Effect of Whey Protein Isolate on Oxidative Stress, Exercise Performance, and Immunity

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In

Human Nutrition, Foods, and Exercise

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Abstract

The purpose of this study was to evaluate the effectiveness of a whey protein isolate (WPI), a reported glutathione (GSH) booster, on exercise performance, immune function, and antioxidant status during weight maintenance and energy restriction in humans. Twenty well-trained, college age, male cyclists performed a cycling exercise test for 45 min, the first 7 min at 70% of VO_{2peak} and the remaining 38 min at 55% VO_{2peak} immediately followed by a performance test set at 90% VO_{2peak} until exhaustion. Blood samples were collected prior to the exercise test, after 45 min of exercise, within 5 min of exhaustion, and 1 h after exercise. Blood samples were analyzed for GSH, GSH/GSSG ratio, glutathione peroxidase (GPx), lipid hydroperoxides (LPO), phagocytosis, oxidative burst, peripheral blood mononuclear cell (PBMC) proliferation, and PBMC phenotyping. Subjects consumed 40g/day of WPI or casein placebo (P) along with their normal diet for 2 wk, repeated the exercise test, and then began a low energy period continuing the same supplementation for 4 d before the final exercise test. WPI was not associated with superior exercise performance or antioxidant status following exercise or weight loss. WPI supplementation did result in 33% greater lymphocyte proliferation capacity following exercise. Following exhaustive exercise for all trials, tGSH and GPx increased 7% and 11%, respectively, while WBCGSH decreased 13%. For WPI, GPx activity was 10% lower than P following exhaustive exercise for all trials combined. Weight loss (2.67 ± 0.26 kg) resulted in increases in phagocytosis (65%), white blood cell (WBC)

GSH (40%), and GPx (35%) while decreasing the GSH/GSSG ratio (55%) and LPO (16%). Exhaustive exercise caused a 28% increase in CD3+CD8+ PBMCs and decreased CD3+CD4+ (34%), CD3+ (15%), the CD4+/8+ ratio (45%), and phagocytosis (8%) with all values returning to baseline after 1 h recovery. Supplementation with WPI did not enhance GSH status or exercise performance in trained cyclists, during weight maintenance or energy restriction. Following exercise, WPI is associated with greater lymphocyte proliferation of PBMCs which may help maintain an athlete's health during heavy training or competition.

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Chapter 1

Introduction

Glutathione (GSH) is a thiol containing tripeptide that serves as the cell's major antioxidant compound. By donating electrons GSH is able to reduce potentially deleterious oxidants thereby allowing the continuation of normal intracellular homeostasis or, during episodes of oxidative insult, attenuate cellular dysfunction. During this process GSH becomes oxidized to glutathione disulfide (GSSG). GSH status, as represented by the GSH/GSSG ratio, decreases during periods of oxidative stress (23, 91). Exercise can dramatically increase oxygen consumption and stimulate several pathways of reactive oxygen species (ROS) generation. Increased ROS production is associated with the onset of fatigue and altered immune function (73). Immune cells rely on GSH for protection against oxidative stress and membrane stabilization (95). Indeed, impaired immunity is often observed following strenuous prolonged aerobic exercise. Therefore, uncompromised intracellular GSH is crucial for proper muscle and immune cell function.

GSH is lipophobic and cannot cross cellular membranes such as those in skeletal muscle cells. Furthermore, oral and injected administration of GSH has been shown to be ineffective in increasing GSH concentrations (75, 94). Therefore, de novo synthesis of GSH is the predominant route of maintaining adequate GSH concentrations. To maintain adequate concentrations of GSH, the cell must be provided with the amino acid cysteine, the rate limiting substrate for GSH synthesis (44). Free cysteine is toxic and spontaneously oxidizes in circulation but it can be delivered intact in the form of γ-glutamylcysteine (7).

Several unique amino acid groups and peptides are present in whey following the precipitation of caseins in the production of cheese (95). One of these components is serum albumin, which, unlike casein, is rich in γ-glutamylcysteine groups. Intact glutamylcysteine dipeptides are rare in the edible portions of both plant and animal food products (7). The delivery of intact glutamylcysteine, the main substrate to glutathione synthetase (GS), results in the intracellular formation of GSH (8).

The heat, acidity, and mechanical stresses of normal food production techniques and pasteurization processes usually result in the denaturing of any glutamylcysteine groups present. Immunocal®, a commercially available whey protein concentrate was developed using a proprietary pasteurization process, which allows glutamylcysteine to remain intact. Absorption of this dipeptide is then mediated by the jejunal H^+/peptide transport system of healthy humans (40). Immunocal® has also been shown to have a high content of cystine, which is two cysteines linked together via a disulfide bond (7, 44). This disulfide bond is pepsin and trypsin resistant allowing cystine to be readily absorbed and subsequently transported safely in the circulation. Upon cellular entry cystine will be reduced to two cysteine molecules which are then available for GSH synthesis (7). Supplementation with Immunocal® has resulted in significant increases in red and white blood cell GSH concentrations (8, 33). Given the role GSH plays in maintaining cellular redox status and proper immune function, we hypothesize that Immunocal® supplementation will modulate exercise performance and the immune and antioxidant response to exercise during weight maintenance or energy restriction.

Purpose

The purpose of this study is to examine whether chronic administration of a GSH boosting supplement (Immunocal®) will influence exercise performance, the antioxidant response to exercise, and the immune response to exercise. As well, this study will examine these same effects following a period of energy restriction.

Significance

The aerobic athlete, through training, subjects their body to daily increases in oxidative stress. If these oxidative insults are not controlled by the body's natural antioxidant defenses the likelihood of contracting illness increases. This means the athlete will lose valuable training time during the recovery period. The results of this study may shed new light on a nutritional intervention, which may boost our antioxidant defenses, thereby enhancing disease resistance. As well, the aerobic athlete may experience increased sports performance by delaying the onset of ROS induced fatigue. The ability to avoid training induced compromises in immune function and the potential to increase exercise performance would be of tremendous interest to the elite level aerobic athlete as well as the non-athlete beginning a training program. Also, athletes and non-athletes alike often wish to lose body fat through caloric restriction. The added stress of dieting, while training, will further increase the opportunity of contracting an illness. GSH boosting, through dietary supplementation of a whey protein isolate, may provide the antioxidant protection the elite level and recreational athlete needs to remain healthy.

Hypotheses

During periods of both weight balance and energy restriction consumption of a glutamylcysteine rich whey protein product (Immunocal®) will result in:

1) Increased resting whole blood and lymphocyte glutathione.
2) Increased aerobic exercise performance.
3) Enhanced antioxidant response to exercise as measured by the GSH/GSSG ratio, whole blood GSH, white blood cell GSH (WBCGSH), lipid hydroperoxides (LPO), and glutathione peroxidase (GPx) activity.
4) Attenuation of immune responses to exercise.
    a) Superior phagocytic capability of neutrophils and monocytes compared to placebo.
    b) Superior oxidative burst capacity of phagocytic cells compared to placebo.
    c) Superior ability of mitogen induced lymphocyte proliferation.
    d) Superior maintenance of CD3+, CD4+, and CD8+ immune cell populations compared to placebo.
5) Consumption of Immunocal® will reduce the effect of energy restriction on antioxidant status, exercise performance, and immune function in athletes compared to placebo.

Delimitatations

The following delimitations were established for this study:

1. Subject selection was limited to males age 19-29 years who were present on the Virginia Polytechnic Institute and State University campus or in the Blacksburg, Virginia surrounding area.

2. Subjects were free from chronic disease or orthopedic limitations that would preclude their involvement in strenuous exercise.
3. Subjects were of trained aerobic fitness status with respect to cycling.
4. The subjects were not housed on-campus for the experimental period. They returned to their homes after testing.

Limitations

The following are potential limitations of this study:

1. The results of this study may be generalized to males of similar age and training status as defined by the subject sample.
2. The timing of blood draws was limited to fasting, immediate prior, after 45 minutes of exercise, after performance test, and 1 hour post on exercise testing days. Therefore, the antioxidant and immune function response is limited to these time points. More frequent sampling was not possible due to subjects' class or work schedules, and limitations set by the Virginia Polytechnic Institute and State University Institutional Review Board.
3. No biochemical assessments of nutritional status were performed on the subjects prior to the start of the experimental period.

Basic Assumptions

The following assumptions were made in this study:

1. The subjects were honest in reporting their daily activity, food, and supplement consumption.

2. The subjects refrained from taking any protein supplements or stimulatory substances during the course of the experimental period.
3. Changes in blood antioxidants and immune function were due to the exercise treatment and/or supplementation and not caused by extraneous environmental factors.
4. All subjects gave a maximal effort during the exercise testing protocols (VO_{2peak} testing, exercise tests, and performance tests).

Definition of Terms

GSH. Glutathione (reduced), a thiol containing tripeptide (L-γ-glutamyl-L cysteine-glycine) which serves as the cell's major antioxidant compound.

GSSG. Glutathione disulfide (oxidized), the product of the glutathione peroxidase reaction in which GSH donates an electron in the reduction of hydrogen peroxides.

GPx. Glutathione peroxidase, the enzyme that catalyzes the reduction of hydrogen peroxide to water using GSH as the electron donor.

LPO. Lipid hydroperoxides, result from the oxidation of polyunsaturated lipids.

Lymphocyte proliferation. The ability of lymphocytes to replicate their genetic material and divide.

Phagocytosis. The process of ingestion and processing by immune cells, neutrophils and monocytes, of solid substances (e.g. bacteria).

Oxidative burst. Following phagocytosis, the ability to kill the ingested material with endogenous microbicides.

Whey protein. The watery portion remaining after the precipitation of caseins during the production cheese. Whey contains serum albumin which, prior to normal pasteurization, contains intact glutamylcysteine groups, a necessary precursor to intracellular GSH synthesis.

Peak Oxygen Uptake (VO_{2peak}). The highest level of oxygen use by skeletal muscle, heart, and lungs during an incremental cycling or running exercise test performed to exhaustion. It is considered an indicator of aerobic or cardiorespiratory physical fitness.

Chapter II

Review of Literature

Overview

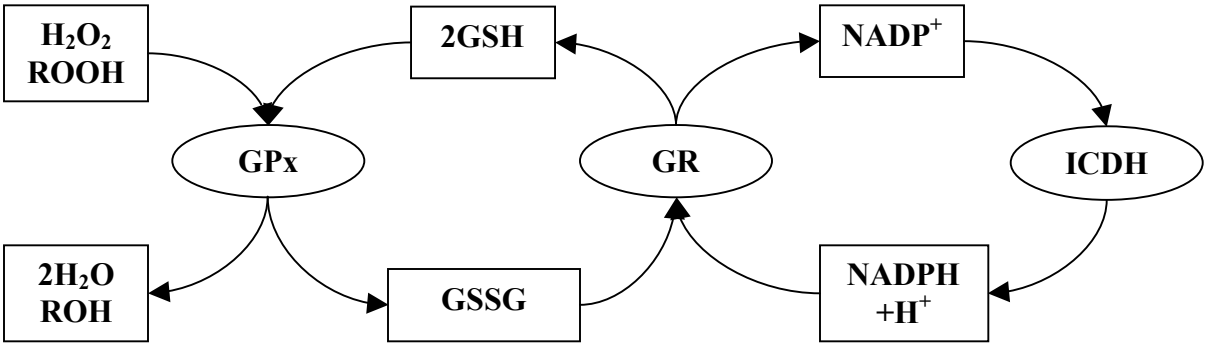
GSH and related enzymes

GSH, a thiol containing tripeptide (L-γ-glutamyl-L-cysteine-glycine), is present in all mammalian cells in varied concentrations and serves a multitude of functions. While the major role of GSH is that of an intracellular antioxidant, additional functions include drug detoxification, regulation of signal transduction, cysteine storage and transport, cell proliferation regulation, synthesis of deoxyribonucleotide, and immune response regulation (73, 95). Depending on the organs function and oxidative capacity, levels of GSH vary correspondingly. For example, the liver has one of the highest GSH concentrations (5-7 mM) second only to the lens of the eye (~10 mM) (77). To protect against oxidative stress, red blood cells, despite a lack of mitochondria, have a relatively high GSH concentration (~2 mM) compared with blood plasma (~0.05 mM). Highly oxidative type I skeletal muscle fibers possess six times the amount of GSH when compared to type IIb fibers (~3 mM vs. 0.5 mM, respectively) (77). GSH concentrations relative to the oxidative capacity of tissues illustrate the antioxidant importance of GSH.

GSH is the main substrate for glutathione peroxidase (GPx) which catalyzes the reduction of hydrogen peroxide or organic hydroperoxide to H_2O or alcohol, respectively (63). In this reaction GSH is used as the electron donor and is thereby oxidized to GSSG. To regenerate GSH from GSSG the enzyme glutathione reductase (GR) harnesses the

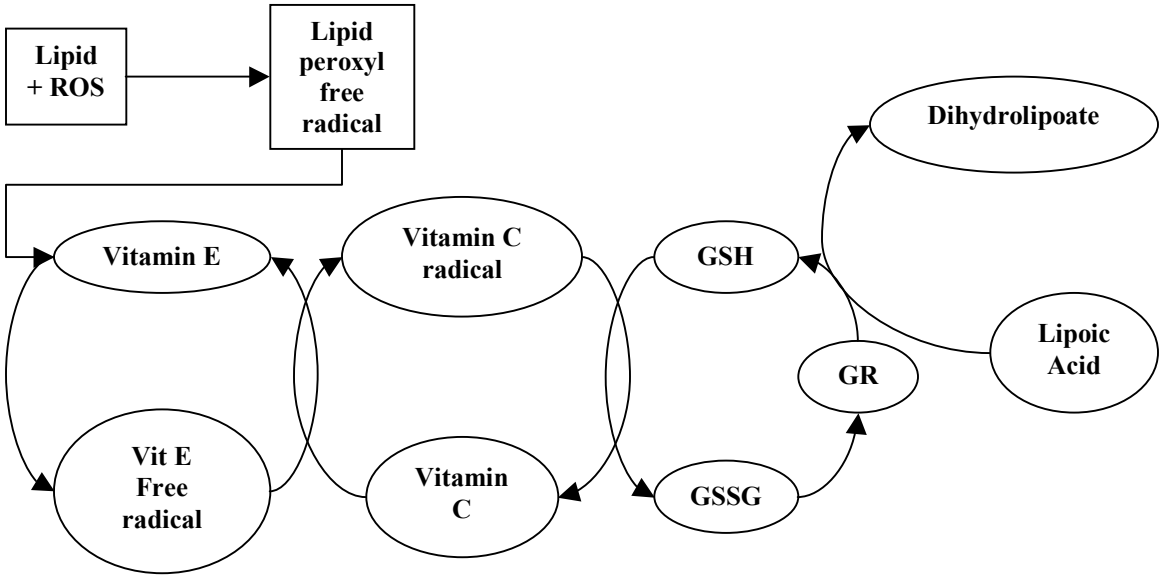
reducing power of NADPH produced via the pentose pathway or, in skeletal muscle, by isocitrate dehydrogenase (ICDH) (see Figure 1) (63). GPx and GR have a similar cellular distribution both of which being most highly concentrated and active in oxidative muscle. In the cell approximately 45% of GPx is located in the cytosol and the remaining 55% in the mitochondria allowing it to be in close proximity of hydroperoxide generation (63). While GR is not considered to be a primary antioxidant enzyme it is crucial in maintaining GSH availability for the reduction of hydroperoxides.

Figure 1: Glutathione redox cycle.



One important function of GSH is to remove hydrogen peroxide and organic peroxides. GSH also serves to reduce other antioxidants back to their biologically active forms (51). Vitamin E oxidation occurs during chain breaking reactions with lipid peroxyl radicals. Semi-dehydroascorbate, the vitamin C radical, is subsequently generated during the recycling of vitamin E. Both the oxidized forms of vitamins E and C are regenerated at the expense of GSH. GSH is also involved in the reduction of lipoic acid to its biologically active form dihydrolipoate (see Figure 2).

Figure 2: Antioxidant interactions and reductions



GSH synthesis and transport

Intracellular concentrations of GSH are determined by intracellular synthesis as well as GSH utilization and subsequent regeneration by GR. GSH is lipophobic and when intact cannot cross cellular membranes (74). De novo synthesis of GSH is accomplished via the enzymes of the γ-glutamyl cycle (62). In order for GSH to enter a cell it must first be broken down to its constituent amino acids. Membrane bound γ-glutamyl-transpeptidase (GGT) controls the cleavage and transport of the three GSH amino acids. Once inside, the rate limiting enzyme glutamylcysteine synthetase (GCS) catalyzes the formation of a peptide bond between cysteine and the γ-carboxylate of glutamate. This peptide is then rejoined with glycine by glutathione synthetase (GS) (62). The rate of GSH synthesis depends on the activity and concentration of the γ-glutamyl cycle enzymes. Skeletal muscle has a relatively high amount of GCS making the availability of

cysteine the rate-limiting component. Because skeletal muscle has a low activity of GGT, intact circulating GSH does not contribute significantly to skeletal muscle GSH concentration (75). Therefore, the abundant glutamylcysteine groups found in whey represent a viable source of amino acid precursors for skeletal muscle GSH de novo synthesis.

The GSH redox system

In an attempt to better monitor quantitative changes in intracellular redox potential, Schafer et al., (72) offered this definition of the redox environment: "The redox environment of a linked set of redox couples as found in a biological fluid, cell, or tissue is the summation of the products of the reduction potential and the reducing capacity of the linked redox couples present". This would differ from the "redox state" which refers to the reduction potential and reducing capacity of a particular linked couple (72). For example, GSH/GSSG represents an important linked redox couple. Because GSH concentrations range from 500-1000 fold higher than other cellular reductive substances (ie NADH), the GSH/GSSG ratio is often used as a direct measure of the intracellular redox environment (72)). The reduced form of glutathione is commonly 10- to 100-fold higher than the oxidized form and changes in the GSH/GSSG have an impact on signal transduction, cellular proliferation, differentiation, and apoptosis.

Periods of oxidative insult are characterized by decreases in GSH/GSSG. However, cells can maintain their GSH redox state by increasing GR activity and/or GSSG export (72). The enzyme γ-GCS can also sense oxidizing conditions and be upregulated to synthesize new GSH. Despite these defense mechanisms, cellular GSH

status can become compromised during prolonged exposure to oxidative stress and lead to cellular dysfunction and death (72). Any decrease in the GSH/GSSG ratio will be reflected in a decreased or unbalanced cellular redox environment which may then serve to activate several nuclear transcription factors (21). Activator protein 1 and nuclear factor-κB (NF-κB) are two such nuclear factors which are sensitive to redox alterations (21). NF-κB is activated by physical and oxidative stress (39) and considered a central mediator of the immune response (57). GSH also plays a role in redox signaling through the ability to remove H_2O_2, which has second messenger properties (16). Therefore, metabolic alterations in the GSH/GSSG redox state will have significant implications on cell function and defense capacities.

Exercise induced generation of reactive oxygen species (ROS)

An exercising human can considerably increase oxygen consumption. The body may increase oxygen intake by as much as 15 fold while oxygen flux through active muscle can increase 100 fold (13). This increase of oxygen flux through the mitochondrial respiratory chain contributes to an increased production of free radicals. This is due to 2-10% of the oxygen consumed not being completely reduced (50). An imbalance between a body's antioxidant defenses and the generation of free radicals leads to a state of oxidative stress. The intensity and duration of the exercise dictates the extent of free radical production and the magnitude of any ensuing oxidative stress. Although cells can withstand a certain amount of oxidative stress, severe levels can compromise membrane integrity, induce apoptosis, cause enzyme dysfunction, and damage DNA (50).

Free radicals are atoms or molecules containing one or more unpaired electrons in their valance shell. This characteristic makes them highly reactive with surrounding tissues which is the basis for their destructive effects on lipids, proteins, and nucleic acids. Several free radicals and ROS have been identified. Superoxide radical ($O_2^{\cdot -}$) is formed when an electron is added to an oxygen molecule. Hydrogen peroxide (H_2O_2), a waste product of metabolism, is not a free radical but can be involved in free radical producing reactions. Hydroxyl radical ($OH^{\cdot}$) formation occurs when H_2O_2 reacts with $O_2^{\cdot -}$ in the presence of free metal ions, such as Fe^{3+} or Cu^{2+}, or when water reacts with $O_2^{\cdot -}$. Singlet oxygen ($^1O_2$) is formed when energy from nearby chemical reactions or radiation exposure causes one of its valence electrons to move into a higher energy orbit (50). These metabolic by-products have several sources and collectively have been implicated in the altered immune function following strenuous exercise.

Potential Sources of ROS

Research indicates that 90-98% of the oxygen consumed is reduced during mitochondrial oxidation (50). The remaining oxygen is incompletely reduced leading to the formation of superoxide radical. Increased oxygen consumption by exercising muscle will further enhance superoxide generation. Although sometimes considered the main source of free radicals, the mitochondria may play a smaller role than once believed (13). The following sources of free radicals and ROS have the potential to be as important or more in exercise induced oxidative stress and its association with immune functions.

The enzymatic activity of xanthine oxidase is an important contributor to superoxide generation. Xanthine oxidase converts hypoxanthine to xanthine and

subsequently xanthine to uric acid. Xanthine oxidase usually exists in its dehydrogenase form. However, disturbances in intracellular calcium homeostasis, heat stress, and thiol group oxidation (ie GSH→GSSG), all of which are normal responses to aerobic exercise, result in the conversion to the oxidase form which produces superoxide (59). Hypoxanthine is the main substrate for xanthine oxidase and along with uric acid has been shown to increase with exercise (97). Increased ATP hydrolysis during exercise leads to the accumulation of AMP, which is degraded to IMP and hypoxanthine. Hypoxanthine is then available as the main substrate for xanthine oxidase. Disturbances of intracellular calcium, during aerobic exercise, further enhance superoxide production by causing the conversion of xanthine dehydrogenase to the oxidase form. Both the dehydrogenase and oxidase forms catalyze the degradation of hypoxanthine to xanthine but only xanthine oxidase produces superoxide in the final step when converting xanthine to uric acid (18).

Metal catalyzed reactions also contribute to ROS formation. Conversion of hydrogen peroxide to hydroxyl radical occurs via the Fenton reaction in the presence of iron (64). How much hydroxyl radical is produced appears to depend on the availability of free iron (35). Following exercise induced muscle damage there is an increased release of free iron from hemoglobin in the blood and myoglobin in the muscle.

