liquid from each well ;
   2) dispense 0.3 ml of washing solution into each well ;
   3) aspirate again the content of each well ;
   4) Repeat steps 2) and 3) two times.

j) Prepare HRP solution just before use: see reagents preparation.

k) Dispense 100 µl of HRP solution into all wells, including the blank wells. Put back the cover plate.

l) Incubate the microwell strips at room temperature for 30 minutes.

m) Remove plate cover and empty wells. Wash microwell strips according to point (i). Proceed immediately to the next step.

n) Pipette 100 µl of ready-to-use TMB substrate solution into all wells, including the blank wells and incubate in the dark for 12-15 minutes at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.

o) Incubation time of the substrate solution is usually determined by the ELISA reader performances: many ELISA readers record absorbance only up to 2.0 O.D. The O.D. values of the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly readable (maximum 20 minutes).

p) The enzyme-substrate reaction is stopped by quickly pipetting 100 µl of 1.8 N sulfuric acid into each well, including the blank wells, to completely and uniformly inactivate the enzyme. Results must be red immediately after the addition of sulfuric acid, or within one hour, if the microwell strips are stored at 2-8°C in the dark.

q) Read absorbance of each well on a spectrophotometer using 450 nm as the primary wavelenght and optionnally 620 nm (610 nm to 650 nm is acceptable) as the reference wavelenght.

TNF-α Assay

ASSAY METHOD

a) Before use, mix all reagents thoroughly without making foam.

b) Determine the number of microwell strips required to test the desired number of samples, plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control samples should be assayed **in duplicate**. Remove sufficient microwell strips from the pouch.

c) Add 100 µl of appropriate standard diluent to standard wells B1, B2, C1, C2, D1, D2, E1, E2, F1, F2. Reconstitute standard vial with the appropriate volume as described in the chapter reagents preparation. Pipet 200 µl of standard into wells A1 and A2 (see Plate Scheme below). Transfer 100 µl from A1 and A2 to B1 and B2 wells. Mix the contents by repeated aspirations and ejections. Take care not to scratch the inner surface of microwells. Repeat this procedure from the wells B1, B2 to wells C1, C2 and from wells C1, C2 to D1, D2 and so on creating two parallel rows of TNFα standard dilutions ranging from 800 to 25 pg/ml. Discard 100 µl from the content of the last microwells used (F1, F2).

d) Add 100 µl of appropriate standard diluent to the blank wells (G1-G2).

e) Add 100 µl of sample to sample wells and 100µl of control to control wells (H1, H2)

f) Preparation of biotinylated anti- TNFα: see reagents preparation.

g) Add 50 µl of diluted biotinylated anti- TNFα to all wells.

h) Cover with a plate cover and incubate for 3 hours at room temperature (18°C - 25°C).

i) Remove the cover plate and wash the plate as follows:
   1) aspirate the liquid from each well ;
   2) dispense 0.3 ml of washing solution into each well ;
   3) aspirate again the content of each well ;
   4) Repeat steps 2) and 3) two times.

j) Prepare HRP solution just before use: see reagents preparation.

k) Dispense 100 µl of HRP solution into all wells, including the blank wells. Put back the cover plate.

l) Incubate the microwell strips at room temperature for 30 minutes.

m) Remove plate cover and empty wells. Wash microwell strips according to point (i). Proceed immediately to the next step.

n) Pipette 100 µl of ready-to-use TMB substrate solution into all wells, including the blank wells and incubate in the dark for 12-15 minutes at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.

o) Incubation time of the substrate solution is usually determined by the ELISA reader performances: many ELISA readers record absorbance only up to 2.0 O.D. The O.D. values of the plate should be monitored and the substrate reaction stopped before positive wells are no longer properly readable (maximum 20 minutes).

p) The enzyme-substrate reaction is stopped by quickly pipetting 100 µl of 1.8 N sulfuric acid into each well, including the blank wells, to completely and uniformly inactivate the enzyme. Results must be red immediately after the addition of sulfuric acid, or within one hour, if the microwell strips are stored at 2-8°C in the dark.

q) Read absorbance of each well on a spectrophotometer using 450 nm as the primary wavelenght and optionnally 620 nm (610 nm to 650 nm is acceptable) as the reference wavelenght.

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