Recently, hemoglobin and myoglobin have been further implicated in the generation of ROS as well as enhancing the reactivity of ROS generated via other mechanisms (81). Superoxide is formed from the auto-oxidation of both oxyhemoglobin and oxymyoglobin: $Fe^{2+} + O_2 = Fe^{3+} + O_2^-$. The superoxide produced may lead to subsequent peroxide formation: $O_2^- + O_2^- + 2H^+ = H_2O_2 + O_2$ (10). These peroxides can

react with ferric heme proteins to produce both ferryl iron and a protein bound free radical leading to lipid peroxidation (81). Evidence exists that catecholamines can also auto-oxidize resulting in free radical production thereby providing an additional source of free radicals during exercise (see Figure 3) (36).

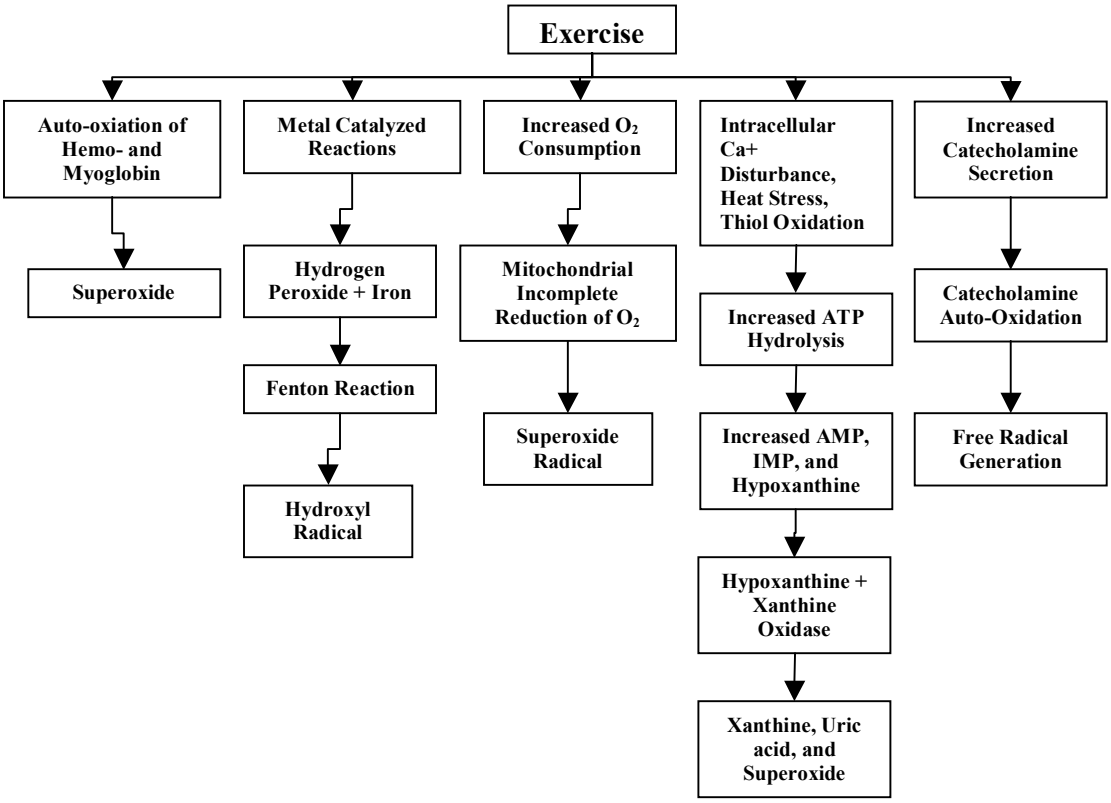
Many studies have observed increased free radical production with contracting muscle (20, 68). However, because skeletal muscle cells contain other potential sources of ROS generation, such as endothelial cells and lymphocytes, the contribution by the muscle cell needed to be further described. McArdle et al., (53) used contracting cultured skeletal muscle myotubes to measure ROS generation. Myotubes released very little or non-detectable amounts of superoxide at rest. When stimulated with low frequency (1 Hz) pulses over 15 minutes superoxide release increased ~40 times over basal levels. This increase in superoxide generation remained significantly higher for 75 minutes following electrical stimulation. If myotubes represent fully differentiated skeletal muscle cells, these results indicate that exercising muscle cells alone have the capacity to be major contributors to the generation of ROS.

Exercise, GSH alterations and Lipid Hydroperoxides

The multiple sources of ROS generated during strenuous exercise affects intracellular GSH concentration. As described earlier GSH is oxidized as the electron donor in the GPx reaction reducing hydrogen peroxide to water. During aerobic exercise the GSH/GSSG ratio drops (31, 92). Early work by Gohil et al., (31) demonstrated GSH oxidation during submaximal exercise. Eight healthy endurance trained males performed two separate cycle ergometer exercise tests, one a continuous graded exercise test to

exhaustion and another being a submaximal 90-minute ride (65% VO_{2peak}). During exhaustive exercise blood GSH did not change significantly. During the submaximal bout blood GSH decreased from ~0.4 mM to 0.15 mM in the first 15 minutes and continued to decrease over the remaining 75 minutes to ~0.10 mM. GSSG rose by 100% to 0.4 mM over the course of submaximal exercise. The differences between the two tests in GSH oxidation may be related to the total amount of oxygen consumed rather than the peak

Figure 3: Potential Sources of ROS



level of oxygen consumed. However, the oxygen consumed was less during the first 15 minutes of submaximal exercise than during the graded test. The authors suggested that this may be explained by differences in blood acidosis during the two trials. Results of the graded test demonstrated a 625% increase in blood lactate compared to a 100% increase during the submaximal test. Other research has indicated that lactic acid

can act as a hydrogen donor, which could have buffered ROS generation (23). Research by Viguie et al., (91) confirmed GSH oxidation during submaximal exercise. Using identical exercise mode, duration, and intensity as the Gohill study, they observed decreases in whole blood GSH from 0.55 mM to 0.27 mM during the first 15 minutes of cycling. No further changes were observed during the remaining 75 minutes. Together, these studies illustrate blood GSH oxidation during submaximal workloads while GSH oxidation during shorter tests, performed to exhaustion, may be attenuated by the increased lactate production buffering ROS production. However, recent animal study has shown greater reductions of blood compartment GSH with high intensity (fast treadmill running) compared to low intensity (treadmill walking) exercise (22). These data suggest that high intensity exercise induced ROS generation is associated with a subsequent increased demand for GSH mediated antioxidant protection. In the blood, GSH plays an integral antioxidant role during sub-maximal and strenuous exercise and GSH oxidation is closely related to exercise intensity.

Alterations of GSSG and total GSH (tGSH) in the blood compartment are affected by intensity, duration, and volume of oxygen consumed during exercise. Sen et al., (79) examined three different exercising intensities on blood GSH fluctuations in untrained males. On separate occasions subjects completed either incremental cycle ergometry until exhaustion (mean duration 14.06 min) or two different 30-minute cycle bouts representing 50% and 77% VO_{2peak}. Exhaustive and 50% submaximal exercise resulted in no change in blood tGSH or GSH, but did produce significant increases in GSSG. The 77% submaximal exercise trial caused a 157% and 56% increase in total oxygen consumption compared to the exhaustive and 50% trials, respectively. This increase in

VO_2 was associated with significant increases in tGSH and GSSG. GSH concentration was unchanged (79). The lack of increase in tGSH following the exhaustive and 50% trials indicates the absence of GSH or GSSG outflow from tissues, such as skeletal muscle or liver. The increase in tGSH, but not GSH, following the 77% trial indicates efflux of GSSG from other tissues. The source of GSSG is likely exercising skeletal muscle (37). Sen et al., (78) have reported rapid loss and subsequent efflux of muscle cell tGSH. Work with hepatectomized rats further supports the contribution of muscle cells to increases in blood tGSH (31). During strenuous exercise, when oxygen consumption is large enough, export of GSSG from working muscle cells can contribute to increases in circulating tGSH (77). This appears to be dependant on the amount of oxygen consumed during the exercise bout.

Cellular membranes, rich in polyunsaturated fatty acids, are primary targets of free radicals and ROS generated during oxidative insult. Exercise stress does result in an increase of LPO generation. Bailey et al., (3) examined the effects of incremental cycling to exhaustion in healthy college age males (n=32). Compared to the resting pre-exercise condition, exhaustive cycling caused a 31% increase in serum LPO. As the many pathways of ROS generation increase their activity, with increases in physical work, the antioxidant defenses of vitamin E and GSH subsequently become burdened. Lipid free radicals are then produced which readily react with oxygen to form peroxyl radicals. These peroxyl radicals are then able to inflict further damage on cellular membranes and continue a cycle of lipid hydroperoxide generation (77, 78, 79). The importance of uncompromised blood GSH status is illustrated by research reporting that higher resting blood GSH is highly correlated with lower resting and exercise induced LPO (32).

ROS generation and fatigue

The generation of ROS may play a causal role in the onset of muscular fatigue (5, 87). Antioxidant supplementation or the indirect boosting of GSH may therefore, theoretically, enhance aerobic exercise performance. In vitro research using highly oxidative mouse and canine skeletal muscle indicates that ROS administration accelerates fatigue. Barclay et al., (5) subjected muscle preparations to 70 Hz stimulated contractions once every minute for 60-minutes. The addition of xanthine oxidase, a major contributor to ROS production during physical work, doubled the rate of fatigue over control muscle preparations treated with saline. Both treatment solutions were perfused at a rate equal to the animal's blood pressure. A second experiment was also performed comparing the effects of allopurinol, a xanthine oxidase blocker, with saline treated samples (5). Muscle was isometrically contracted at 4 Hz for 30-minutes with saline. This was followed by a 45-minute saline perfused recovery and subsequent 30-minute 4 Hz contraction treated with either saline or allopurinal. With saline the rate of fatigue increased over the 30-minutes of contractions by 26% from the first to the second stimulation. With allopurinal administration fatigue rate decreased by 37% from the first to second stimulation (5). These results demonstrate ROS induced impairment of muscle function and their contribution to fatigue in oxidative skeletal muscle.

Antioxidants, GSH manipulation and fatigue

If ROS plays a role in the onset of fatigue then antioxidant reduction of these ROS and free radicals could prolong the ability to do physical work. Studies examining

in vivo models further elucidate the relationship between ROS and muscle fatigue and indicate antioxidant administration may increase muscular performance. Supinski et al., (87) assessed the effect of free radical scavengers on the fatigue rate of electrically stimulated contracting canine diaphragm muscle. Animals were administered saline, denatured superoxide dismutase (SOD), or undenatured SOD before a 2-hour period of electrophrenic stimulation. Muscle samples were taken at the conclusion of each trial for measurement of thiobarbituric acid reactive substances (TBARS), a marker of ROS mediated lipid peroxidation. The rate of diaphragm fatigue was greater in the dogs given saline or denatured SOD than those given undenatured SOD with force falling to 23%, 21%, and 50% of initial values, respectively. TBAR concentration was also significantly higher in saline and denatured SOD treated dogs than the SOD group (87). This study suggests that lipid peroxidation associated with muscle contraction can be attenuated with free radical scavenger administration and that this results in greater muscular performance.

Human studies involving antioxidant supplementation and muscular performance have mostly examined dietary vitamin E and have found no effect (35, 66, 69, 82, 86). Animal studies contradict the human data usually finding improvement (5, 52, 65, 80). However, one antioxidant, N-acetylcysteine (NAC), a known GSH booster, has improved performance in both animal and human models (see Table 1) (66, 80). Indeed, direct GSH supplementation is ineffective in raising GSH because of its lipophobic properties and inability to cross cellular membranes (89). Exogenous injection of GSH in rats (1g GSH/kg body weight) resulted in an immediate dramatic increase in plasma tGSH with excess GSH being rapidly oxidized to GSSG (75). This single dose injection did not

Table 1: Effects of antioxidants on human and animal skeletal muscle performance

Study	Subjects	Treatment	Test	Performance
Lawrence, 1975	Human	Vitamin E	500 m swimming	No effect
Sumida, 1989	Human	Vitamin E	VO_{2peak}	No effect
Rokitzki, 1994	Human	Vitamin E	Incremental exercise	No effect
Snider, 1992	Human	Vitamin E, Coenzyme Q	Time to exhaustion @ 70% VO_{2peak}	No effect
Akova, 2001	Human, menstruating females	Vitamin E	Time to exhaustion of isokinetically exercised knee	No effect
Reid, 1994	Human	NAC	Low-freq. muscle stimulation	Improved
Novelli, 1990	Animal	Vitamin E	Swimming, in vivo	Improved
Barclay, 1991	Animal	Allopurinol	Soleus, in vitro	Improved
Reid, 1992	Animal	SOD, Catalase	Diaphragm, in vitro	Improved
Piercy, 2001	Animal	Vitamin E	Sled dogs performance	Improved
Shindoh, 1990	Animal	NAC	Diaphragm, in situ	Improved
Supinski, 1997	Animal	NAC	Diapragm, in vivo	Improved

result in any changes of GSH in skeletal muscle, lung, kidney, or heart tissues. Furthermore, there was no significant difference in exercise induced lipid peroxidation for all tissues between GSH treated rats and placebo rats. Time to exhaustion for treadmill running was also the same between GSH and saline treated rats. Oral GSH administration, in a dose of 0.15 $mmol.kg^{-1}$ in humans, has also failed to increase plasma GSH (94). Therefore, it appears exogenous GSH is ineffective in raising tissue GSH levels and has no effect on exercise induced oxidant stress and muscular performance.

Buthionine sulfoximine (BSO) is known to block the synthesis of intracellular GSH. Rats administered BSO resulted in an 80-90% decrease in the tGSH of skeletal and heart muscle and a 50% reduction in plasma, liver, and lung tGSH. BSO treated rats displayed a 50% reduction in exhaustive treadmill running time when compared to controls (75). Other studies using GSH synthesis blockers have observed similar results. Kramer et al., (29) used 1 ml/kg diethyl maleate to block GSH synthesis and observed a subsequent decrease in swim performance. These results show that, although exogenous GSH administration is ineffective in increasing muscular performance, decreased intracellular GSH results in the early onset of fatigue.

As described earlier exogenous GSH is not effective in increasing intracellular GSH. Synthesis of GSH within the cell depends on the availability of cysteine which, in its reduced form, is highly unstable. Thus, virtually all of the cysteine in circulation is in the more stable and non-toxic form of cystine, which is two cysteines linked together with a sulfide bond (23, 91). One agent capable of increasing intracellular GSH via increasing the availability of cysteine is NAC. Rats subjected to exhaustive treadmill exercise 30-minutes after a 1g/kg body weight dose of NAC had significantly less blood GSH oxidation than control rats or rats given GSH. NAC also provided protection against exercise induced GSH oxidation in the lung but failed to improve endurance time during an exhaustive treadmill run (75). Other studies feeding NAC to rats (1g/kg/day) showed similar decreases in blood GSH oxidation following exhaustive exercise (71). Shindoh et al., (80) observed an increase in muscular performance with NAC supplementation in rabbit diaphragm muscle. Rabbits were treated with either intravenous NAC or saline. In situ diaphragm muscle was then rhythmically stimulated to contract at a rate of 30/min

for 20 min. The rate of development of fatigue was significantly greater in saline treated animals. Over the 20 minute protocol reductions in tension were 55% and 34% in saline and NAC treated animals, respectively (80). Similar research using dogs has also shown decreases in diaphragmatic fatigue with NAC (87). Providing biologically available cysteine increases intracellular GSH, reduces oxidative stress, and is associated with increased muscular performance in animals.

Human research on NAC supplementation and fatigue, although limited, has shown positive results. Reid et al., (65) administered a 150 mg/kg intravenous dose of NAC to 10 healthy males (32 y, 75 kg). The tibialus anterior of each subject was then subjected to repetitive tetanic 10 Hz stimulations over 30 minutes. Use of this low frequency stimulus is claimed to closely resemble the biochemical changes that normally occur during volitional exercise. During the fatiguing contractions NAC increased force output by ~15% after the first 3 minutes and force remained elevated throughout the 30-minute protocol (66). Results of this study suggest that muscular performance is increased by indirectly boosting GSH concentrations.

The intended use of NAC is in treating acetominophen hepatic toxicity, which is achieved by providing cysteine to increase hepatic GSH concentration (66). Due to many side effects associated with NAC supplementation NAC is not a realistic option for increasing intracellular GSH for ergogenic aid purposes (66). Within 30 minutes, intravenous administration of 150 mg/kg in humans caused several unpleasant to debilitating side effects. These included: dysphoria, sleepiness, lightheadedness, nausea, and, in some subjects, loss of motor control severe enough to make walking impossible (66). Therefore, the potential increased muscular performance associated with increased

GSH cannot, in a practical manner, be achieved through direct GSH or NAC supplementation. Efforts have recently been made to find tolerable doses of NAC in humans. Medved et al., (43) found that intravenous infusion of NAC at a rate of 125 $mg.kg^{-1}.h^{-1}$ induced no serious adverse reactions but did not effect time to fatigue in exercising healthy males. Therefore, NAC remains impractical and ineffective at tolerable doses in humans. Unlike NAC, the serum albumin portion of whey protein may provide the intact cysteine needed to augment GSH levels.

Exercise and immune function

The effect of physical work on immune function has been studied sporadically over the past one-hundred years. Larrabee first reported changes in white blood cells that appeared similar to diseased conditions in four athletes completing the 1901 Boston Marathon (34). Three decades later Baetjer noticed that exercise decreased immune function and was a "predisposing factor to infectious disease" (2). Recently the field of exercise immunology has seen a resurgence of attention due to epidemiological studies linking physical exertion with increased risk of upper respiratory tract infections (URTI) (48). It is now realized that immune function can be manipulated through physical work. Generally speaking, moderate exercise appears immuno-stimulatory while exhaustive work may be followed by varying lengths of immuno-supression (60).

The literature defines aerobic exercise intensity in excess of 70% VO_{2peak} as intensive while intensities between 50-70% are usually labeled as moderate (48). Both moderate and intense exercise are associated with changes in leucocyte, granulocyte, and lymphocyte counts (46). A competitive sprint triathlon (400 m swim, 25 km cycling, 4

km run, mean duration = 68 min) in trained males resulted in leucocytosis one hour post event. This was predominantly due to a 4-fold and 2-fold increase in granulocytes (neutrophils, eosinophils, basophils) and monocytes, respectively. Lymphocyte count decreased to less than half of pre-exercise values by one hour post (92). Mooren et al., (46) observed significant increases in total leucocytes, granulocytes, and lymphocytes following exhaustive exercise performed at 80% VO_{2peak}. Similar but less prominent changes were observed in a 60% VO_{2peak} trial except for lymphocytes which increased without reaching significance. Lymphocytes fell below baseline by one hour post in the 80% trial. Specific subpopulations of lymphocytes were not measured in these experiments but results do indicate that both intense and moderate aerobic exercise performed to exhaustion, in both lab and real world settings, result in marked immune changes, within one hour of exercise, as measured by alterations in immune cell counts.

Different lymphocyte subpopulations are affected following an intense exercise challenge. Vider et al., (90) attempted to expand on the relationship between exhaustive exercise, oxidative stress, and the cellular immune response. Nineteen male endurance trained athletes (22.21 ± 5.5 years, VO_{2peak} 69.79 ± 8.2 ml/kg/min) performed incremental treadmill exercise until volitional exhaustion. Blood samples were collected prior, immediate post, and 30 min post exercise for analysis of glutathione peroxidase (GPx), TBARS, and tGSH. Lymphocyte subpopulations (CD3+, CD4+, CD8+, and CD69+, a T-cell activation marker) were determined by flow cytometry. Mononuclear cell proliferation was measured by incubating cells with the T-cell mitogens, phytohemagglutinin (PHA) and concanavalin A (Con-A). The exhaustive exercise increased total T-cells (CD3+), decreased the CD4+/CD8+ ratio and decreased

lymphocyte mitogenic response to both T-cell mitogens. Total CD3+ cells fell below baseline by 30 minutes post exercise. Exercise induced a decreased expression of CD69 on both CD4+ and CD8+ cells immediate post. Also, exhaustive exercise increased lipid peroxidation immediately following exercise. Interestingly, there was a strong positive correlation between total GSH and lymphocyte mitogenic response (90). Mooren et al., (47) observed a 74% increase in CD3+ T-cells following treadmill running at 80% maximal oxygen uptake until exhaustion (mean running time = 30.5 ± 2.5 min). By one hour post exercise the CD3+ T-cell count had dropped to 24% below baseline. The CD4+/CD8+ ratio fell significantly immediate post but recovered to baseline values by one hour post (47). Other studies indicate that the CD4+/CD8+ ratio decreases following one hour of moderate intensity aerobic work (46). While most research indicates leucocytosis immediately following both moderate and intense exercise specific lymphocyte subsets appear to react differently depending on duration and intensity. A transient lymphocyte depression is usually observed after one hour of recovery. Whether this is due to decreases in CD4+ or CD8+ as depicted by changes in the CD4+/CD8+ ratio also appears dependant on exercise duration and intensity. These alterations of immune function, following both exhaustive and moderate intensity exercise, indicate the plasticity of lymphocytes to exercise stress. CD4+ T-cells, also known as T-helper cells, are crucial in activating B-cells and thereby initiating a healthy immune response. The decrease in CD4+ T-cells following exercise may help explain the increased vulnerability to viral and bacterial pathogens following strenuous aerobic exercise. By increasing antioxidant defenses and decreasing oxidative stress subsequent alterations in T-helper

cells may be attenuated. Theoretically this could diminish the chances of opportunistic infectious agents gaining a foothold.

Exercise, Phagocytosis, and Oxidative Burst

The process of phagocytosis is carried out by neutrophils in the blood and macrophages in the tissues. Together they represent our body's first line of non-specific defense against invading organisms. The phagocyctic process is carried in ordered stages consisting of adherence, chemotaxis, attachment, ingestion, and killing (56). The attachment through ingestion stages are generally termed phagocytosis while the actual killing is accomplished by subjecting the ingested organism to oxygen radicals and microbicides during oxidative burst activity. The stages of the phagocytic process, adherence through killing, appear to be affected differently by exercise. As well, the types of phagocytic cells (neutrophils, macrophages) react differently to exercise stress.

Several studies have investigated the influence of exercise on both phagocytosis and oxidative burst capacity (see Table 2 and 3) (14, 18, 19, 25, 38, 54, 55, 68, 81). Animal studies indicate that intensity of exercise plays a role in the phagocytic capacity in macrophages. Su et al., (85) subjected mice to either an exhaustive treadmill run or moderate treadmill running for 30 minutes. Macrophage phagocytic capacity increased ~100% immediately after exhaustive exercise and was maintained through one hour of recovery. Moderate exercise did not affect macrophage phagocytic capacity (85). Lewicki et al., (38) examined the effect of maximal cycle ergometry on the phagocytic process in both trained and untrained male cyclists. Baseline values for absolute and percent number of neutrophils and monocytes, and neutrophil oxidative burst capacity

were similar in both groups. Neutrophil adherence was lower in the trained cyclists. Exhaustive cycling induced significant increases in absolute numbers of neutrophils and monocytes in both populations. Neutrophil and monocyte adherence and neutrophil oxidative burst activity significantly decreased in the trained cyclists following exercise while neutrophil phagocytic activity remained unchanged. In untrained subjects maximal exercise did not result in changes in neutrophil and monocyte adherence or oxidative burst activity in neutrophoils, while neutrophil phagocytic activity increased (29). Research indicates that training state of the individual and intensity and duration of exercise influence the phagocytic process. Exercise appears to have potent stimulatory effects on phagocytosis and oxidative burst capacity in both neutrophils and macrophages. However, some macrophage functions can be diminished following exercise. For example, Woods et al., (96) observed reductions in macrophage MHC II expression and subsequent antigen presenting capacity following exercise. It is possible that exercise may enhance many non-specific effector functions while downregulating the ability of macrophages to induce specific immune reactions. These alterations, both stimulatory and inhibitory, seem to occur most prominently following exhaustive exercise with changes observed through one hour of recovery.

Energy restriction, antioxidant status, and immunity

Animal studies have shown increased GPx gene transcription, as well as other proteins involved in free radical scavenging, with energy restriction (83). Reduced food intake will also decrease the amount of ROS produced via normal nutrient metabolism (44). Indeed, caloric restriction has been associated with decreased oxidative stress in

mammals (18). Therefore, energy restriction may lead to increased antioxidant protection for two reasons: 1) an increased expression of antioxidant enzymes and 2) decreased oxidative metabolism of fuels which could decrease ROS formation. Sreekumar et al., (83) reported significant decreases in expression of several antioxidant enzyme genes, including GPx, following 36 w of energy restriction (60% of control) in rat.

Table2: Effect of exercise on phagocytosis (ingestion).

Author	Cell type	Exercise	Subjects	Results
Lewicki, 1987	Neutrophils	Maximal incremental cycle of approximately 16 minutes	Sedentary humans _____ Trained Cyclists	27% increase _____ 11% decrease
Rodriguez, 1991	Neutrophils	Exhaustive t-mill running (6-19 min)	Sedentary humans	4.5% and 9.5% increase in M and F, respectively
Ortega, 1993	Neutrophils	Moderate cycle ergometry (60% VO_2, 30 minutes)	Sedentary humans	~46% increase
Fehr, 1989	Macrophages	Exhaustive t-mill running (10-17 min)	Handball and soccer players	~12% increase
Fehr, 1988	Macrophages	Exhaustive running	Trained and sedentary mice	Both groups had twice the phagocytic activity as non-exercised controls
De al Fuente, 1993	Macrophages	Exhaustive swimming (avg time = 182 minutes)	Trained and sedentary mice	25% increase
Ortega, 1992	Macrophages	Exhaustive swimming	Old and young sed. guinea pigs	~37% increase

Table 3: Effect of exercise on oxidative burst capacity.

Author	Cell type	Exercise	Subjects	Results
Fehr, 1988	Macrophages	Exhaustive running	Trained and sedentary mice	~33% increase in both groups
Fehr, 1989	Macrophages	Exhaustive t-mill running (10-17 min)	Handball and soccer players	~14% increase
Lewicki, 1987	Neutrophils	Exhaustive incremental cycling (~16 min)	Trained and sedentary humans	5% decrease in trained, no change untrained
Ortega, 1993	Neutrophils	Moderate cycling	Sedentary humans	No change
Rodriguez, 1991	Neutrophils	Exhaustive t-mill running (6-19 min)	Sedentary humans	15% and 26% increase in M and F respectively
Smith, 1990	Neutrophils	Moderate cycling (1h at 60% VO_{2max})	Elite cyclists and sedentary humans	2 fold increase maintained through 6 h post

Severe energy restriction is associated with decreased immunocompetence and resistance to infection (8, 63). Short term fasting has also been linked to decreased lymphocyte proliferation with mitogen stimulation (19, 68). Decreased erythrocyte GSH synthesis, coinciding with maintained tGSH levels, have been reported following VLCD in humans, even when the diet supplied adequate amino acid GSH precursors (13). Animal research has suggested that, during times of energy restriction, erythrocyte GSH is used as a cysteine reservoir which could allow relocation of GSH to the lymphocyte compartment or other tissues (9). Total energy consumed, irregardless of amino acid composition, may play a significant role in GSH maintenance during weight loss.

Reducing caloric intake in order to lose body weight is a common practice among both athletes and non-athletes. While decreasing fat mass may increase sports

performance the process of enduring caloric restriction may compromise immune function. Severe energy restriction as seen with anorexia nervosa or long term fasting is associated with decreased immunocompetence and enhanced susceptibility to infection (12, 84). Short term fasting has also been linked to decreased lymphocyte proliferation following mitogen stimulation (24, 93). An athlete attempting to lose weight with caloric restriction can expect a certain level of immunosuppresion depending on the magnitude of caloric deficit endured and the duration of the diet plan.

Much of our knowledge concerning energy restriction and immune alterations originated from studying obese subjects. Very low calorie diets (VLCD), consisting of 400-800 kcal/day, produce dramatic weight loss and are associated with various indicators of immunosupression. McMurray et al., (42) examined the effects of a 3 month VLCD on immune function in 13 obese subjects (avg wt loss = 14 kg). Compared to baseline there was a 30-40% decrease in mitogen stimulated lymphocyte proliferation. Neutrophil phagocytosis was not altered, but a significant decrease in oxidative burst capacity was observed (42). Research by Field et al., (20) also observed decreased mitogen stimulated lymphocyte proliferation following six weeks of a VLCD (avg wt loss = 13 kg). Proportions of specific lymphocyte subpopulations did not change over the course of the diet. However, there was a trend for a decrease in CD4+ T-cells. Contradicting the above mentioned findings, Tanaka et al., (88) found an increase in mitogen stimulated lymphocyte proliferation following a VLCD (avg wt loss = 22.5 kg). These findings indicate that while VLCDs are effective in decreasing body weight the associated immune changes are not consistent in the obese population. The immunocompetence of obese individuals does not reflect that found in non-obese.

Research has found decreased mitogen stimulated lymphocyte proliferation in obese subjects when compared to normal weight subjects (88). Recent research on hyperlipidemic males and females (N=7, avg age = 63 years, BMI = 27.7 females, 25.1 males) reported no effect on lymphocyte proliferation after losing 3.6 kg over 12 weeks (70). Therefore, more thorough examination is needed to elucidate the immune alterations associated with weight loss in normal weight and athletic populations. For athletes in training VLCDs may not provide enough energy to maintain an adequate training regime and may compromise immunity to the extent of allowing establishment of infectious agents. A more modest caloric restriction, while slowing the rate of weight loss, may be more favorable from an immunological and training perspective. Neiman et al., (49) studied the effects of three months of a moderately low calorie diet (MLCD) (1200-1300 kcal/day) on immune function in obese females. After completion of the dietary intervention and having lost an average of 9.9 kg, total leukocyte, neutrophil, and monocyte counts decreased significantly. A decrease in lymphocyte proliferation to ConA and PHA stimulation was also observed. Phagocytosis was not affected by the MLCD but monocyte oxidative burst capacity decreased 24% (49). Despite the slower rate of weight loss and increased caloric intake compared to a VLCD an MLCD is still associated with compromised immune function. To an athlete in training MLCD would represent severe caloric restriction as their physical activity far exceeds the normal population. Furthermore athletes may wish to opt for rapid weight loss in order to get back to intense training as soon as possible. At present there is little information regarding the acute changes in immunity with rapid weight loss that athletes may undergo.

Immune alterations following caloric restriction in athletes is far less investigated. Kono et al., (28) examined the effect of a MLCD in nine female athletes on monocyte phagocytic function and mitogen induced lymphocyte proliferation. The athletes (18-24 years) were four gymnasts, two swimmers, two volleyball players, and one tennis player who all trained at least 2 hours a day 5 days a week. Ten healthy sedentary age matched females consuming their usual diet served as controls. Athletes underwent a 1300 kcal/day diet for 2 weeks. Following diet intervention monocyte phagocytic activity was significantly decreased (~36%). Lymphocyte proliferation, while not significant, did decrease ~18%. Of interest is that athletes, at baseline, possessed over 3.5 times the lymphocyte proliferation capacity than sedentary controls (28). These results indicate that athletes may exhibit enhanced immune function when compared to non-athletes. This level of immune function is still subject to immunosuppression following a MLCD followed for as little as two weeks.

As mentioned, GSH is essential for healthy immune function. Animal studies have observed decreased GSH with food deprivation. Leeuwengurgh et al., (36) subjected rats to 48 hours of starvation and observed significantly lower liver GSH and GSH/GSSG ratio. These effects were reversed with refeeding. Muscle GSH was also significantly lower in food deprived rats compared to refed rats (36). Although not studied, decreased GSH may help explain the immunosuppression observed with caloric restriction and weight loss. This may be counteracted by providing a protein source rich in cysteine such as that found in whey protein.

Whey protein and immunity

Whey protein supplementation increases both humoral and cell mediated immunity (95). As discussed previously, whey protein represents a significant source of intact glutamylcysteine, the necessary precursor for GSH synthesis. Indeed, when compared to casein or soy proteins the lactalbumin portion of whey contains more than five times the amount of available cysteine (58). GSH stabilizes lysosomal and other immune cellular membranes and protects against oxidation. Therefore, GSH is crucial for the functional state of immune cells including both T and B lymphocytes (95).

Animal studies comparing different protein sources on immune function indicate that the proteins found in whey have the greatest positive impact on certain immune parameters. Many early studies found humoral and cell mediated immune responses five times higher in whey supplemented mice when compared to casein, soy, or wheat proteins (11). Later studies confirmed these findings and detailed the superior immunoenhancing properties of whey over other protein sources such as corn, egg albumin, beef, and fish protein (6). Very recent research by Minehira et al., (45) compromised immune function in rats with administration of oxidized cholesterol. Compared to casein or soy protein the whey protein isolate suppressed the disturbance in immune function (45). The amino acid composition, unique to whey, appears to be associated with greater immunocompetence.

Parker et al., (58) compared whey, casein, and soy proteins on the immune function of mice. Standard purified diets were supplemented with 20% of each respective protein in a powder form. Normal growth was sustained on all diets. Whey supplemented

mice exhibited significantly higher plaque forming cells to a T-dependent antigen (sheep red blood cells). This indicates increased T-helper function possibly reflected in the increased thymic weight and thymic cellularity observed in the whey supplemented group. Macrophage function as a measure of complement secretion was also enhanced with whey protein (58). This study indicates potentially meaningful immunoenhancing properties of whey protein. Although GSH was not measured, these properties may be related to enriched GSH levels within immunocytes due to the delivery of intact cysteine. Other research confirms and further describes the association of whey supplementation and immuno-enhancement. Wong et al., (95) observed higher leukocyte counts in mice fed whey protein concentrate compared to a soybean protein isolate after five and remaining through 8 weeks of dietary intervention. The secondary anti-ovalbumin response was also significantly higher in the whey protein concentrate fed mice. As well, by 8 weeks the T-cell response to ConA was higher in whey protein fed mice (95). These studies demonstrate whey proteins superior immuno-enhancing effects. Whether this is due to the unique amino acid profile of whey or to specific portions of whey remains to be clarified.

Human studies on the immuno-modulatinig effects of whey protein supplementation often use HIV infected patients, a population with compromised GSH status and immune function. Whey protein, prepared as to not denature the thermosensitive glutamylcysteine rich serum albumin portion, was given as a dietary supplement to three HIV positive males for three months. Dosage was increased form 8.4 to 39.2 g/day. By week six the CD4+ percentage, the absolute number of T helper cells, and the CD4+/CD8+ ratio had increased 16.5%, 31.6%, and 20.2%, respectively. At

baseline, GSH content of blood mononuclear cells for all three patients was below normal values. Over the 3 month intervention mononuclear cell GSH concentration rose for all subjects with one case showing a 70% increase reaching normal values (8). Although definitive evidence cannot be gathered from such a small subject number this study does demonstrate the potential for increased GSH levels in immune cells as well as enhanced immune function from whey protein supplementation. Micke et al., (44) found a 25-44% increase in plasma tGSH in HIV positive patients after only 2 weeks of 45 g/day whey protein supplementation. This increase was maintained over a 6 month period. By 3 months of whey protein supplementation absolute CD4+ cell count increased from 243 ± 102 /µl to 286 ± 131 /µl, a 17.7% increase (32). Unlike NAC, whey protein causes few and only mild side effects. Only occasional slight gastrointestinal disturbance was reported by 4 of 18 subjects who completed the entire 6 month trial. These studies indicate increased plasma and immune cell GSH results with whey protein supplementation. The whey protein should be prepared as to not denature the available glutamylcysteine groups present in the serum albumin portion.

Immunocal®, a glutamylcysteine rich whey protein concentrate

Immunocal® is a bovine whey protein concentrate prepared using a proprietary microfiltration and low-temperature pasteurization technique that allows the retention of intact γ-glutamylcysteine groups. Glutamylcysteine has been shown to be efficiently transported into cells providing the main substrate for GSH synthesis (9, 10). Lands et al., (33) examined the effect of Immunocal® supplementation on muscular performance and lymphocyte GSH in 18 healthy young adults (9 males, 9 females). Subjects consumed 20

g/day of either Immunocal® or casein placebo for 3 months. Casein is known to have ~0.3% cystine compared to 2.5% in the whey protein of Immunocal® (7). Muscular performance was assessed during a 30-second isokinetic cycle sprint. Immunocal® subjects experienced a significant 13.3, 12.7, and 35.5% increase in peak power, 30-second work, and lymphocyte GSH, respectively. Side effects were mild consisting of occasional bloating and queasiness, but were well tolerated overall. The mechanisms behind improved muscular performance with Immunocal® supplementation are not clear, but could be attributed to the increase in intracellular GSH and its attenuation of oxidant induced muscular dysfunction. Although this study demonstrates increased muscular performance with whey protein supplementation the lack of GSSG measurement during exercise leaves one only to speculate on the role increased GSH played. To better demonstrate Immunocal®'s effectiveness in increasing antioxidant capacity and increasing muscular performance an exercise test of longer duration is warranted. An exercise test of this nature has been shown to induce xanthine oxidase activity and other sources of free radical production.

Whey protein in the form of Immunocal® raises GSH concentrations in both whole blood and the white blood cell compartment. The dose and duration of Immunocal® feeding to elicit meaningful changes in humans has yet to be clearly defined. The delivery of intact cysteine, which is most often destroyed during the normal pasteurization of whey, is a key step in the production of intracellular GSH. Through a proprietary pasteurization process involving micro-filtration and low heat, according to the company producing Immunocal®, this process retains the vital glutamylcysteine groups necessary for de novo synthesis of GSH.

Moderate and intense aerobic exercise, for varied lengths of time, result in GSH oxidation dependent on the amount of oxygen consumed. Exercise induced production of ROS has been clearly observed and is the primary cause of GSH oxidation and subsequent alterations in immunity. Similar to exercise, periods of energy restriction, whether moderate or severe, have a profound impact on immuno-comptetence. ROS have also been implicated with the early onset of fatigue and methods of raising GSH levels are associated with increased muscular function. Unfortunately, these methods include side effects rendering them ineffective as an ergogenic aid. Due to the lipophobic nature of GSH, exogenous supplementation is not effective. Immunocal®, which is well tolerated, may represent a viable nutritional supplement capable of increasing muscular performance and attenuating post exercise and post energy restriction immune alterations. The efficacy of Immunocal® is yet to be thoroughly evaluated using an aerobic exercise test known to elicit oxidative stress severe enough to result in immune alterations. Furthermore, Immunocal® has never been examined during periods of energy restriction. The purpose of this study is to examine the effects of Immunocal® supplementation in trained athletes on exercise performance and immunity following periods of both weight maintenance and caloric restriction. Because of the glutamylcysteine groups that Immunocal® provides, we hypothesize that supplementation will result in increased aerobic exercise performance and an attenuation of immmune alterations following exercise and energy restriction.

Summary

GSH is critical for cellular protection against oxidative stress. It serves to protect against damaging H_2O_2 and other organic hydroperoxides and also helps regenerate other antioxidants (eg vit C and E) into their biologically active form. As oxygen consumption increases the GSH/GSSG ratio will drop indicating GSH oxidation. Indeed aerobic exercise does result in GSH oxidation. As well, strenuous exercise also results in several immune alterations, some of which may contribute to a period of immunosuppression, leading to an increased likelihood of infection. An increased incidence of URTI is associated with severe exercise such as marathon runs. Like exercise, periods of caloric restriction also result in negative alterations of immune function. These alterations appear to be immuno-inhibitory seen as a decrease in lymphocyte proliferative capacity, phagocytosis, and CD4+ cell number. An athlete seeking to lose fat mass may diet even while attempting to maintain their training regime. With the additive affects of training and caloric restriction the athlete may be in jeopardy of contracting illness. Discovery of a nutritional intervention that could reduce the suppression of immunity caused by strenuous exercise and by energy restriction would be welcomed by athletes and coaches.

GSH is critically important to immunocytes by protecting them against oxidative stress as well as playing a role in membrane stabilization. Not only does GSH serve as our most important cellular antioxidant substance, it also is needed to keep immune cells functioning properly. Severe oxidative stress leads to GSH oxidation and several potentially negative immune alterations. Therefore, to the aerobic athlete adequate intracellular GSH is critical for protection against oxidative stress and in initiating an uncompromised immune response.

Oxidative stress is associated with the onset of fatigue. By increasing our antioxidant defenses via boosting GSH levels, fatigue may be delayed leading to increased muscular performance. Indeed, GSH boosting drugs have resulted in increased performance in both animals and humans. While these are exciting findings, the side effects associated with these drugs precludes their ability to be effective and practical ergogenic aids.

Exogenous supplementation of GSH is commercially available and marketed as an ergogenic aid capable of increasing performance and aiding recovery. However, because of its lipophobic properties GSH can not effectively cross biological membranes. Research has convincingly demonstrated the inability of exogenous GSH to increase intracellular levels of GSH in several tissues, including skeletal muscle. Intracellular manufacture of GSH is limited by the availability of the amino acid cycteine, which is highly unstable in circulation. The delivery of intact cysteine to the cell results in increased intracellular GSH. This is possible by providing intact glutamylcysteine groups, a compound abundant in whey protein. However, conventional pasteurization processes destroy these glutamylcysteine groups negating any benefit. Immunocal® is a commercially available whey protein concentrate which utilizes a proprietary pasteurization technique which retains the intact glutamylcysteine groups. Supplementation with Immunocal® appears to be effective in increasing intracellular GSH levels in both healthy and sick human populations.

Chapter III

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Effect of Whey Protein Isolate on Oxidative Stress, Exercise Performance, and Immunity

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Running Head: Whey Protein and Immunity following Exercise

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Abstract

Purpose: The purpose of this study was to evaluate the effectiveness of a whey protein isolate (WPI), a reported glutathione (GSH) booster, on exercise performance, immune function, and antioxidant status during weight maintenance and energy restriction in trained athletes. **Methods:** Twenty well-trained, college age, male cyclists performed a cycling exercise test for 45 min, the first 7 min at 70% of VO_{2peak} and the remaining 38 min at 55% VO_{2peak} immediately followed by a performance test set at 90% VO_{2peak} until exhaustion. Blood samples were collected prior to the exercise test, after 45 min of exercise, within 5 min of exhaustion, and 1 h after exercise. Blood samples were analyzed for GSH, GSH/GSSG ratio, glutathione peroxidase (GPx), lipid hydroperoxides (LPO), and several immunity measures (phagocytosis, oxidative burst, peripheral blood mononuclear cells (PBMC) proliferation, and immunophenotyping). Subjects consumed 40g/day of WPI or casein placebo (P) along with their normal diet for 2 wk, repeated the exercise test, and then began a low energy period continuing the same supplementation for 4 d before the final exercise test. **Results:** WPI was not associated with superior exercise performance or antioxidant status following exercise or weight loss. WPI supplementation resulted in 33% greater lymphocyte proliferation capacity following exercise. Following exhaustive exercise for all trials, tGSH and GPx increased 7% and 11%, respectively, while white blood cell (WBC) GSH decreased 13%. For WPI, GPx activity was 10% lower than P following exhaustive exercise for all trials combined. Weight loss (2.67 ± 0.26 kg) resulted in increases in phagocytosis (65%), white blood cell (WBC) GSH (40%), and GPx (35%) while decreasing the GSH/GSSG ratio (55%) and LPO (16%). Exhaustive exercise caused a 28% increase in % CD3-CD8+ T-cells and

decreased CD3-CD4+ (34%), CD3+ (15%), the CD4+/8+ ratio (45%), and phagocytosis (8%) with all values returning to baseline after 1 h recovery. **Conclusions:** Supplementation with WPI did not enhance GSH status or exercise performance in trained cyclists, during weight maintenance or energy restriction. Following exercise, WPI is associated with greater lymphocyte proliferative ability which may help maintain an athletes health during heavy training or competition.

Keywords: Immunocal®, Antioxidants, Immune Function, Weight Loss

Glutathione (GSH) is a thiol containing tripeptide that serves as the cell's major antioxidant compound (4, 25). During episodes of oxidative insult such as exercise, (7, 31, 40, 65), GSH donates electrons to reduce harmful oxidants thereby attenuating cellular dysfunction. Throughout this process, GSH becomes oxidized to glutathione disulfide (GSSG) so that the GSH/GSSG ratio decreases during periods of oxidative stress. For example, Gohill et al. found an 81% decrease in GSH/GSSG following submaximal cycling (13). Increased ROS production has been associated with the onset of fatigue and altered immune function in several studies (50). Barclay et al., using highly oxidative mouse and canine skeletal *in vitro* muscle preparations, observed twice the rate of fatigue with ROS exposure as well as a 37% decrease in fatigue rate when ROS were blocked (2). Immune cells rely on GSH for protection against oxidative stress and membrane stabilization (63). Indeed, impaired immunity is often observed following strenuous prolonged aerobic exercise. Therefore, uncompromised intracellular GSH is crucial for proper muscle and immune cell function. However, GSH boosting drugs (i.e. N-acetylcysteine) have proven to be impractical or ineffective as ergogenic aids (46, 32).

GSH is lipophobic and cannot efficiently cross skeletal muscle cell membranes. De novo synthesis of GSH requires the amino acid cysteine, the rate limiting substrate for GSH synthesis, to maintain adequate GSH concentrations. Cysteine is highly unstable in circulation but can be delivered intact in the form of glutamylcysteine (34). Several unique amino acid groups and peptides are present in whey following the precipitation of caseins in the production of cheese (63). One of these components is albumin, which, unlike casein, is rich in glutamylcysteine groups. Normal food production techniques and pasteurization processes usually result in the denaturing of any glutamylcysteine groups

present. Immunocal®, a commercially available whey protein isolate was developed using a proprietary pasteurization process, which claims to retain intact glutamylcysteine (34). Supplementation with Immunocal® (20-40 g/day, 3 months) resulted in significant increases in red blood cell and white blood cell (WBC) GSH concentrations in both healthy and HIV+ males and females (3, 25). Given the role GSH plays in maintaining cellular redox status and proper immune function, we hypothesized that Immunocal® supplementation will improve glutathione status and thus exercise performance and the immune response to exercise.

Reducing caloric intake in order to lose body weight is a common practice among both athletes and non-athletes. While decreasing fat mass may increase sports performance, caloric restriction may compromise immune function. Research indicates that severe and moderate energy restriction or long term fasting**,** in both obese and trained females, is associated with decreased immunocompetence and resistance to infection (5, 20, 37, 57). Short term fasting has also been linked to decreased lymphocyte proliferation with mitogen stimulation (16, 62). Even so, oxidative stress markers, such as lipid hydroperoxides (LPO), decrease with energy restriction, possibly because of decreased oxidative metabolism-induced ROS generation resulting in a reduction in overall metabolic rate (14, 64). The link between energy restriction, immune function, and oxidative stress requires additional evaluation, especially in athletes.

The purpose of this study was to evaluate the effectiveness of Immunocal®, a reported glutathione (GSH) booster, on exercise performance, immune function, and antioxidant status during weight maintenance and energy restriction in male aerobic athletes.

METHODS

Study design and subjects. The protocol for this study was reviewed and approved by the Institutional Review Board for Research Involving Human Subjects. Twenty well-trained, college age, male cyclists were recruited from the Virginia Tech Cycling and Triathlon Clubs. Subjects were engaged in 168.9 ± 10.9 km of weekly cycle training (343.3 ± 25.2 min), all signed an informed consent, and filled out a medical screening form prior to being accepted. Baseline body weight was measured and VO_{2peak} testing was performed using progressive cycle ergometry (described below). Body composition was measured using a 3-site skinfold test (18). Within a week of baseline testing all subjects underwent a cycling exercise test consisting of 45 min with the resistance set to elicit 70% of VO_{2peak} for the first 7 min and 55% for the remaining 38 min. This was immediately followed by a performance test set at 90% VO_{2peak} until exhaustion. Oxygen consumption was measured at the beginning of the exercise test to verify intensity and every 15 min there after. Blood (8 ml) were collected in sodium heparinized tubes prior to the exercise test (EX1), after 45 min of exercise (EX2), within 5 min of exhaustion (EX3), and 1 h after exercise (EX4). The first three blood samples (EX1, EX2, EX3) were analyzed for GSH/GSSG ratio, GPx, and LPO. EX1, EX3, EX4 blood samples were used for immunity measures consisting of oxidative burst, phagocytosis, and lymphocyte T-cell subsets. Lymphocyte proliferation was measured at EX1 and EX3 (pre-exercise and post exhaustion). After 1 h of recovery, each subject performed a 30 s Wingate power test on a Monarch cycle ergometer. Following this baseline exercise test (T1), subjects consumed 40 g/day of Immunocal® (WPI) or casein placebo (P) along with their normal diet for two wks. This dose was based on a pilot

study performed on eight healthy males who consumed either 20 or 40 g/day of WPI over 4 wks. Pilot study results showed a 25% and 13% increase in whole blood and WBCGSH, respectively, after 2 w of 40 g/day WPI supplementation. Subjects were instructed to hand mix, without heat, WPI with juice, milk, or water in 20 g morning and evening doses. Following the protein supplementation period, subjects repeated the exercise test (T2) as described above and began a low energy period.

Low Energy Experimental Period. All subjects were provided with an energy restricted, formula diet (Ensure®, Ross Laboratories, 20 kcal/kg, 54.7% CHO, 21.3% PRO, 24% Fat) designed to cause weight loss for four days. Each subject supplemented the low energy diet with 40 g/day of WPI or P as during the weight maintenance phase. The exercise test with blood sampling was repeated at the end of the weight loss period (T3).

Exercise Testing. Peak oxygen consumption was determined via a progressive cycle ergometer test. Tests were performed on a Sensormedics 800 Cycle Ergometer, which utilizes computer controlled eddy current braking (eddy current runs opposite the main electrical current) with torque measurement technology to provide a constant workload independent of pedal speed (Sensormedics, Yorba Linda, CA). Subjects pedaled at their preferred rpm beginning at 75 W of resistance for 2 min. Resistance increased 15 W every 30 s until exhaustion defined as pedal rpm falling below 30 or volitional exhaustion. Oxygen consumption was monitored throughout using a Sensormedics Vmax 229 metabolic cart (Sensormedics, Yorba Linda, CA). After determination of peak oxygen consumption, 55%, 70%, and 90% of peak oxygen

consumption was matched to the corresponding wattage. These power outputs were used for the submaximal exercise and performance test, respectively.

Antioxidant measures. Both whole blood and lymphocyte samples were frozen at -70º for future analysis. Whole blood for GSSG analysis was initially snap-frozen in a 50/50 ethyl alcohol dry ice solution for immediate freezing. For GPx, the whole blood sample was centrifuged at 2500 x g for 5 min at 4ºC. Supernatant was discarded and the packed cell pellet frozen at -70º for future analysis. Whole blood GSH/GSSG was measured using a commercially available spectrophotometric assay (GSH/GSSG-412 assay, OxisResearch, Portland, OR). WBCGSH was determined after lymphocyte separation using Vacutainer Cell Preparation Tubes (Vacutainer CPT, Becton Dickinson and Co., Franklin Lakes, NJ). After washings, lymphocytes were counted (Beckman Coulter, Inc., Fullerton, CA) and resuspended in 200 µl PBS before being assayed using a commercially available spectrophotometric assay (GSH/GSSG-412 assay kit, OxisResearch, Portland, OR). In order to achieve detectable WBCGSH concentration, the prescribed amount of assay buffer was decreased while pH was maintained. Whole blood was analyzed for total protein using a detergent free protein assay (Bio-Rad Laboratories) in order to report GSH per mg protein. Red blood cell GPx was measured using a commercially available spectrophotometric assay (Oxyscan cGPx-340 assay kit, OxisResearch, Portland, OR) and a proprietary automatic rotating spectrophotometer (OxyScan Instrument, OxisResearch, Portland, OR).

Plasma was measured for LPO using a commercially available spectrophotometric assay (Bioxytech LPO-560 kit, OxisResearch, Portland, OR). All antioxidant parameters were measured in duplicate.

Immune measures. The CD3+, CD3-CD4+, and CD3-CD8+ lymphocyte populations were determined by flow cytometry. Data was acquired using the Coulter EPICS-XL MCL instrument (Coulter, Miami, FL). Mouse anti-human CD3 (IgG2a Heavy chain, Kappa Light chain) (S4.1 clone), mouse anti-human CD4 (S3.5 clone), and mouse anti-human CD8 (IgG2a Heavy chain, Kappa Light chain) (3B5 clone) monoclonal antibodies (Caltag Laboratories, Burlingame, CA) conjugated with specific fluorochromes (CD3, TRI-COLOR, CD4, Phycoerythrin (PE), CD8, Fluorescin (FITC)) were incubated with 100 µl whole blood and 180µl phosphate buffered saline (PBS) for 15 min allowing binding of the monoclonal antibodies to the receptor sites. Following incubation, red blood cells were lysed (100 µl of a NH_4Cl, $KHCO_3$, Na_4EDTA solution), samples were centrifuged (400 x g/10 min), and the supernatant discarded. Samples were washed twice in PBS, centrifuged (400 x g), and the supernatant discarded. Samples were resuspended in 500 µl of a 2% paraformaldehyde solution and refrigerated in the dark until final analysis.

Prior to the study the ability of paraformaldehyde to crosslink and maintain cell structure was verified by comparing unfixed and fixed samples. This comparison showed no difference, therefore paraformaldehyde was used to 'fix' samples for this assay prior to flow cytometry analysis throughout the study.

Mitogen-induced lymphocyte proliferation was measured via the Alamar Blue colorometric assay. Blood was collected in Vacutainer CPT tubes, centrifuged (1100 x g/10 min) and the WBC layer removed. After multiple washings in RPMI 1640 media (Irvine Scientific, Santa Ana, CA), WBCs were mixed with complete media (RPMI 1640, 10% fetal calf serum, L-glutamine, gentamycin) to obtain a final concentration of 1×10^6

cells/ml. WBCs were cultured in sterile 96 well plates as follows: BLANK containing media only (no WBCs), UNSTIMULATED CONTROLS containing WBCs with no mitogenic stimulation, and STIMULATED containing 100 µl of the WBC suspension and 100 µl of one of the following concanavalin A (Con-A) mitogenic concentrations: 5, 10, and 20 µg/ml (final volume 200 µl). Plates were then incubated in a 37ºC, humidified 5% CO_2 chamber for 48 h. After incubation, 20 µl of Alamar Blue was added to each well and plates were returned to the incubator for an additional 24 h. Plates were then measured for absorbance at 570 and 600 nm on an ELISA plate reader (EL311 S, Bio-Tek Instrumaents, Inc.). The absorbance at 570 and 600 nm determines the optical density (OD) of the Alamar Blue color indicator. To determine the true absorbance (specific absorbance) the OD at 600 nm is subtracted for the OD at 570 nm. This reflects proliferation. Specific absorbances of unstimulated controls were subtracted from specific absorbance of Con-A stimulated samples to yield Δ-specific absorbance.

The ability of neutrophils to phagocytize carboxylated fluorescent microspheres (1 micron in diameter) (Polysciences Inc., Warrington, PA) was measured flow cytometrically. A 500 µl WBC suspension of 1 x 10^6 cells (suspended in KRH-gelatin) was pipetted into both cold (C) and warm (W) labeled polypropylene test tubes and mixed with 50 µl fetal bovine serum and 10 µl fluorescent beads. Tubes C and W were incubated in ice and 37ºC water baths, respectively, for 60 min. Reactions were stopped by adding 1.5 ml cold PBS-gelatin. Samples were then spun at 1200 rpm for 10 min, supernatant discarded, resuspended in 300 µl of paraformaldehyde, and refrigerated in the dark until analysis. Each sample's mean channel fluorescence, which represents neutrophils' uptake of fluorescent beads, was analyzed on a flow cytometer emitting 525

nm. The C samples reflect random binding of beads to neutrophils and were subtracted from the % fluorescence of the W samples yielding net % fluorescence.

Oxidative burst capacity of activated WBCs, as measured by H_2O_2 production, was measured via flow cytometry on the same day as sample collection. A 500 µl suspension of 1 x 10^6 cells was pipetted into one "control" and three "stimulated" labeled polypropylene test tubes. All tubes received 1µl/ml of 5 mM dichlorofluorescin diacetate (DCF-DA) and tubes were then incubated for 15 min in a 37ºC water bath. After incubation, 10 µl of phorbol 12-myristate 12-acetate (PMA) was added to "stimulated" labeled tubes. An equal volume of HBSS was added to the "control" tube. Both "control" and "stimulated" samples were incubated in a 37ºC water bath for an additional 10-15 min. Mean fluorescence was measured via a flow cytometer emitting at 525nm. Net % fluorescence was calculated by subtracting the % fluorescence emitted by "control" samples from the % fluorescence emitted by "stimulated" samples. The triplicate reading for each subject sample was averaged for the final measure of oxidative burst.

Statistics.

Antioxidant and immune measures were analyzed using a completely randomized 2 x 3 x 3 (group x trial x exercise time point) repeated measures factorial arrangement. Main effects included: Group (WPI x P), Trial (T1 x T2 x T3), and Exercise time point (EX1 x EX2 x EX3 or EX4). Interaction effects included: Group x Trial, Group x Exercise time point, and Group x Trial x Exercise time point. Performance measures were analyzed using a completely randomized 2 x 3 (group x trial) repeated measures factorial arrangement. Main effects included: Group (WPI x P) and Trial (T1 x T2 x T3).

The interaction effect was Group x Trial. Significant interactions were further investigated using paired-samples t-tests to locate differences. A $P < 0.05$ was considered significant. Statistical calculations were processed using the statistical package SPSS 11.0 for Windows (SPSS, Chicago, IL). Missing values were not used in any statistical analysis.

RESULTS

Subject characteristics. There were no significant differences between groups for baseline age, height, weight, body composition, VO_{2peak}, or training volume (Table 1).

While there was no difference in body weights at T1 and T2, the energy restriction caused a significant decrease of 2.7 ± 0.3 kg over the four days. There was no difference between groups for change in body weight with WPI losing 2.4 ± 0.4 kg and P losing 2.9 ± 0.3 kg (Figure 1).

Performance measures. Time to exhaustion at 90% VO_{2peak} was highly variable and did not differ between groups over the trials (Table 2). There was a strong trend for time, with both groups combined, to decrease time to exhaustion following energy restriction (260 ± 16 seconds prior and 200.5 ± 28.5 seconds post, P=.051). Because of extraneous circumstances that resulted in too long of a post submax rest period five test were thrown out over the three trials.

There was no difference between groups or over trials for peak power, total work, or fatigue rate during the Wingate test (Table 3). For both groups combined, total work was 219 ± 7.9, 221 ± 7.9, and 219 ± 8.3 kJ for T1, T2, and T3, respectively. Fatigue rate was 33.5 ± 3.3, 34.4 ± 3.1, and 35.5 ± 3.3% for T1, T2, and T3, respectively.

Immune function.

Effect of Supplementation. WPI did not influence immune function as measured by CD3+ (TRI-COLOR), CD3+CD4+ (PE), CD3+CD8+ (FITC) PBMC subsets, the CD4+/8+ ratio, oxidative burst capacity, or phagocytic ability (Table 4**).** Lymphocyte proliferation was 33% greater, following exercise, for WPI with all trials combined (WPI=2112 ± 192, P=1584 ± 166, *P*=0.024).

Effect of Acute Exercise. For both groups combined and all trials collapsed, exhaustive exercise decreased levels of CD3+ and CD3+CD4+ T-cells by 15% and 34%, respectively (*P*<0.01). CD3+CD8+ lymphocytes increased following exhaustive exercise (28%, *P*<0.01). Consequently, the CD4+/8+ ratio fell post exercise (45%, *P*<0.01). All T-cell subsets returned to baseline after 1 h of recovery. Immediately following exercise, phagocytosis decreased 8% (*P*<0.01) while oxidative burst capacity and lymphocyte proliferation were unchanged. Following 1 h of recovery phagocyctosis rebounded to baseline values while oxidative burst increased 4% above baseline (*P*<0.05) (Table 5).

Effect of Weight Loss. For both groups combined and all exercise time points collapsed, energy restriction resulted in a 65% increase in phagocytosis (*P*<0.01). T-cell subsets, lymphocyte proliferation, and oxidative burst capacity were unaffected by weight loss.

Antioxidant measures.

Effect of Supplementation. WPI did not significantly affect antioxidant status as measured by tGSH/mg protein, WBCGSH, GSH/GSSG ratio, GPx, or LPO (Table 7). Although not statistically significant, WPI had a 9.9% increase in tGSH/mg protein from baseline T1 to baseline T2 (T1 = 1288 ± 39, T2 = 1416 ± 54μmol/mg protein) while P

had a more modest, 2% increase (T1 = 1359 ± 35, T2 = 1399 ± 54µmol/mg protein) (observed power = 0.079 [Wilks Lambda]). Following exhaustive exercise (EX3), with all trials collapsed, P had significantly higher (10%) GPx activity (WPI = 6.8 ± 1.8 µmol/ml, P = 7.5 ± 1.8 µmol/ml) ($P<0.05$).

Effect of Acute Exercise. For both groups combined and all trials collapsed, there was no effect of submaximal exercise on tGSH but a 7% increase in tGSH/mg protein after exhaustive exercise (EX1 = 1368 ± 47, EX2 = 1400 ± 52, EX3 = 1470 ± 56 µmolGSH/ mg protein) ($P<0.05$). Following submaximal exercise (EX2), GPx increased 23% and remained 11% above baseline immediately following exhaustion (EX1 = 6.5 ± 1.6, EX2 = 8.0 ± 1.6, EX3 = 7.2 ± 1.5) ($P < 0.01$). WBCGSH decreased 14% ($P<0.05$) following submaximal exercise and remained 13% ($P<0.05$) below baseline immediate post exhaustion. The GSH/GSSG ratio and LPO were unaffected by exercise (Table 8).

Effect of Weight Loss. For both groups combined and all exercise time points collapsed, energy restriction resulted in several alterations to antioxidant status. The GSH/GSSG ratio decreased 55% following weight loss (T1 = 135 ± 12, T2 = 142 ± 19, T3 = 63 ± 7) ($P<0.001$). LPO decreased 16% ($P<0.01$) from T1 to T2, during weight maintenance, and then decreased a further 16% ($P<0.01$) from T2 to T3. Energy restriction increased WBCGSH and GPx 40% and 35%, respectively, but did not affect tGSH/mg protein (Table 9).

DISCUSSION

The results of this study do not support a value of WPI for exercise performance or antioxidant status over a casein placebo. Lymphocyte proliferation was enhanced with

WPI following exercise. No other immunity measure was affected by the WPI treatment. We found that supplementation with WPI did not significantly boost whole blood or WBC glutathione levels. Antioxidant enzyme activity, GPx, was also unaffected. However, we observed a non-significant increase in whole blood antioxidant status as measured by GSH with WPI which was not matched by the casein treatment. Exercise resulted in dramatic decreases in indicators of specific immune function (ie CD3+ immunocytes) while moderately increasing cytotoxic capabilities (ie CD3-CD8+ immunocytes). This was in spite of a lack of clear oxidative stress induced by the exercise.

GSH boosting and exercise performance. Improving antioxidant status in animals has been associated with *in vivo* and in vitro increases in muscular performance (2, 39, 45). Although less established, human research indicates GSH boosting improves the ability to do physical work. In one study, WPI supplementation was associated with increases in power output during cycle ergometry (25). Lands et al. observed a 13.3 % increase in peak power and a 12.7% increase in total work during a 30-second isokinetic cycle sprint after 3 months of WPI supplementation in healthy males. While Lands et al. (25) did not measure whole blood or tissue GSH they observed a 35.5% increase in lymphocyte GSH concentrations with WPI supplementation and suggested that this improvement in antioxidant capacity explained the ergogenic effect. The lack of improvement in performance in our study could be attributed to our shorter supplementation period (2 wks), which did not cause significant increases in GSH or could represent a true lack of benefit of this supplement on performance. We observed higher baseline GSH status in our aerobically trained subjects which may have limited

further increases (9, 22, 47). The subjects consuming whey protein in the study by Lands et al (25) reported more activity during the supplementation period which could have explained their improved performance independent of the supplement. Although some human and animal studies have observed increased skeletal muscular performance with the GSH boosting drug NAC (46, 55), other research does not show an increase in performance, even when antioxidant status improves (32). Despite some research indicating a positive link between antioxidant status and muscular performance our study does not support this link.

Effects of acute exercise on immune function. Our study showed that an acute bout of submaximal exercise immediately followed by exhaustive cycling elicited a dramatic effect on several immune parameters. We observed a substantial decrease in percentages of CD3+ and CD3+CD4+ T-lymphocytes and a more modest, yet significant, increase in CD3+CD8+ T-cells. This was reflected in a decrease in the CD4+/CD8+ ratio immediately following exercise. Other research using cycling as the exercise mode has also found decreases in CD3+ and CD3+CD4+ T-cells following exhaustive exercise (24). In contrast, both Vider et al. and Mooren et al. observed 1.6 and 1.7 fold increases in total T-cells (CD3+) following exhaustive running which fell below baseline by 30 minutes and one hour post, respectively (35, 60*).* While the effect of different modes of exercise on CD3+ T-cells remains unclear, both running and cycling appear to decrease CD3+CD4+ T-cells and elicit a subsequent decrease in the CD4+/CD8+ ratio. The mechanisms behind these changes may relate to exercise induced lymphocyte apoptosis. Mars et al., found that apoptosis occurs in 63% of lymphocytes immediately following high-intensity exercise (29). This apoptotic response may be initiated by exercise induced

metabolic and hormonal changes that damage cells (35). Increased cytosolic calcium has also been shown to be an important intracellular signal involved in apoptosis (66). Finally, generation of ROS and subsequent changes in cellular redox status can damage DNA and initiate apoptosis (35, 60). Our observed decrease in WBCGSH may reflect a change in the lymphocyte intracellular redox environment triggering the apoptotic process. Recent research has associated decreases in intracellular GSH with cell death (11). The results from the present study, as well as previous research, indicates that exhaustive exercise compromised CD3+CD4+ T-cell status at least for the short term (~1 h post exercise). This may represent a brief lapse in the ability to recruit B-cells and therefore decreased specific immune capabilities. Our research, as well as others (35, 60), measured immune changes following exhaustive exercise. Whether similar or more modest changes occur following various submaximal intensities remains untested.

We observed a modest decreased capacity for phagocytosis and a non-significant decrease in oxidative burst immediately following exercise. Lewicki et al., found decreased phagocytosis and oxidative burst, of similar magnitudes, in trained cyclists following exhaustive cycling (27). Other research using aerobic athletes have reported decreased phagocytic activity following long- and ultra-distance running (12, 36). Contrastingly, animal and untrained human data predominantly find increases or no change in phagocytosis and oxidative burst following moderate and exhaustive exercise (27, 41, 48). By one hour post exercise we found that oxidative burst recovered to values above baseline while phagocytosis began to recover but remained below baseline. While the post recovery-enhanced oxidative burst capacity indicates increased immune function, the decreased phagocytic ability following exercise would limit the amount of foreign

protein subjected to oxidative burst killing**.** It is possible that aerobic athletes, who are known to have enhanced resting immune function (20), respond differently than other subject populations to exercise stress. Indeed, when compared to untrained individuals, trained athletes exhibit significantly greater immune defense capacity and less muscle damage following strenuous aerobic exercise (42, 43, 59). The frequently reported enhanced non-specific immune function observed in animals and sedentary humans, following exercise, may be in response to increased cellular debris from localized muscle damage (49). Overall, the strenuous aerobic exercise in our study compromised specific as well as non-specific immune responses putting the competing athlete, theoretically, at increased risk of infection.

There appears to be a relationship between exercise-induced alterations in immune function and antioxidant defenses. Immune cells rely on GSH for protection against oxidative stress and membrane stabilization (63). We observed an exercise induced decrease in WBCGSH status which was associated with a decreased post exercise total lymphocyte population. Specifically, CD4+ T-cells fell following exercise possibly due to less available antioxidant defenses reflected in decreased WBCGSH. To our knowledge, we are the first group to measure WBCGSH alterations following exercise in humans. Compromised WBCGSH status may play a role in the compromised specific immune function following strenuous exercise. This decreased ability to activate specific B-cell populations may provide a window of opportunity for infection.

Effects of acute exercise on antioxidant status. We found a non-significant 9% and 7% decrease in the GSH/GSSG ratio following submaximal and exhaustive exercise, respectively. Other research, using exhaustive and submaximal cycling exercise, has

typically observed greater decreases in the GSH/GSSG ratio (13, 54, 61). Previous research has demonstrated that it is difficult to induce oxidative stress in trained subjects. Following a competitive triathlon, Margaritas et al., found no evidence of lipid hydroperoxides or increases in GSSG. Interestingly, basal GSH levels correlated significantly with cycling training volume, indicating that cycling may be associated with greater antioxidant defense training adaptations than other modes of aerobic exercise (28). Extremely long durational exercise (>6.5 h) may be required to elicit oxidative stress in aerobic athletes (30). Shorter graded exercise tests to exhaustion, on a cycle ergometer, have not induced oxidative stress in trained subjects (19). Therefore, a longer more intense exercise protocol may have produced more meaningful changes in levels of oxidative stress in our subjects.

Our exercise protocol stimulated a non-significant increase in LPO. It is possible that the handling of ROS generated during exercise by the enzymatic conversion of GSH to GSSG, mediated by GPx, was sufficient to protect our subjects from statistically significant oxidative damage as measured by LPO.

Effect of supplementation. Our supplementation protocol of 40 g/day over a 3 week period, which included 4 d of energy restriction, failed to elicit meaningful performance and antioxidant differences between WPI and casein placebo. WPI, which has been shown to boost GSH levels in healthy untrained (25) and diseased (34) populations, may not elicit the same response in trained individuals. Research indicates that intense aerobic training increases whole blood and red blood cell GSH concentration and may limit the opportunity to further increase this capacity (9, 22, 47). For example,

when compared to sedentary healthy males (n=265, 941 ± 115 μmol) (33) our subjects had a 42% higher concentration of tGSH (1347 ± 33 μmol).

It is possible that a longer supplementation period may have yielded improved GSH status with WPI. Micke et al., found significant 25-44% increases in plasma tGSH after only 2 weeks of 45 g/day whey protein supplementation in HIV+ patients (34). However, these diseased subjects had severely compromised baseline GSH levels. In healthy untrained populations, 3 months of 20 g/day whey protein supplementation produced significant increases in GSH (25). No data was presented to show GSH at earlier time points. Our pilot study, which compared weekly 20 g and 40 g WPI supplementation, over 4 weeks in healthy untrained males, showed a 25% peak increase after 2 weeks of 40 g WPI/day. These pilot subjects had baseline GSH levels 63% lower than those recruited for the principle study. Because of our trained subject's initially high GSH levels, WPI may either be ineffective or require a longer supplementation period to produce meaningful increases.

We observed increased ability for lymphocyte proliferation, following exercise, in those subjects supplemented with WPI (Figure 2). Increased lymphocyte proliferation with whey protein supplementation, but not casein, has been reported in animals (3). These previous findings were attributed to the much higher cysteine content of whey, compared to casein, and subsequent enhanced splenic GSH synthesis. Furthermore, when the GSH level of the whey supplemented animals was reduced by half, a 4-5 fold decrease in lymphocyte proliferation ensued. Other research has reported a high correlation between whole blood tGSH and lymphocyte proliferation in humans (60). Although we did not measure splenic GSH, it is possible that splenic concentrations were

affected by our supplementation protocol. While not significantly different, WPI increased WBCGSH by 49% over the course of the study while P resulted in a 32% increase. This numeric increase in WBCGSH, with WPI, may offer a protective mechanism behind the increased lymphocyte proliferation in WPI treated subjects. Although whole blood tGSH did not significantly increase with WPI, the 9.9% increase that was observed may also reflect greater antioxidant protection resulting in enhanced immune function.

Effects of weight loss on antioxidant status and immunity. The present study demonstrated several changes in antioxidant status following energy restriction. While tGSH did not change, the GSH/GSSG ratio dropped dramatically along with significant decreases in LPO and increases in GPx activity. Animal studies have shown increased GPx gene transcription, as well as other proteins involved in free radical scavenging, with energy restriction (56). Reduced food intake will also decrease the amount of ROS produced via normal nutrient metabolism (38). Our subjects had increased GPx activity and GSSG indicating a more active GSH/GSSG redox cycle. This was reflected in decreased LPO production, as GSH may be more easily sacrificed to handle the present oxidant burden. Periods of energy restriction are not characterized by increased ROS but rather by an enhancement of ROS metabolism (56).

We observed a 40% increase in WBCGSH in humans after losing 2.6 kg of body weight over 4 d of energy restriction. To our knowledge we are the first to measure WBCGSH following weight loss. Given the importance GSH plays in maintaining the health of immune cells, increased WBCGSH, during caloric restriction, may reflect a prioritized defense mechanism while the body endures a physiologically vulnerable

period. Decreased erythrocyte GSH synthesis, coinciding with maintained tGSH levels, has been reported following very low calorie diets (VLCD) in humans, suggesting a change in the distribution of GSH. Animal research has suggested that, during times of energy restriction, erythrocyte GSH is used as a cysteine reservoir which could allow relocation of GSH to the lymphocyte compartment or other tissues (6). Total energy consumed, irregardless of amino acid composition, may play a significant role in GSH concentration and distribution.

We observed increased phagocytic ability following weight loss. Overall, data on the effect of energy restriction on phagocytosis is very limited. To a large extent most of our knowledge comes from research performed on obese populations, a group known to have relatively decreased immunocompetence (58). Nieman et al., reported no change in phagocytic activity following moderate weight reduction (9.9 ± 1.4 kg) in obese females over 12 w of energy restriction (37). Research on athletes is extremely limited. One study indicated decreased phagocytic activity following 2 wk of a moderately low calorie diet (N = 9,1300 kcal/day, 25% protein, 25% fat, 50% carbohydrate) in females from a variety of sports (20) while another observed no change or decreased in two groups of male judoists after 20 d of moderate energy restriction (25 kcal/kg/d) resulting in 2.7 kg of weight loss (21). Our aerobically trained study subjects endured a more restrictive diet with more rapid but brief weight loss. In non-diseased individuals the magnitude of the phagocytic response is governed by the amount of microorganisms, cellular debris, and injured or dead cells present that require processing (8). Although not measured in ours or the previously mentioned studies, the cortisol response to energy restriction may have been of a higher magnitude in our subjects. Diets of 1200/kcal/d are associated with

increased circulating cortisol and closely resemble that of the present study (15). High serum cortisol concentrations initiate excessive protein breakdown precluding an increased phagocytic need to clean up cellular debris. Indeed, adrenocorticotropic hormone, which stimulates the release of cortisol from the adrenal cortex, is associated with increased phagocytosis (17). The increased WBCGSH supports healthy immunocyte status allowing an uncompromised or heightened phagocytic response. Considering the lack of research and varied protocols, more study is needed utilizing homogenous groups with standardized diets and intervention periods to fully understand the effect weight loss has on the phagocytic process.

Conclusions. Previous research has shown that WPI supplementation has promise in the treatment of GSH depleting, pro-oxidant diseases such as AIDS (34). Our data did not indicate a benefit of two weeks supplementation of 40 g/d in aerobically trained athletes, possibly due to an already enhanced GSH status. However, the numerical increase in whole blood and WBCGSH suggests that further research could explore different doses or duration of supplementation in athletes. WPI was associated with a greater ability for lymphocyte proliferation following an exercise bout. This finding could help keep athletes healthy during heavy training or competition. Energy restriction increased the antioxidant enzyme activity, GPx, and immunocyte antioxidant defenses while simultaneously reducing LPO generation. Therefore, acute bouts of exercise in well trained athletes compromised WBCGSH status and immune function while brief energy restriction stimulated immunocyte antioxidant defenses and reduced evidence of oxidative stress. Overall, our study does not support the value of WPI to increase

antioxidant defenses or physical performance but may increase immunocompetence following physical work.

TABLE 4. Subject characteristics.

	WPI (N=10)	**P (N=10)**
Age	21.7 ± 0.6	23.4 ± 1.0
Height (cm)	175.3 ± 2.7	178.6 ± 2.5
Body mass (kg)	71.9 ± 1.8	73.0 ± 1.6
Body fat (%)	10.0 ± 1.3	11.4 ± 1.1
VO_{2peak} ($ml \cdot kg^{-1} \cdot min^{-1}$)	60.7 ± 2.2	56.3 ± 2.3
Training volume (min/wk)	304 ± 30	381 ± 37

Values are expressed as mean ± SE.

TABLE 5. Time to exhaustion at 90% VO_{2peak}.

	WPI (N=9)	**P (N=7)**
	seconds	
Trial 1	257 ± 89	179 ± 55
Trial 2	244 ± 61	272 ± 66
Trial 3	183 ± 11	214 ± 121

Values are expressed as mean ± SE.

TABLE 6. Anaerobic performance measures from Wingate test.

	WPI (N=10)	**P (N=10)**
Peak power (w)		
Trial 1	711.8 ± 45	709.8 ± 45
Trial 2	712.4 ± 33	718.1 ± 34
Trial 3	722.7 ± 35	717.4 ± 35
Total work (kJ)		
Trial 1	219 ± 7.9	219 ± 7.9
Trial 2	220 ± 8.0	222 ± 8.0
Trial 3	219 ± 8.3	221 ± 8.3
Fatigue rate (%)		
Trial 1	34.4 ± 3.3	32.6 ± 3.3
Trial 2	33.3 ± 3.1	35.5 ± 3.1
Trial 3	34.0 ± 3.3	33.0 ± 3.3

Values are expressed as mean ± SE.

TABLE 7. Immune function measures baseline, immediate post exercise, and 1 h post exercise for trials 1, 2, and 3.

	WPI (N=10)			P (N=10)		
	EX1	EX3	EX4	EX1	EX3	EX4
Percentage CD3+CD4+						
Trial 1	37.3±2.1	26.1±2.3	39.9±1.8	38.7±2.3	24.7±2.5	42.5±1.7
Trial 2	26.1±2.0	24.3±1.8	40.0±1.8	24.7 ±2.1	24.6±2.1	41.1±2.2
Trial 3	39.9±2.1	24.4±1.7	39.6±1.9	42.5±2.3	26.7±2.8	38.8±2.4
Percentage CD3+CD8+						
Trial 1	27.9±2.0	32.0±2.0	25.4±1.2	28.8±2.1	35.2±2.2	25.5±2.1
Trial 2	29.7±1.8	34.9±2.4	27.1±1.2	27.4±2.1	35.1±2.1	25.7±2.0
Trial 3	27.9±2.4	33.1±1.8	26.2±1.4	26.8±1.6	34.7±2.4	24.6±1.4
Percentage CD3+						
Trial 1	67.3± 2.6	57.9±3.2	67.6±2.3	65.1±2.1	53.6±3.3	68.1±2.1
Trial 2	67.9±2.4	58.2±3.2	68.8±2.6	63.2± 2.6	53.0±3.2	67.0±2.8
Trial 3	67.1±2.3	57.0±3.4	67.7±3.2	63.9±2.5	55.4±3.5	63.1±3.8
Oxidative burst (% cells)						
Trial 1	96.3± 1.9	92.1±2.3	97.3±1.3	92.9±1.8	93.9±2.2	96.0±1.3
Trial 2	91.9±3.1	91.4±5.4	95.9±3.1	91.0±3.1	86.9±5.4	90.8±3.1
Trial 3	91.9±2.2	93.2±1.5	94.0±1.5	94.1±2.2	94.7±1.5	96.3±1.5
Phagocytosis (% cells)						
Trial 1	37.8±7.6	34.9±6.4	37.0±6.9	36.9±7.6	34.9±6.4	36.7±6.9
Trial 2	27.0±4.6	24.8±4.7	29.0±5.9	26.2±4.6	22.6±4.7	21.5±5.9
Trial 3	50.9±6.8	46.7±6.2	45.7±6.7	37.0±6.8	34.9±6.2	35.0±6.7
Lymphocyte proliferation						
Trial 1	921±59	932±51		930±62	958±54	
Trial 2	2279±236	2747±200		2282±250	2126±212	
Trial 3	2309±203	2754±277		2200±216	1606±294	

Values are expressed as mean ± SE.
EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
See Table 5 for statistical results of main effects of exercise and Table 6 for main effects of trial on immune factors

TABLE 8. Effects of exercise on immune factors.

	EX1	EX3	EX4
CD3+ (%)	65.7±2.1	55.8±2.3[a]	66.9±2.5
CD3+CD4+	37.8±1.8	25.1±2.0[a]	40.3±2.3
CD3+CD8+	28±1.6	34.1±2.0[a]	25.7±1.8
CD4+/8+	1.4±0.4	0.76±0.2[a]	1.62±0.4
Oxidative burst (% cells)	93±4.1	91.9±3.9[b]	95.2±4.1
Phagocytosis (% cells)	35.9±2.3	33.1±2.2[c]	34.2±2.3
Lymph. Prol. (abs)	1800±203	1837±210	

Values are expressed as mean ± SE.
Values are collapsed averages for groups and trials.
EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
[a] $P < 0.05$ from EX1 and EX4
[b] $P < 0.05$ from EX4
[c] $P < 0.05$ from EX1
abs = absorbance

TABLE 9. Effects of trial on immune factors.

	T1	T2	T3
CD3+ (%)	63.2±2.0	62.9±2.5	62.3±2.2
CD3+CD4+	34.8±1.8	34.0±2.3	34.3±2.2
CD3+CD8+	29.1±1.8	29.9±2.1	28.8±1.7
CD4+/8+	1.26±0.6	1.20±0.3	1.22±0.6
Oxidative burst (% cells)	94.7±3.9	91.3±3.6	94.0±3.7
Phagocytosis (% cells)	36.4±2.1	25.2±2.3[a]	41.7±2.0
Lymph. Prol. (abs)	944±58[b]	2390±221	2123±208

Values are expressed as mean ± SE.
Values are collapsed averages for groups and exercise time points.
T1=trial 1
T2=trial 2
T3=trial3
[a] $P < 0.05$ from T3
[b] $P < 0.05$ from T2 and T3
abs = absorbance

TABLE 10. Antioxidant measures

	WPI (N=10)			P (N=10)		
	EX1	EX2	EX3	EX1	EX2	EX3
			tGSH/mg protein			
Trial 1	1288±39	1482±94	1492±134	1359±35	1562±84	1514±120
Trial 2	1416±54	1357±58	1473±89	1399±54	1313±58	1424±89
Trial 3	1347±30	1334±70	1411±62	1383±30	1344±70	1507±62
			WBCGSH (μmol/10^6cells)			
Trial 1	.84±0.9	.72±.11	.62±.10	.87±.09	.73±.10	.82±.10
Trial 2	.96±.10	.84±.13	.74±.05	.77±.10	.77±.13	.74±.05
Trial 3	1.23±.11	.98±1.3	1.03±.13	1.15±.11	.96±.13	1.09±.13
			GSH/GSSG ratio			
Trial 1	154±20	129±14	149±26	140±18	121±13	128±23
Trial 2	142±20	129±40	134±26	143±20	162±40	142±26
Trial 3	75±10	60±5	70±12	61±10	59±5	56±12
			LPO (μmol)			
Trial 1	10.3±1.4	10.2±1.4	12.9±1.6	9.6±1.4	11.8±1.4	9.1±1.6
Trial 2	8.2±1.1	7.1±1.1	10.2±2.1	8.9±1.1	8.1±1.1	11.4±2.1
Trial 3	9.3±1.0	6.5±1.2	7.4±1.0	7.4±1.0	8.6±1.2	6.0±1.0
			GPx (μmol/ml)			
Trial 1	6.5±0.8	8.3±0.6	5.5±0.4	5.8±0.8	7.7±0.5	7.7±0.7
Trial 2	6.0±0.6	7.5±0.6	5.9±.05	4.8±0.5	7.1±0.6	6.6±0.6
Trial 3	7.9±0.4	8.9±0.7	9.0±.08	8.1±0.6	8.9±0.7	8.4±0.6

Values are expressed as mean ± SE.
EX1=baseline
EX2= immediate post submaximal
EX3=immediate post exercise
See Table 8 for main effects of exercise and Table 9 for main effects of trial.

TABLE 11. Effects of exercise on antioxidant status.

	EX1	EX2	EX3
tGSH (μmol/mg protein)	1368±47	1400±52	1470±56 [a]
GSH/GSSG	118±15	108±12	114±13
WBCGSH (μmol)	.97±.16	.83±.20 [b]	.84±.18 [b]
GPx (μmol/ml)	6.5±1.6	8.0±1.6 [b]	7.2±1.5
LPO (μmol)	8.9±1.1	8.7±1.0	9.5±1.3

Values are expressed as mean ± SE.
Values are averages of collapsed groups and trials.
EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
[a] $P < 0.05$ from EX1 and EX2
[b] $P < 0.05$ from EX1

TABLE 12. Effects of trial on antioxidant status.

	T1	T2	T3
tGSH (µmol/mg protein)	1452±60	1397±52	1387±43
GSH/GSSG	135±15	142±12	63±7 [a]
WBCGSH (µmol)	.74±.12	.76±.18	1.07±.18 [a]
GPx (µmol/ml)	6.9±1.4	6.3±1.4	8.5±1.5 [a]
LPO (µmol)	10.6±1.1	8.9±1.2	7.5±1.2 [a]

Values are expressed as mean ± SE.
Values are collapsed averages for groups and exercise time points.
EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
[a] $P < 0.05$ from T1 and T2

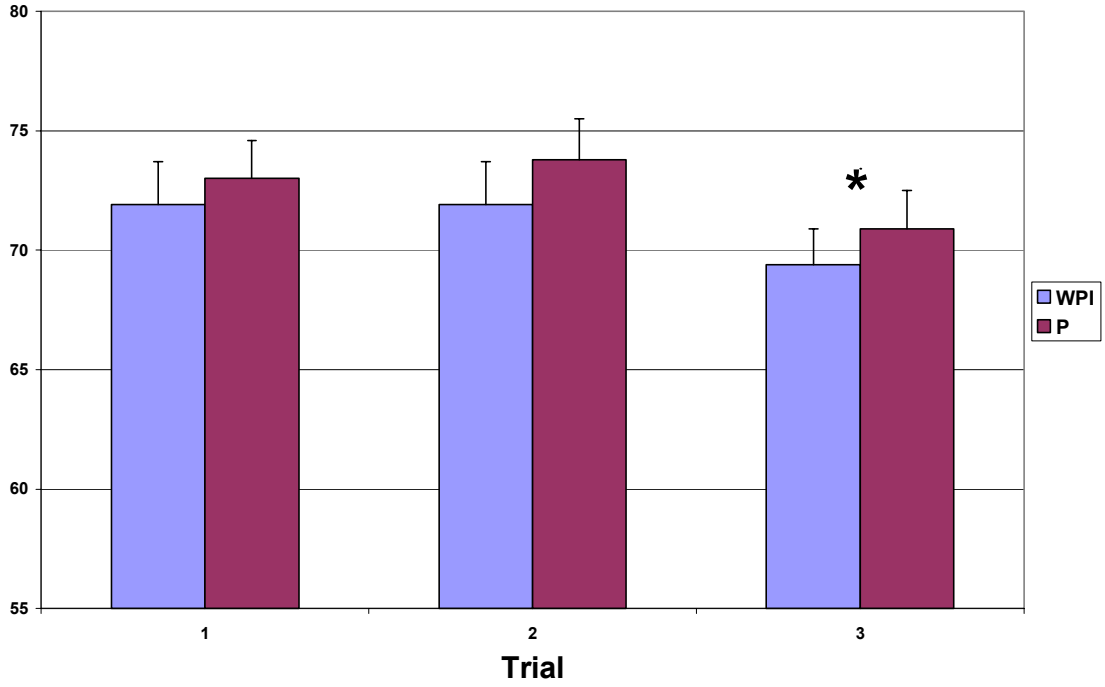

FIGURE 4-Body weight at baseline (T1), after 2 weeks of supplementation (T2) and after energy restriction (T3). * Significant difference from Trial 2 to Trial 3 for both groups combined.

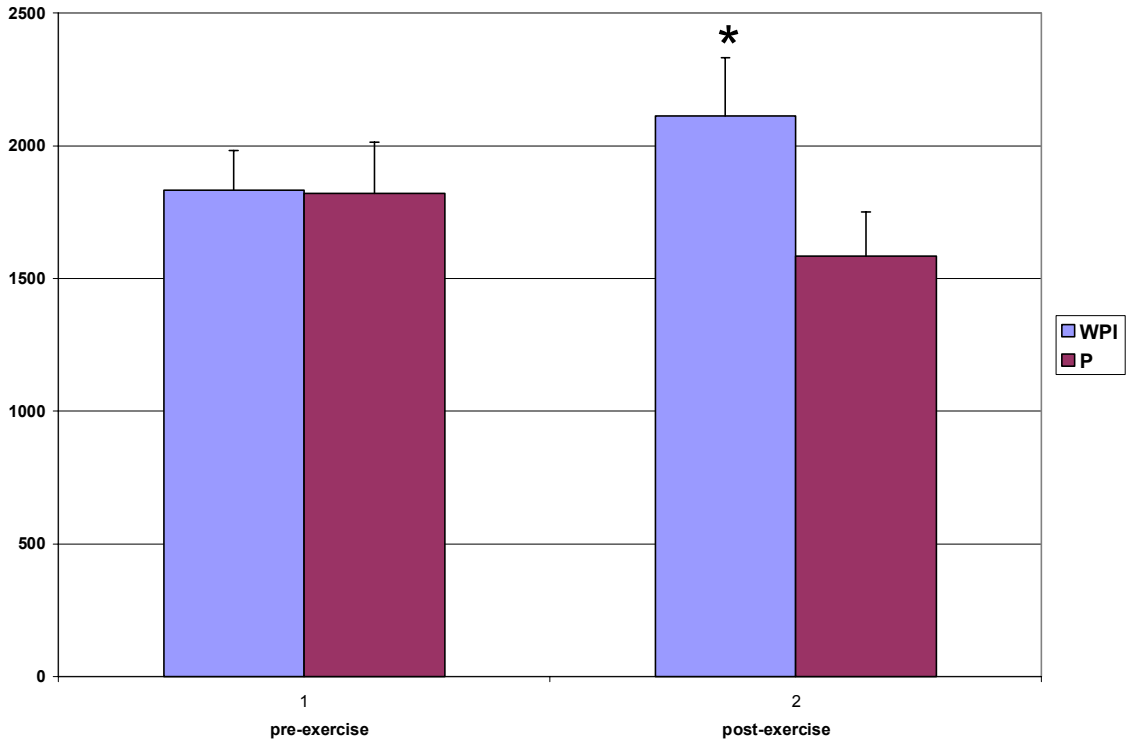

FIGURE 5-Lymphocyte proliferation before and after exhaustive exercise
*** Significant difference between WPI and P ($P < 0.05$)**

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Chapter IV
Summaries, Recommendations for Future Research, Theoretical Issues, and Implications

This chapter contains overall summaries of research results pertaining to the influence of a why protein isolate (WPI), Immunocal®, or casein placebo (P) on immune function, antioxidant status, and exercise performance following exercise and energy restriction. Possible modifications to the current design and recommendations for future research are also discussed. Finally, theoretical and practical implications are presented.

Summaries

A main purpose of this study was to evaluate the potential GSH boosting capabilities of WPI and subsequent effects on performance and immunity when compared to a casein placebo. WPI has been previously shown to increase plasma and WBC GSH in healthy young males and females when consumed for several months (33, 44). Despite these positive results, the effect of WPI on the antioxidant status of trained aerobic athletes and effects on processes dependant on antioxidant status are unknown.

Therefore, one purpose of this study was to evaluate antioxidant status in trained cyclists following WPI versus casein placebo supplementation following aerobic exercise. Because many athletes desire to lose weight while attempting to maintain a training regimen, the effect of WPI supplementation following rapid weight loss was also examined. Two potential time points for treatment effects were proposed: (a) on exercise test #2 which followed two weeks of supplementation during weight maintenance and (b) exercise test #3 which followed four days of energy restriction.

Twenty well-trained, college age, male cyclists were recruited for the study. Within a week of baseline testing all subjects underwent a submaximal cycling exercise test designed to elicit oxidative challenge and modification of immune response. This was immediately followed by a performance test set at 90% VO_{2peak} until exhaustion. Blood samples were collected prior to the exercise test, after 45 min of exercise, within 5 min of exhaustion, and 1 h after exercise. Blood samples were analyzed for tGSH, GSH/GSSG ratio, glutathione peroxidase (GPx), and lipid hydroperoxides (LPO), and immunity measures (T-cell subsets, oxidative burst, phagocytosis, lymphocyte proliferation). Subjects then consumed 40g/day of WPI or casein placebo (P) along with their normal diet for two weeks. Subjects repeated the exercise test and began a protein supplemented low energy period (20kcal/kg) for four days. The exercise test with blood sampling was then repeated at the end of the weight loss period.

Antioxidant and immune measures were analyzed using a 2 x 3 x 3 RMANOVA (group x trial x exercise time point). Performance measures were analyzed using a 2 x 3 RMANOVA (group x trial). Significant main effects and interactions were further investigated using paired-samples t-tests to locate differences. An α-level of $P < 0.05$ was considered significant. Statistical calculations were processed using the statistical package SPSS 11.0 for Windows (SPSS, Chicago, IL).

Antioxidant Status. For both groups combined and all trials collapsed, exercise caused a 7% increase in tGSH/mg protein at EX3 (EX1 = 1368 ± 47, EX2 = 1400 ± 52, EX3 = 1470 ± 56 μmolGSH/ mg protein) ($P < 0.05$). GPx activity was also increased with exercise. Following submaximal exercise (EX2), GPx increased by 23% and

remained 11% above baseline immediately following exhaustion (EX1 = 6.5 ± 1.6, EX2 = 8.0 ± 1.6, EX3 = 7.2 ± 1.5) ($P < 0.01$). WBCGSH decreased 14% ($P < 0.05$) following submaximal exercise and remained 13% ($P < 0.05$) below baseline immediate post exhaustion (Table 7).

For both groups combined and all exercise time points collapsed energy restriction resulted in a 65% increase in phagocytosis ($P < 0.01$). T-cell subsets and oxidative burst capacity were unaffected by weight loss.

WPI did not result in superior antioxidant status as measured by tGSH/mg protein, WBCGSH, GSH/GSSG ratio, GPx, or LPO. Although not statistically significant, WPI resulted in a 9.9% increase in tGSH/mg protein from baseline T1 to baseline T2 (T1 = 1288 ± 39, T2 = 1416 ± 54μmol/mg protein) while P resulted in a more modest 2% increase (T1 = 1359 ± 35, T2 = 1399 ± 54μmol/mg protein). Following exhaustive exercise (EX3), with all trials collapsed, P had significantly higher (10%) GPx activity (WPI = 6.8 ± 1.8 μmol/ml, P = 7.5 ± 1.8 μmol/ml) ($P < 0.05$).

Immune Function. Another purpose of this study was to examine if WPI supplementation was superior to casein in attenuating the normal immune alterations following exercise and energy restriction. Several studies have observed dramatic changes in immune parameters following aerobic exercise (14, 18, 19, 38, 54, 55, 68, 90). Subjects, methods, and statistics were identical to those described previously.

For both groups combined and all trials collapsed, exercise resulted in a decreased proportion of CD3+ and CD4+ T-cells by 15% and 34%, respectively ($P < 0.01$). CD8+ lymphocytes were increased following exhaustive exercise (28%, $P < 0.01$).

Consequently the CD4+/8+ ratio fell post exercise (45%, $P < 0.01$). All T-cell subsets returned to baseline after 1 h of recovery. Immediately following exercise phagocytosis decreased 8% ($P < 0.01$) while oxidative burst capacity remained unchanged. Following 1 h of recovery, phagocyctosis rebounded to baseline values while oxidative burst increased to 4% above baseline ($P < 0.05$).

For both groups combined and all exercise time points collapsed energy restriction resulted in several alterations to antioxidant status. The GSH/GSSG ratio decreased 55% following weight loss (T1 = 135 ± 12, T2 = 142 ± 19, T3 = 63 ± 7) ($P < 0.001$). LPO decreased 16% ($P < 0.01$) from T1 to T2, during weight maintenance, and then decreased a further 16% ($P < 0.01$) from T2 to T3. WBCGSH and GPx increased significantly 40% and 35%, respectively, following energy restriction.

WPI did not provide superior immune function as measured by CD3+, CD4+, CD8+ T-cell subsets, the CD4+/8+ ratio, lymphocyte proliferation, oxidative burst capacity, or phagocytic ability.

Exercise Performance. Another purpose of this study was to examine if consumption of WPI was superior to the casein placebo in improving exercise performance. Previous research has associated WPI supplementation with increased power outputs (33). We, however, did not find any increases in peak power, rate of fatigue, or total work after two weeks of WPI supplementation. The lack of improvement in performance in our study could be attributed to our shorter supplementation period (2 wks), which did not cause significant increases in GSH or could represent a true lack of

benefit of this supplement on performance. We observed higher than normal baseline GSH status in our aerobically trained subjects which may have limited further increases.

Aerobic exercise performance also was not different between groups. Time to exhaustion was highly variable and did not differ between groups over the trials. There was a strong trend for time, with both groups combined, to decrease time to exhaustion following energy restriction (260 ± 16 seconds prior and 200.5 ± 28.5 seconds post (P = .051). To our knowledge, no other research has evaluated the effect of WPI ingestion on aerobic exercise test performance. It is possible that the two week supplementation period was too short to elicit high enough increases in GSH levels to attenuate muscular fatigue during an aerobic test. Additionally, our subject population, through training adaptation, may already had optimal levels of GSH and the GSH boosting effects of WPI were ineffective. Other research has observed increased levels of GSH after endurance training in runners (17, 31, 67).

These results do not support the hypothesis that supplementation of a glutamylcysteine rich whey protein improves whole blood or lymphocyte GSH in trained aerobic athletes. WPI also did not enhance the antioxidant response to exercise as measured by the GSH/GSSG ratio, LPO, or GPx activity.

Contrary to previous research (33) WPI supplementation did not increase anaerobic power as measured during a 30 second Wingate cycle test. We observed no differences between groups for peak power, fatigue rate, or total work. The same lack of treatment effect can be said for aerobic exercise performance. Time to exhaustion, when pedaling at 90% $VO_{2peak,}$ was not different between WPI and P either before or after energy restriction.

Suggested Modifications of Research Design

There are a few modifications to the design of this study that could have been improved upon in order to lessen variability in the data or to provide more information about the observed responses.

Because the study timeline fell in with the athletes racing season the previous to the test day's activity could not be controlled. This means that some subjects, especially those coming in for Monday tests, may have raced the day before. This may have directly affected their performance and consistency during the performance tests. Future research on trained cyclists should coincide with their off-season as to allow more control over subject's activity levels.

Practice performance tests should have been conducted prior to the study. Performance data were highly variable and some subjects may have experienced a learning curve eliciting longer performance on subsequent trials. Although timing the study during the racing season decreased access to subjects, efforts to conduct practice performance tests may have increased test reliability.

The exercise test did not clearly elicit oxidative stress in our trained subjects. While pilot work on a moderately trained male and one other trained female did indicate decreases in the GSH/GSSG ratio, following the exercise test, other indicators of oxidative stress could not be measured for lack of funding at the time. The exercise test should have been piloted on trained males more representative of the study population and assayed all measures of oxidative stress.

Recommendations for Future Research

The potential for WPI supplementation to increase antioxidant status, delay fatigue, and attenuate the immune response to exercise remains a worthy topic of exploration. The aerobic athlete population represents a group of healthy individuals who consistently tax their antioxidant defenses with daily training-induced oxidative insults. As well, these individuals strive to improve muscular performance and require avoidance of illness to maintain training and to compete at a high level.

One unexpected finding from this study was a drop in the GSH/GSSG ratio following energy restriction. This was accompanied by an increase in GPx activity and a decrease in LPO. Increased GPx gene transcription has been previously observed in animals enduring periods of weight loss (83). If glutathione reductase (GR), an enzyme not measured in our study, is not also simultaneously upregulated the decrease in GSH/GSSG ratio could be explained. Future research examining the effect of weight loss on GSH status should measure both GPx and GR to gain insight on the GSH redox cycle during energy restriction.

Aerobic athletes may characteristically possess increased GSH levels due to training adaptation. This may mean that GSH boosting techniques that work in diseased and healthy untrained populations may be ineffective in trained individuals. Therefore, future research must focus on finding a WPI supplementation regimen that elicits meaningful increases in GSH levels in this specific population. A higher daily dose consumed over a longer time period may be required to observe such increases.

The GSH boosting effects of WPI supplementation were not clearly demonstrated within our subject population. Therefore, it was not possible to observe or attribute any

changes in immune function, antioxidant status, or exercise performance to WPI. It would be interesting to examine annual fluctuations in GSH levels in trained aerobic athletes. If athletes, during their competitive season, have achieved optimal GSH levels because of a training adaptation then GSH boosting at that time may be fruitless. However, if a cyclist coming off a winter break has reduced GSH levels they then may be at increased risk for opportunistic infection and may benefit from GSH boosting during the early parts of the season as they begin to train hard once again. The end of a demanding season could also potentially leave the aerobic athlete with compromised antioxidant defenses. This may be another time point in which the athlete may benefit from GSH boosting interventions. Overall, our understanding of the GSH status and seasonal fluctuations of aerobic athletes needs further attention. Then it may be possible to improve the health of athletes and their athletic performance through seasonally timed nutritional GSH boosting techniques.

During oxidative insult, a particular tissue will attempt to extrude GSSG into the circulation in order to maintain the internal cellular redox environment. As a substrate for the GPx enzymatic reaction, GSH will be oxidized to GSSG in working skeletal muscle. This process will be influenced by cellular GSH concentration and muscle fiber type. Therefore, tissue and whole blood GSH dynamics is a complicated issue. To gain further understanding of how certain muscles deal with reductions in the GSH/GSSG ratio, during aerobic exercise, future research must integrate muscle biopsies with current whole blood GSH assay techniques

Theoretical Issues Related to Antioxidant Status, Immunity, Exercise and Weight Loss

Submaximal and exhaustive exercise elicited a non-significant 9% and 7% decrease in the GSH/GSSG ratio, respectively. This indicates some level of GPx mediated oxidation of GSH in the presence of exercise induced ROS. Other studies have reported similar or greater decreases in GSH/GSSG following submaximal and exhaustive exercise (23, 79, 91). Furthermore, exercise induced increases in GPx, along with decreases in GSH and the GSH/GSSG ratio, have also been observed (4). Ultimately, how effectively GSH and GPx counteract the deleterious effects of exercise induced ROS is paramount to avoiding significant oxidative stress and subsequent cellular damage. Increases in LPO serve as an indicator of oxidative stress magnitude. Our subjects, at least from a statistical viewpoint, were able to keep LPO generation to a minimum through the concerted efforts of GSH and GPx. Enhancement of antioxidant status, either through nutritional techniques or training adaptation, should increase protection during oxidative insult. This will allow the athlete to perform higher intensity work for a longer period of time while attenuating ROS induced cellular dysfunction.

Following exhaustive exercise, levels of tGSH increased. This may be accounted for by intramuscular export of GSSG and GSH during exercise (37). While the increased GPx activity indicates intracellular conversion of GSH to GSSG, some GSSG is likely being exported from exercising skeletal muscle. Work by Sen and colleagues demonstrated that hepatic release of GSH into circulation, although accelerated during exercise, should have little effect on blood GSH or tGSH levels (76). Sen et al have also reported rapid loss and subsequent efflux of muscle cell tGSH (78). Work with hepatectomized rats further supports the contribution of muscle cells, rather than liver, to

increases in blood tGSH (31). Other research has demonstrated that, when oxygen consumption is large enough, export of GSSG from working muscle cells can contribute to increases in circulating tGSH (77). In order to maintain a favorable cellular redox environment, a working muscle cell would need to export GSSG into the circulation. As oxidative stress increases with increased oxygen consumption GSSG efflux is likely to occur and be reflected in increased whole blood tGSH as well as a decrease in the GSH/GSSG ratio.

An interesting and novel finding from our study was the concurrent decreases in both WBCGSH and CD3+ T-lymphocytes following exercise. GSH is critically important to immunocyte health. Therefore, the exercise induced decrease in WBCGSH may offer a mechanistic explanation for the compromised immuno-competence often observed following strenuous exercise. The cellular redox environment is reflective of the GSH/GSSG ratio. While this ratio could not be measured in our harvested immunocytes, the decrease in WBCGSH indicates a decrease in the immunocyte redox environment. A decreased redox environment has been shown to trigger the apoptotic process and reduced GSH concentration is associated with cell death (21). Our decrease in total lymphocytes (CD3+) following exercise was primarily because of decreased CD4+ T-cells, which are responsible for initiating specific B-cell responses. The exercise-induced compromised immunocyte antioxidant status, which likely creates a decreased redox environment, may be responsible for the increased occurrence of illness often observed following prolonged, strenuous endurance events (48).

We found lower phagocytic and oxidative burst capacity following exercise in our trained subjects. After 1 h of recovery, phagocytosis remained depressed while oxidative

burst function improved above baseline. This indicates compromised non-specific immune function following exercise. Although increased oxidative burst capacity after 1 h of recovery would be associated with enhanced killing capabilities this enhancement would be preceded by a decreased ability to engulf foreign or no longer needed proteins. The athlete enduring hard training and/or competition can expect both decreased specific and non-specific immune function following acute exercise. Increasing immunocyte health by maintaining or enhancing the cellular redox environment, through GSH boosting nutritional techniques, should aid in keeping athletes healthy following bouts of strenuous work.

This study suggests that periods of energy restriction are associated with increased antioxidant enzyme activity, decreased production of LPO, and decreased GSH/GSSG. Taken collectively, these data indicate more efficient and active antioxidant defenses. While the decrease in GSH/GSSG may suggest an increased oxidant burden, periods of caloric restriction are not characterized by increased ROS production but rather by enhanced ROS metabolism (83). This stimulated GSH redox cycle is reflected in an increased GPx activity. If glutathione reductase is not equally upregulated then a drop in the GSH/GSSG ratio should, theoretically, ensue. The athlete in training must contend with the added oxidative stress of chronic exercise but weight loss alone appears to enhance the efficiency of antioxidant defenses.

In addition to enhanced antioxidant capabilities, we also observed some indication of increased immunocyte health following weight loss. WBCGSH increased as well as phagocytic capabilities. Professional phagocytic cells, such as neutrophils and monocytes, represent our body's first line of defense against invading foreign pathogens

and microorganisms. Increased WBCGSH should help increase the immunocyte redox environment resulting in enhanced immuno-competence and an uncompromised or heightened phagocytic response. Considering the exercise induced decrease in specific immune function the dieting athlete, in training, may experience enhanced non-specific immunity during times of energy restriction. These alterations may provide some level of protection from the deleterious effects of training while in a catabolic state.

Practical Implications of Exercise, Antioxidant Status and Immunity

Aerobic exercise leads to alterations in both antioxidant status and immune function in trained aerobic athletes (23, 28, 74-77). A submaximal bout lasting as little as 45 minutes followed by a short (1-7 min) exhaustive exercise trial increased tGSH and GPx activity while effecting several T-cell subsets and phagocytic capabilities. Chronic aerobic training is also associated with a training-induced increase in intracellular GSH (17, 31, 67). Competent antioxidant defenses will be crucial for adequate protection from oxidative stress and subsequent ill health ramifications. However, within the parameters of this study, WPI cannot be considered a viable GSH boosting agent in trained individuals. Although significant increases in whole blood GSH were not realized a longer intervention may have yielded meaningful benefits.

Immune function is also altered with acute exercise. CD4+ as well as total T-cells in peripheral blood decrease immediately following exercise. Although they returned to baseline values within one hour of recovery this still represents a window of opportunity for infectious agents to gain a foothold. The decreased CD4+ T-cells in blood indicate a potential decreased ability to activate specific B-cell populations. While CD4+ T-cells are

diminished following exercise, CD8+ cytotoxic T-cells are increased. Taken together, these two immune function alterations mean increased elimination of antigen displaying cells (ie virus-infected cells by CD8+ T-cells) with decreased specific CD4+ T-lymphocyte B-cell activation. WBCGSH decreased with exercise, possibly contributing to the decrease in total T-cell number. Overall, the athlete in training should be concerned about immunocyte health as well as maintaining competent antioxidant defenses to combat the consistent oxidative insults of a regular training program and/or competition.

WPI supplementation resulted in superior lymphocyte proliferative capacity, following exercise, when compared to casein. The ability of lymphocytes to proliferate when exposed to an antigen is critical in the healthy processing of foreign proteins and subsequently avoiding illness. The athlete involved in heavy training or competition is at an increased risk for opportunistic infection following an exercise bout. Therefore, the athlete may consider adding WPI supplementation to their normal diet in order to boost the ability of lymphocytes to proliferate and, theoretically, decrease the chances of becoming sick.

Overall Conclusions

Although we did not observe clear evidence of oxidative stress, the generation of ROS associated with aerobic exercise training and competition places the athlete at increased risk of infection. GSH plays a critical role in antioxidant protection and keeping immunocytes healthy. Furthermore, attenuation of exercise–induced oxidative stress has enhanced muscular performance in the lab and has potential to increase sports performance in a real world setting. The particular WPI used in the present study failed to

elicit statistically higher whole blood tGSH than a casein placebo following 2 wk supplementation. However, WPI was able to enhance one measure of immune function following exercise. At this point, the GSH boosting and sports performance enhancing attributes of WPI supplementation has potential but remains unrealized in aerobic athletes.

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Pilot study

Pilot Methods

Previous research has included Immunocal® supplementation to subjects for varied lengths of time and doses to achieve increases in GSH (24, 29). Thus, little is known about the necessary dose and time required to boost blood or lymphocyte GSH levels. To address this issue a pilot study was performed to determine the dose and time required to elevate GSH concentrations of whole blood and white blood cells. Eight healthy males were recruited to ingest Immunocal® for 28 days. Four subjects consumed 20 g/day and the other four 40 g/day. Baseline and weekly blood draws were performed for the analysis of whole blood and white blood cell GSH concentrations.

Subjects were provided with mixing instructions and preparation recommendations. Compliance and adverse effects forms were turned in weekly at the time of blood collection. No prior (within 6 months) or current consumption of protein supplements or any other product (i.e. lipoic acid) known to possibly effect GSH levels was allowed. Blood was drawn by a certified medical technician skilled in phlebotomy or by a researcher trained by the lab technician.

Pilot Results

Consumption of Immunocal® was well tolerated by most subjects. Two subjects consuming 40 g/day experienced consistent uncharacteristic headaches soon after consumption and withdrew from the study. These two were replaced and no other problems were expressed except for occasional annoyance of the mixing procedure. In general the taste of Immunocal® was not enjoyed but was well accepted as the study

progressed. Results for whole blood and white blood cell GSH concentrations are presented in Figures 4-7. Results are discussed as averages without statistical evaluation of differences due to the low subject number within group. In the 20 g/day group whole blood GSH increased 5.7% within one week and increased by 10.5% by week two which was maintained throughout the remaining two weeks. White blood cell GSH increased 11.4% within one week and was elavated throughout the remainder of the study. The 40 g/day group displayed a 25% increase in whole blood GSH within the first week of supplementation. This steadily decreased to a 17.7% increase by week four. White blood cell GSH concentration increased by 13.6% within one week and further increased by 21% by week three.

Adapting the commercial whole blood GSH kit (GSH/GSSG-412 assay, OxisResearch, Portland, OR) was required to measure GSH in a WBC suspension. After WBC harvest using Vacutainer Cell Preparation Tubes (Vacutainer CPT, Becton Dickinson and Co., Franklin Lakes, NJ), WBC were counted and suspended in 200 µl PBS. After several manipulations of assay protocol it was determined that to obtain detectable GSH levels 100 µl of sample must be used, twice the amount used for a whole blood sample. Then 1.5 ml of provided assay buffer was used instead of the normally required 3 ml. These customized samples were pH tested and were equivalent to non-customized samples. These changes allowed adequate concentration of GSH to be detected by the assay. Examples and further explanation are provided in Appendix A.

Figure 6:

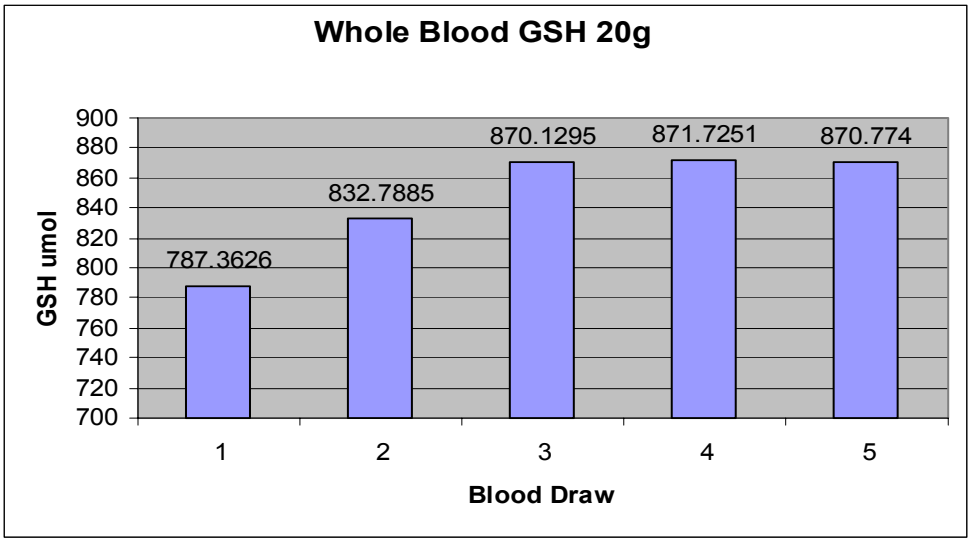


Figure 7:

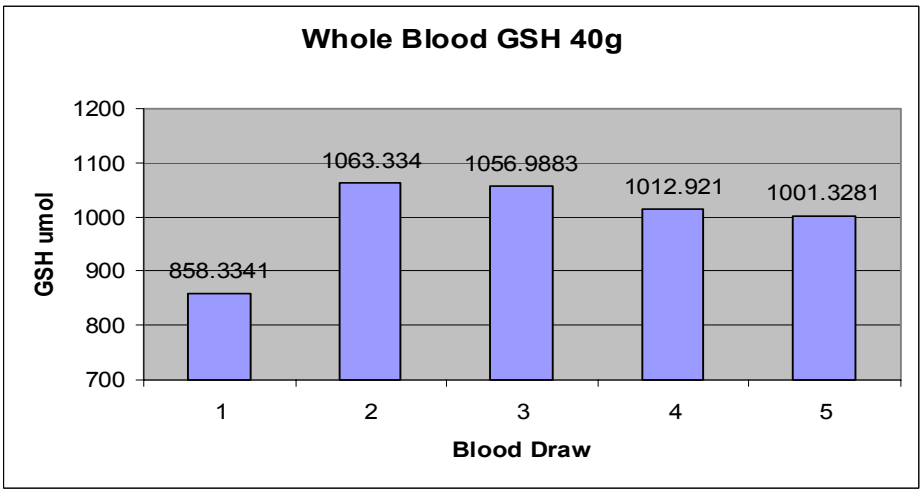


Figure 8:

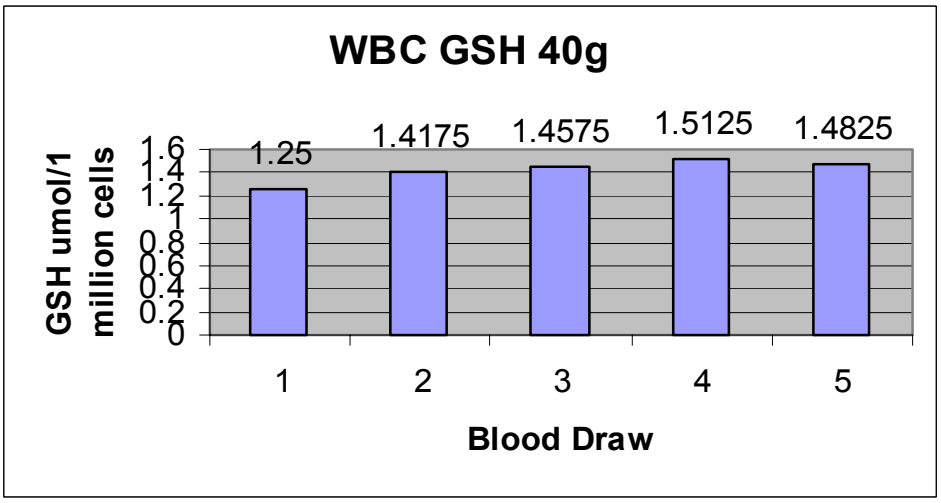
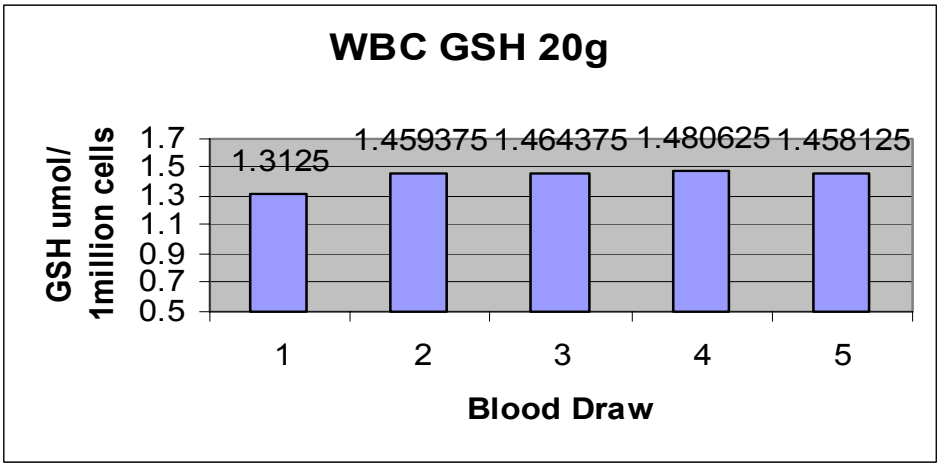


Figure 9:


WBC GSH 20g
GSH umol/ 1million cells
1.7
1.5
1.3
1.1
0.9
0.7
0.5
1.3125
1.459375
1.464375
1.480625
1.458125
1
2
3
4
5
Blood Draw

Appendix A

Sample Handling Procedures

Labeling Procedures

*All storage and microcentrifuge (mc) tubes labeled as follows:

-Intended assay (ie GSH/GSSG, GPx)
-Subject ID #
-Test # (1, 2, or 3)
-blood draw # (BD 1, 2, 3, or 4)

GSH/GSSG 412 WHOLE BLOOD ASSAY:

GSSG aliquot-

1) 10 µl of M2VP placed at bottom of mc tube.
2) From green top tube carefully but quickly pipette 100 µl whole blood into mc tube, gently mix (try to avoid air bubbles).
3) Carefully placed in 50/50 dry ice-ethanol solution to "snap" freeze. Try to leave mc tube in upright position if possible.
4) Store at -70°C.

GSH aliquot-

1) From green top tube place 200 µl whole blood in mc tube.
2) Carefully placed in 50/50 dry ice-ethanol solution to "snap" freeze. Try to leave mc tube in upright position.
3) Store at -70°C.

White Blood Cell (WBC) GSH:

1) Following cell separation (see specific instructions), washing, and counting (see specific instructions) cells are suspended in 200 µl PBS in mc tube.
2) Store at -70°C

Example and Explanation of Buffer Manipulation for GSH/GSSG 412 Assay

std1	**std2**	std3	std4	std5	std6	blank
0	**0**	0.002	0.012	0.019	0.053	0
0.012	**0.009**	0.025	0.049	0.117	0.22	0.011
0.023	**0.021**	0.048	0.085	0.211	0.382	0.024
0.033	**0.037**	0.072	0.122	0.305	0.547	0.035
0.044	**0.054**	0.094	0.159	0.4	0.711	0.041
0.056	**0.07**	0.117	0.196	0.495	0.871	0.055
0.067	**0.086**	0.141	0.233	0.592	1.029	0.066
0.078	**0.103**	0.165	0.27	0.688	1.19	0.078
0.09	**0.12**	0.19	0.307	0.785	1.354	0.09
0.1	**0.138**	0.213	0.346	0.882	1.509	0.098

50ulw/1.5AB		50ulw/1AB		50ulw/.5AB		**100ul w/1.5AB**	
-0.017	-0.014	-0.013	-0.018	-0.01	-0.014	**-0.015**	**-0.024**
0	0.005	0.007	0.002	0.017	0.015	**0.006**	**-0.003**
0.017	0.022	0.027	0.023	0.042	0.044	**0.024**	**0.018**
0.035	0.042	0.046	0.045	0.069	0.073	**0.045**	**0.039**
0.052	0.06	0.067	0.065	0.095	0.102	**0.064**	**0.06**
0.069	0.079	0.087	0.087	0.122	0.131	**0.085**	**0.081**
0.085	0.098	0.108	0.109	0.15	0.16	**0.107**	**0.103**
0.103	0.118	0.129	0.131	0.177	0.19	**0.128**	**0.126**
0.121	0.137	0.15	0.153	0.204	0.22	**0.151**	**0.148**
0.138	0.156	0.171	0.176	0.232	0.25	**0.173**	**0.171**

- Tables provide a sample of a set of standards and four examples of sample and assay buffer manipulations to achieve safe detectable GSH WBC levels while maintaining proper pH. Standard 2 (std2 in bold) is the lowest standard with a concentration of GSH. The 100µl of sample with 1.5 ml of assay buffer (100ul w/1.5AB in bold) provided easily detectable levels with no change in pH. Other examples were either close to undetectable and/or lowered pH levels.

GPx OXYSCAN ASSAY:

1) From green top tube pipette 1ml of whole blood into mc tube.
2) Centrifuge @ 2500 x g for 5 minutes @ 4°C.
3) Discard supernatant.
4) Wash cells 3 times in ice cold 0.9% NaCl.
5) Resuspend packed cells in 4 volumes of ice cold deionized water
6) Vortex for ~5 seconds.
7) Store at -70°C.

LPO ASSAY:

1) From CPT tube remove 2 ml of plasma after first spin.
2) Aliquot plasma in 1ml amounts in 2 mc tubes.
3) Store at -70°C.

PROTEIN ASSAY:

1) From green top tube pipette ~50 µl whole blood into mc tube (5 µl needed).
2) Store at -70°C.

HEMATOCRIT:

1) From green top tube pipette ~300-500 µl into mc tube (25-35 µl needed).
2) Leave at room temperature or on ice until run (must be run the same day, do not freeze).
3) See specific hematocrit procedure sheet for instructions.

IMMUNE MEASURES

LYMPOCYTE PHENOTYPING:

1) From green top tube pipette ~500 µl whole blood into mc tube (100 µl needed).
2) Store at room temperature until fixing.

PHAGOCYTOSIS AND OXIDATIVE BURST:

1) From green top tube pipette 2 ml whole blood into mc tube.
2) Store at room temperature or on ice until fixing (phagocytosis) or DCF assay (oxidative burst).

Appendix B

Detailed Methods

GSH/GSSG 412 Assay (Oxisresearch, Portland, OR)

GSSG sample:

1. Pipette 10µl M2VP to a microcentrifuge tube.
2. Collect blood sample in lithium heparin treated tube.
3. Immediately add 100µl whole blood to the bottom of microcentrifuge tube and mix gently.
4. Snap freeze in 50-50 dry ice/ethanol mixture
5. Store at -70°C
6. Thaw sample and immediately mix, incubate at room temperature for 2-10 minutes.
7. Add 290µl cold 5% MPA to the tube (1/4 dilution of original sample).
8. Vortex sample for 15-20 seconds.
9. Centrifuge at 1000 x g or greater for 10 minutes.
10. Add 50µl MPA extract to 700µl GSSG buffer (1/15 dilution of the acid extract).
11. Place the diluted extract on ice until use (final sample dilution is 1/60).

GSSG Blank:

1. Add 50µl MPA to 700µl GSSG buffer (1/15 dilution of the acid extract).
2. Place the diluted MPA on ice until use (final sample dilution is 1/60).

GSH Sample:

1. Carefully add 50µl of whole blood to the bottom of a microcentrifuge tube.
2. Freeze the sample at -70°C. (sample is stable for at least 30 days at -70°C).
3. Thaw sample and immediately mix.
4. Add 350µl cold 5% MPA to the tube (1/8 dilution of original sample).
5. Vortex the sample for 15-20 seconds.
6. Centrifuge at 1000 x g or greater for 10 minutes.

7. Add 50µl MPA extract to 3 ml Assay Buffer (1/61 dilution of the acid extract).
8. Place diluted extract on ice until use (final sample dilution is 1/488).

Assay:

Reagent preperation:

NADPH: Just prior to use, reconstitute the lyophilized NADPH reagent with 7.5ml of assay buffer. The reconstituted NADPH reagent is stable for 6 hours at room temperature.

Assay Buffer: Reconstitute the lyophilized powder with 650ml of deionized water. The reconstituted reagent is stable at 4°C for the life of the kit.

Metaphosphoric Acid (MPA): Prepare fresh daily. Weigh 1g MPA and dissolve in 20ml deionized water.

MPA and NADPH reagents are intended for same day use following reconstitution.

Standards are ready to use.

1. Add 200µl of standards, blank, or samples to the cuvettes.
2. Add 200µl of chromogen to each cuvette.
3. Add 200µl of enzyme to each cuvette.
4. Mix and incubate at room temperature for 5 minutes.
5. Add 200µl of NADPH to each cuvette.
6. Record the change of absorbance at 412nm for 3 minutes.

Calculations:

The calculation of the GSH and GSSG concentrations and the GSH/GSSG ratio requires four steps:

1. Determination of the reaction rate.
2. Construction of calibration curves.
3. Calculation of the analyte concentration.

4. Calculation of the GSH/GSSG ratio.

Determination of the reaction rate:

The change in absorbance at 412nm is a linear function of the GSH concentration in the reaction mixture is described by the following equation of a line:

$$A412 = \text{slope} \times \text{minutes} + \text{intercept}$$

where the slope of the regression equation is equal to the rate.

Construction of calibration curves:

The GSH/GSSG-412 assay uses a six-point standard curve for both GSH_t and GSSG determinations. The net rate is the difference between the rate at each concentration of GSH and the Blank rate.

Calculation of the analyte concentration:

The general form of the regression equation describing the calibration curve is:

$$\text{Net Rate} = \text{slope} \times \text{GSH} + \text{intercept}$$

Therefore, to calculate the analyte concentration from the GSH calibration curve:

$$\text{GSH} = \text{Net Rate} - \text{intercept/slope} \times \text{dilution factor}$$

Determination of the GSH/GSSG ratio:

The GSH/GSSG ratio is then calculated by dividing the difference between the GSH_t and GSSG concentrations (reduced GSH) by the concentration of GSSG.

$$\text{Ratio} = GSH_t - 2GSSG/GSSG$$

Adaptation of GSH/GSSG 412 assay to measure white blood cell GSH:

White blood cell GSH will be determined after lymphocyte separation using Vacutainer Cell Preparation Tubes (Vacutainer CPT, Becton Dickinson and Co., Franklin Lakes,

NJ). After washings, lymphocytes will be resuspended in 200µl PBS before being assayed. Follow directions for GSH assay with the following modifications:

1. Use 100µl of WBC suspension instead of 50µl sample.
2. Use 1.5ml of assay buffer instead of 3ml.

Lipid Hydroperoxide LPO-560 Assay (Oxisresearch, Portland, OR)

Store reagents tightly closed at 2-8°C in the dark. Do not freeze. The color developer is stable for 10 days after opening if stored tightly closed at 2-8°C. when stored properly, the remaining reagents are stable until expiration date printed on the box label. The prepared working reagent is stable for 24 hours at 2-8°C. Keep on ice during use. Prepare working reagent by mixing 1 volume of color developer with 100 volumes of chromogen. Prepare only the volume needed for the day's assays.

1. For each sample (including controls), label two microcentrifuge tubes, one as TEST, the other as BLANK.
2. Add 10µl of enzyme to each tube.
3. Add 90µl of sample Note: for reagent control use buffer as sample.
4. Mix gently and incubate at room temperature for two minutes.
5. a) Add 10µl of reducing agent into each sample BLANK tube.

   b) Add 10µl of deionzed water to each sample TEST, reagent control.
6. Cover and mix the sample by vortexing. Incubate at room temperature for 30 minutes.
7. Add 900µl of working reagent to each tube
8. Cover and vortex the sample for 30 seconds, then incubate at room temperature for 60 minutes.

9. Centrifuge at 10,000-12,000 x g for 10 minutes to remove all flocculated materials.
10. Zero spectrophotometer with deionized water.
11. Transfer the supernatents to specrophotometric cuvettes and measure the absorbance at 560 nm.

Calculations:

1. Subtract each sample BLANK absorbance value from the sample TEST value to obtain the net absorbance (Net A_{560}).
2. The concentration of hydroperoxides in the reaction mixture is calculated from the net absorbance and the hydroperoxide apparent molar extinction coefficient of 4.31×10^4 M^{-1} cm^{-1} using the equation:

$$[LOOH] = (\text{Net } A_{560}/0.0431\ \mu M^{-1}\ cm^{-1}) \times \text{dilution factor} \quad (11.2)$$

GPx-340 Oxyscan Assay (Oxisresearch, Portland, OR):

Sample collection procedures (erythrocyte lysates):

1. Draw blood into an anticoagulant tube and mix by inversion.
2. Centrifuge at least 500 ml whole blood at 2500 x g for 5 minutes at 4°C.
3. Discard plasma supernatant, wash cells 3 times in ice cold 0.9% NaCl
4. Resuspend the packed cells in 4 volumes of ice cold deionized water, vortex well. Pellet can now be stored at -70°C for late use.
5. Centrifuge at 10,000 x g for 5 minutes at 4°C.
6. Collect the supernatant for assay.
7. Dilute supernatant 1/100 with sample diluent provided.

Oxyscan Pump Preparation:

1. Insert reagents into the proper wells:

t-BOOH Working Solutio	Pump #1
Empty	Pump #2
Reconstituted NADPH Reagent	Pump #3
Empty	Pump #4

2. Insert proper tubing in to the reagents bottles.
3. Lift the reagent arm and place the waste cup beneath the tubing exit.
4. Ensure the pump cover is in place. Press PUMP ENGAGE/RELEASE to engage the pumps. Press PRIME. Select PRIME ALL.
5. When the screen changes to the step pumps. Press each pump number to pipet 100 ml of fluid to confirm tubing is primed.
6. Press ESC.

Instrument preparation:

1. Press UTILITY and scroll down to REPLICATE SELECTION.
2. Choose number of replicates.
3. If running an assay calibration curve with the samples, perform steps 4 and 5.
4. Scroll down to the ASSAY CAL screen and choose the CONC.
5. Choose GPx, enter the calibration concentration values for the Oxyscan GPx-340 Calibrator Kit from its package insert.
6. Return to the main menu.

Cuvette/carousel preparation:

1. Pipet 200 ml deionized water into the first two p[ositions of the carousel to serve as blanks.
2. If running an assay calibration curve with the samples, perform step 3.
3. Starting with position 3, pipet 50 ml of each calibrator level into the appropriate number of wells equivalent to the replicates to run.
4. Pipet 50 ml of each dilute sample in the replicates specified into the following wells.
5. Insert cuvettes into carousel, starting with the first blank position 1.

Assay run:

1. Press ASSAYS and select YES or NO for assay calibrators during run.
2. Select GPx.
3. Enter the number of samples to test, not total number of replicates.
4. Press SAMPLE ID to enter numerical IDs for the samples including calibrators. Press ESC to exit the menu.
5. Ensure cover is down.

Calculation of results:

1. Oxyscan instrument reports the concentration of the sample placed in the cuvette.
2. The result may be corrected for sample preparation dilution.

White Blood Cell Separation

1 Invert CPT tubes 5 times to mix following venipuncture

2 Store upright at room temperature (2 h max)

3 Spin tubes at ~2800-2900 rpm (brake off throughout) for 20 min (if no band appears spin additional 5 minutes)

4 Draw off plasma layer carefully, do not disturb white buffy coat. Leave a few mls above band.

5 Carefully remove white blood cell layer and place into a 15ml conical centrifuge tube

6 Fill tube to 15 ml with PBS (remember to reconstitute, 10 ml of concentrated PBS + 90 ml deionizd water)

7 Invert 8 times

8 Spin at 1200 rpm for 15min

9 Pour of supernatent

10 Fill tube up to 10 ml with PBS (mix with glass pipette)

11 Spin at 1200 rpm for 10 min

12 Pour of supernatent and carefully remove remainder with glass pipette

13 Resuspend to 200 µl with PBS and place in 2ml labeled storage mc tube

14 Count cells using dilution set up from data collection sheet or Coulter Counter

Coulter Counter Directions

1 Remove blue washing vial (pull down w/ lever)

2 Get new vial, fill with 10 ml Isoflow (1 squirt) and push into up position

3 Turn machine on (START, wait 1-2 min)

4 One squirt of Isoflow in each vial (10 ml)

5 mix in 40 ul of sample to vial

6 Add 4 drops of Zap Oglobin (small yellow solution in bottle)

7 Add one more squirt of Isoflow

8 Check initial count (background) (should be < 100,000, if not wash)

9 Install sample vial and hit START (gives count per ml)

10 Run plain Isoflow to clean (until background count is < 100,000)

11 Run with blue solution to clean (count doesn't matter)

12 Turn machine off

**** NEVER DO ANYHTING WHREN SCREEN SAYS: "RESETTING MEASURING SYSTEM"****

Reagents needed for Immune Measures

Red Blood Cell Lysing Reagent:
8.26 g NH_4Cl
1.0 g $KHCO_3$
0.037 g Na_4 EDTA
1.0 L deionized distilled H_2O
pH to 7.2
store at 4ºC for up to 3 months

Phosphate Buffered Saline:
PBS, pH 7.4 order #P3813 (.01 M PBS at 25ºC) (0.138 M NaCl, 0.0027 M KCl)
(Sigma-Aldrich, St. Louis, MO)

Monoclonal antibody cocktail:
Mouse Anti CD3 Tri-color labeled (TC)/
Mouse Anti CD4 R-phycoerythrin labeled (R-PE)/
Mouse Anti CD8 Fluorescein (FITC)
Catalog # CD3-4-8-B (200 tests, 3.0 ml)
(Caltag Laboratories Inc, Burlingame, CA)

KRH Gelatin:
97 ml H_20
10 ml 9% NaCl
616 µl 1M KCl
600 µl 0.5M $MgSO_4$
20 ml 0.1M Hepes-TEA pH 7.4
0.108 g Knox unflavored gelatin

PBS Gelatin EDTA
100 ml PBS (Sigma)
0.1 g Knox unflavored gelatin
0.1 g EDTA disodium salt

T-cell subset (CD3+, CD4+, CD8+) analysis (Caltag Laboratories, Burlingame, CA)

1. Pipette 100µl whole blood into 12 x 75 polypropylene tubes.

2. Add 2.5 µl Caltag CD3/CD4/CD8 monoclonal antibody and 180 µl PBS.

3. Gently mix.

4. Incubate at room temperature for 15 min in the dark (cover with aluminum foil).

5. Add 100µl of lysis solution.

6. Incubate at room temperature for 10 min in the dark.

7. Add 3ml of deionized water (room temperature) and mix.

8. Centrifuge at 400 x g/10 min and remove supernatant (pour out and gently tap on paper towel).

9. Add 3ml PBS and repeat step #8.

10. Resuspend in 500µl of fixing solution (paraformaldehyde).

11. Cover with foil and refrigerate until flow cytometric analysis.

WBC Isolation Protocol (WIP)

1. Add 500µl whole blood to 15 ml conical tubes.
2. Add 3 ml of lysing solution to each sample, mix, and incubate at room temperature for 5 min.
3. Centrifuge at 480 x g/5 min.
4. Pour off supernatant.
5. Add 15 ml PBS to each tube, mix well.
6. Centrifuge at 480 x g/5 min.
7. Pour off supernatant.
8. Repeat steps 5-7.
9. After pouring off supernatant add 500 µl PBS.

Phagocytosis Assay Protocol

1. Obtain phagocytic cells from whole blood as described in WIP.

2. Resuspend cells to a final concentration of 1 x 10^6 cells/ml in HBSS.

3. Place 500 µl (1 x 10^6 cells/ml) into:
   -Tube labeled cold (C)
   -Tube labeled warm (W)

4. Centrifuge C and W tubes at 480 x g/10 min.

5. Pour off supernatent, add back 500µl KRH gelatin.

6. To each tube add:
   -50 µl FBS
   -10 µl beads
   - vortex gently

7. Incubate C in ice bath (covered with parafilm) for 60 min.
   Incubate W in 37^0C water (covered with parafilm) bath for 60 min.

8. Remove from ice and water bath; add 1.5 ml cold PBS gelatin.

9. Centrifuge at 480 x g /10 min.

10. Pour off supernatent; add 300 µl paraformaldehyde.

11. Place parafilm securely over tubes and refrigerate until flow analysis.

Oxidative Burst (DCF) Assay Protocol

1. Obtain phagocytic cells from whole blood as described in WIP.

2. Resuspend cells to a final concentration of 1 x 10^6 cells/ml in HBSS.

3. Add 500 µl (1 x 10^6 cells/ ml concentration) into:
-1 tube labeled C for "control"
-3 tubes labeled S for "stimulated"
per subject sample.

4. Add 1µl/ml of 5 mM DCF-DA to each tube.

5. Incubate cells in a 37ºC water bath for 15 minutes.

6. After incubation, add 10 µl of PMA to tubes labeled "S". Place an equal volume of HBSS into tubes labeled "C".

7. Incubate tubes in a 37ºC water bath for 10-15 minutes.

8. Analyze cells on a flow cytometer set to emit at 525 nm.

9. Calculate net % fluorescence by subtracting the % fluorescence emitted by "C" samples from the % fluorescence emitted by "S" samples.

Lymphocyte Proliferation Assay Protocol

1. Collect whole blood in Vacutainer® CPT tubes (w/sodium heparin) and centrifuge at 1120 x g/20 min.

2. Remove WBC layer and place in 50 ml conical tubes. Centrifuge at 600 x g for 10 min. Discard supernatant (plasma).

3. Wash remaining WBC pellet by adding 10 ml of RPMI 1640 medium and centrifuge at 600 x g for 8 min. Discard supernatant.

4. Repeat step 3.

5. Add complete media to the precipitated WBCs to obtain a final concentration of 1 x 10^6 cells/ml.

6. A sterile 96 well plate is needed to culture the WBCs.

7. Design the plate as follows:

-In the first column of the plate (BLANK) add 200 µl of complete media /well and no WBCs.

-In the second column (UNSTIMULATED CONTROL) add 100 µl of the 1 x 10^6 cell suspension and 100µl complete media/well with no mitogen.

-In columns 3-12 add 100 µl of the 1 x 10^6 WBC suspension and 100 µl of the following mitogenic (Con-A) concentrations (final volume = 200 µl):

-5, 10, and 20 µg/ml prepared in complete media/well.

8. Incubate plates in a 37ºC, 5% CO_2 chamber under humidified conditions for 48 h.

9. After 48 h incubation add 20µl of Alamar Blue to each well, return to CO_2 chamber, and incubate an additional 24 h.

10. Measure absorbance at 570 and 600 nm using an ELISA plate reader. The absorbance at 570 nm and 600 nm determines the optical density (OD) of the Alamar Blue color indicator.

11. Subtract the OD at 600 nm from the OD at 570 nm. This determines the true absorbance (specific absorbance) and reflects proliferation.

12. Subtract the specific absorbance of unstimulated cells (media alone) from the specific absorbance of cells incubated with Con-A to yield Δ-specific absorbance.

Appendix C

Request for Approval of Research Proposal
Department of Human Nutrition, Foods, and Exercise
Virginia Tech

TITLE: Value of Whey Protein Isolate on Glutathione Status, Exercise Performance, and Immunity in Men During Energy Balance and Restriction

FACULTY INVESTIGATORS:

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STUDENT INVESTIGATORS:

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Doctoral Graduate student
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I. Literature Review and Justification of Project

Free radicals are produced during exercise and may be associated with fatigue.

The elevated use of oxygen that occurs during aerobic exercise has been shown to cause an increase in production of reactive oxygen species (ROS, e.g. hydrogen peroxide, superoxide) within the active muscle (McArdle, 2001). These compounds can be managed by antioxidants within the body such as glutathione, ascorbate, etc. However, production of ROS at a level beyond the capacity of endogenous antioxidants can result in damage to various lipid, protein, and genetic compounds.

Production of ROS is often measured indirectly, using the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG). Most studies show that this ratio falls during aerobic exercise (e.g. Gohill et al., 1988; Viguie et al., 1993) indicating ROS generation.

A side effect of ROS within the muscle may be acceleration of fatigue. Barclay and Hansel (1991) showed that free radicals enhanced the rate of fatigue of an isolated rodent muscle. Several other studies performed in rodents showed that drugs used to alter cellular GSH influenced rate of fatigue from exercise (Kramer et al 1993, Sen et al 1994a). A single study has an intravenous drug known to increase GSH content in humans; electrically but not voluntary muscle fatigue was delayed (Reid et al, 1994). However, the drug caused multiple side effects and is not well tolerated.

Whey protein isolate may increase glutathione and delay fatigue.

These findings suggest that increasing cellular glutathione could improve the body's ability to squelch ROS and delay fatigue. As an increase in glutathione cannot be achieved through consumption or even intravenous injection, because the compound cannot pass through cell membranes, most efforts to enhance glutathione quantity has focused on boosting the blood concentration of cysteine, as intracellular synthesis of glutathione is limited by the availability of this amino acid. A dietary cysteine donor, whey protein isolate, has been shown in clinical situations to boost plasma glutathione levels between 25 and 44% over just 2 weeks of supplementation (Micke et al, 2001).

A study by Lands et al (1999) showed a 35.5% increase in lymphocyte GSH as well as a 13% increase in peak power and total work performed during a maximal cycling bout of 30 s for subjects consuming 20 g of a whey protein isolate (Immunocal) for three months. Although these are exciting results with a dietary supplement, there are several questions that remain due to the design and measurements used in that study. For example, the use of a 30 s maximal exercise test is an unusual one to use for an evaluation of a product thought to be effective due to elevation of antioxidant capacity. Most studies do not find that a brief, intense exercise like this is likely to be limited by oxidative products. Oxidation of GSH is not observed with brief, intense exercise (Sastre et al 1992). Although this study validated an increase in total GSH, they did not include a measure of oxidation of GSH caused by the exercise. Thus, the mechanism for an improvement in performance cannot be clearly ascribed to higher GSH. A more appropriate exercise test (i.e. more prolonged) would be one that has been shown by others to cause oxidative stress, as illustrated by oxidation of GSH. In order to identify glutathione boosting as the mechanism for a performance effect, it would be important to measure GSH/GSSG ratio to determine whether this ratio is changed during the exercise and if the change is muted by the Immunocal supplement.

Whey protein isolate may affect lean tissue mass.

Lands et al. (1999) also reported that the Immunocal supplement increased lean tissue mass in the healthy men over 3 months. However, the subjects consuming the Immunocal also reported increased physical activity during the experimental period compared to the placebo, potentially confounding the improved body composition finding. Limited other research supports the potential value of increased blood cysteine on lean tissue. Low plasma cysteine concentrations predicted losses in lean tissue consequent to a weight training program in healthy individuals (Kinscherf et al, 1996). Observations in HIV patients also show that low cysteine concentrations exist prior to tissue wasting (Droge and Holm, 1997; Hack et al, 1997). More research is warranted to determine the effect of a cysteine donor like whey protein isolate on lean tissue mass.

Many people self-impose a catabolic state with energy restriction to lose weight. Studies from our laboratory have shown that dieting causes loss of lean tissue as measured by net nitrogen loss in the urine (Walberg et al, 1988). The potential value of whey protein isolate on lean tissue loss during energy restriction has not been studied.

Whey protein isolate may affect immunity

Whey protein has been shown by several research groups to enhance the immune system in mice (Parker and Goodrum, 1990; Wong and Watson, 1995). The mechanism for improved immunity is hypothesized to be an increase in glutathione levels in immunocytes. Patients with HIV have reduced glutathione levels, along with impaired immunity. Three months of whey protein supplementation has been shown to improve immune status (i.e. CD4+/CD8+) in such patients (Bounous et al, 1993). Strenuous exercise, in addition to depleting GSH stores via elevated oxygen usage, has been shown to reduce immune response and increases susceptibility to illness in humans (Neiman and Pederson, 1999). Although, whey protein isolate has been used in clinical situations to boost glutathione in order to improve immunity, whether whey protein can reduce the depression in immunity resulting from hard exercise has not been adequately studied.

Other studies have shown a reduction in tissue GSH content during food restriction in rats, suggesting an increased vulnerability to oxidative stress (Leeuwenburg and Ji, 1996). This oxidative stress may be linked to immunity. Limited but provocative studies have shown an impairment of immune response in overweight individuals (Nieman et al, 1996) and athletes (Kono et al, 1988) who underwent energy restriction.

Statement of the problem

In summary, whey protein isolate has been shown to enhance immunity (Bounous et al, 1993), boost blood cysteine, and increase cellular glutathione levels (Bounous, 2000). Although mounting concentrations of ROS have been suggested to accelerate fatigue, increased glutathione levels via whey protein isolate consumption have yet to be adequately investigated as a mechanism for delaying the onset of fatigue or as a way to reduce the depression in immunity resulting from strenuous exercise. While various studies suggest that the elevation of cysteine may reduce loss of lean tissue in catabolic states (Kinscherf et al, 1996), none have investigated whether whey protein consumption may have special value to individuals who are dieting but want to maintain lean body mass. This study will examine the value of whey protein isolate on glutathione status, exercise, and immunity during energy balance and restriction.

II. Procedures

Subject selection

Twenty-eight well-trained, healthy men or women (likely cyclists) will be recruited for participation as subjects (eight for the pilot study, twenty for the experimental study). We anticipate recruiting subjects via posted fliers and e-mail announcements. Those who respond will be told of the general plan for the study. Those still interested will be invited to a group or an individual session to hear the details of participation and potential risks. They will be given a chance to ask any questions. Those still interested will receive a copy of the informed consent. Those who return this

signed document will be considered during subject selection. Subjects will undergo a health screening and will be excluded using the criteria of the American College of Sports Medicine (ACSM) for "low risk" for exercise participation and testing. According to ACSM, individuals at low risk can undergo moderate or vigorous exercise without medical evaluation and approval. "Low risk" is defined as men less than 45 years and women less than 55 years who have no symptoms of cardiopulmonary disease and have no more than one risk factor (defined by ACSM) for heart disease (family history, cigarette smoking, hypertension, high blood cholesterol, diabetes, obesity- BMI>30, or sedentary lifestyle). Additional exclusionary criteria related to the low calorie diet and supplement consumption include: eating disorders, food allergy. Subjects who have injuries or orthopedic limitations that affect their ability to do the exercise will be excluded. Finally, those who have a fear of blood withdrawal with needles will not be included in the study (medical screening forms attached).

Pilot study

A pilot study will be performed to determine the amount of time and dose required to significantly boost plasma GSH content with Immunocal supplementation in this population. Eight subjects (men or women aged 18-25 who participate in regular exercise) will consume Immunocal each day for 28 days. Four subjects will consume 20 g/d while four will consume 40 g/d of Immunocal. A baseline and weekly blood samples will be taken for analysis of total GSH in whole blood and in lymphocytes. The subjects will be queried for any side effects or difficulties with the protein supplement. The results of this pilot study will determine the dose (20 or 40 g/d) as well as the duration of feeding required to elicit an increase in blood GSH for the experimental trial.

Experimental study

Baseline measurements will be performed on all subjects: aerobic fitness and body composition (see below). Within a week of the baseline aerobic capacity test, each subject will perform a baseline submaximal exercise test designed to stress the oxidative and immunity systems, entailing 45 minutes of cycling on an ergometer at workload determined from the maximal exercise test to elicit 70% of peak oxygen consumption followed by a timed test to exhaustion at 90% of peak oxygen consumption. Oxygen consumption, VO_2, will be measured at the start of the exercise test to verify correct intensity and every 15 minutes thereafter for the duration of the test. Blood samples will be taken prior to the exercise test, after 45 minutes of exercise, within 5 minutes of exhaustion, and 1.5 hours after the completion of exercise.

Following completion of baseline testing, subjects will consume Immunocal or casein (both whey protein and casein are natural proteins found in milk and other dairy products) placebo, in addition to their normal diet, in an amount and for a duration to be determined by the pilot study. One week prior to the completion of the supplementation period, subjects will begin a controlled dietary period. During the controlled dietary period, all subjects will be provided with an energy restricted, formula diet (Ensure High Protein, 20 kcal/kg, 54.7% CHO, 21.3% PRO, 24% Fat) designed to cause weight loss, for 7 days. Each subject will also continue to consume Immunocal or placebo during this period. During this period, subjects will be given containers to collect all of their urine

(used to assess body protein loss). They will bring urine collections in daily and receive a new collection container for the next day.

At the end of the 7 day controlled dietary period, subjects will again complete the submaximal exercise test with blood sampling to determine effects of energy restriction and supplementation on performance, antioxidant status, and immunity.

Peak Oxygen Consumption (VO_{2peak}) will be determined for all subjects using a graded exercise test on a cycle ergometer (Monark 818E Ergomed C). Prior to VO_2 peak testing subjects will be familiarized with bicycle exercise at a constant pedaling rate while breathing through the mouthpiece and breathing valve. The test will begin at a low intensity, 75 watts, while pedaling at 70 rpm and will increase 25 w every 2 min until the subject can no longer maintain 70 rpm (defined as maximal capacity). Continuous indirect calorimetry will be performed using a metabolic cart system (Sensormedics). This requires subjects to breath into a mouthpiece during the entire exercise test. Their expired air will be analyzed in order to calculate their oxygen consumption. Excluding warm up and cool down, the test will likely last between 10 and 15 minutes.

Body composition will be determined by skinfold measurement at three sites: thigh, chest, and abdomen (men) or tricep, thigh and suprailiac (women). This involves using a caliper to measure the thickness of a fold of skin picked up at the listed sites.

Blood collections will be performed at baseline and weekly during the pilot study (5 samples/subject) and on four occasions (pre-exercise, after 45 minutes of exercise, immediately post-exercise, and 1.5 hours post-exercise) during each of the two submaximal exercise tests during the experimental study (8 samples/subject). The amount in each sample is about four teaspoons (20 ml). Blood samples will be tested for HIV if an experimenter becomes exposed to blood during collection or later during analysis. Blood samples will be analyzed for indicators of oxidative stress (lipid hydroperoxides, glutathione and oxidized glutathione) and immunity [*in vitro* lymphocyte proliferation, lymphocyte populations (CD4, CD8, CD3), phagocytosis and oxidative burst of granuloctyes and monocytes to bacteria challenge].

Urine Collections. Each day of the low calorie diet, subjects will be given several jugs to be used to collect all their urine for the day. They will be asked to bring in the jug(s) to the experimenters each day and pick up more for the next day. The urine volume will be measured and samples analyzed for nitrogen and creatinine to determine body protein change during the weight loss.

III. Risks and Benefits

VO_2 peak testing increases the short-term risk of cardiovascular events such as sudden death and myocardial infarction. The American College of Sports Medicine states that the risk of death during or immediately after a maximal exercise test is less than 0.01% and that the risk of myocardial infarction (heart attack) is less than 0.04% (American College of Sports Medicine Guidelines 2000). As most of the studies that

contribute to these statistics have involved testing of individuals at risk of disease, it is likely that testing of the young, healthy subjects in our study is of even lower risk. The subjects we intend to use are at very low risk because of their young age, trained condition, and screening to eliminate those with elevated risk. Subjects selected will be in the "low risk" stratification according to ACSM guidelines (2000), i.e. men less than 40 years of age, asymptomatic of disease, and positive for no more than one of the following risk factors: family history, cigarette smoking, hypertension, hypercholesterolemia, impaired fasting glucose, obesity, and sedentary lifestyle. Subjects will be monitored throughout the exercise tests by the experimenters for signs and symptoms of cardiovascular problems (e.g. abnormal gait, pale, shortness of breath, angina). Fatigue, muscle soreness, and muscle strains could result from the exercise tests but the latter is unlikely due to the high activity level of the subjects and the safety of stationary biking.

Whey protein is consumed marketed for athletes who wish to increase their lean body mass as well as to patient populations (e.g. HIV, cancer). As whey protein is a constituent of foods normally consumed (dairy products) side effects are unusual. Those subjects with allergy to milk will be excluded. Studies in which subjects consumed 20 g/d whey protein for 3 months (Lands et al, 1999) and 45 g/d for 6 months (Micke et al, 2001) reported no severe adverse side effects of consumption. The most common mild side effect was gastrointestinal disturbance. We will instruct subjects to consume the supplement directly with a meal and water to decrease gastrointestinal problems.

Consumption of a 20 kcal/kg/d formula diet, as will be used in this study, has been used by our group multiple times in the past (e.g. Walberg Rankin et al, 1988, Walberg Rankin et al, 1996, Rockwell et al, 2001) for from 3 to 10 days. We expect that subjects will lose 2-4 kg over the week, although some of this is fluid weight. This is not a dangerous rate of weight loss for healthy subjects over a 7 day period. They may experience fatigue due to weight loss or constipation due to low fiber intake over the 7 d. Appropriate laxatives will be recommended for those experiencing the latter.

Risks of blood withdrawal include bruising and very limited risk of infection. All blood draws will be taken using sterile equipment by a Certified Medical Laboratory Technician (Janet Rinehart) or one of the experimenters trained by Ms. Rinehart (M. Shute) experienced in the procedure. Universal precautions will be taken in collection and handling of all blood samples. Subjects will be told that their blood will be analyzed for presence of HIV if an experimenter is exposed to their blood.

IV. Compensation

Subjects will be financially compensated $50 for completion of the pilot study ($5/wk + $30 for completion) or $100 for completion of the experimental study ($20 for baseline testing + $80 for completion of the study). Additionally, they will receive information on their body composition and aerobic capacity.

V. Confidentiality

The data from this study will be kept strictly confidential. No data will be released to anyone but the principal investigator and graduate students involved in the project without written consent of the subject. Data will be identified by subject number.

Proposed timetable:

September - October, 2002	-pilot study recruitment, sample collection and analysis
November - December, 2002	-subject recruitment -orientation and informed consent meeting
January - February, 2002	-baseline testing -experimental trial and data collection
March - April, 2003	-sample analysis
April - July, 2003	-statistical analysis of data and development of manuscript

Budget:

Subject honorarium
Pilot study, 8 subjects @ $50 $ 400
20 subjects @ $100 $2000

Formula, low energy diet
20 subjects @ $9/d X 7 d $1260

Immunocal supplement Donation from company

Protein (casein/soy) powder placebo supplement Donation from company

Blood sampling and analysis supplies (needles, vacutainers, gloves, pipette tips, hematocrit tubes, test tubes, blood storage tubes)

$ 700

Tubes for isolation of lymphocytes ($440/60 tubes) $1760

Preliminary work to validate assays $ 500

Lymphocyte Glutathione analysis (GSH and GSSG)
Pilot study: 8 subjects X 5 samples/subject = 40 samples
Experimental study: 20 subjects X 3 samples/test X 3 ex tests = 180 samples
Total is 220 blood samples (525 with duplicates plus blanks, stds, repeats)
6 kits of 100 assays @ $595 plus need cuvettes specific to assay, polypropylene tubes, 1.5 ml tubes, and metaphosphoric acid)

	$4010
Protein analysis (express glutathione per mg protein)	$ 400
Whole blood Glutathione analysis (GSH and GSSG)	
Matches cost of lymphocyte analysis above	$4010
Protein analysis (express glutathione per mg protein)	$ 400
Glutathione peroxidase (using OxyScan instrument GPx-340 assay)	$ 840
Lipid hydroperoxides (LPO) (Oxis kit @ $415/100 assays, 6 kits)	$2490
Cell-mediated immunity	
Lymphocyte proliferation	$4215
Lymphocyte subsets (CD4/CD8/CD3)	$4812
Monoclonal antibodies	$1200
Innate immunity	
Phagocytosis	$ 456
Oxidative burst	$ 510
Exercise testing supplies @$10/test, 20 max tests + 60 submax	$ 800
TOTAL	$30,763

Biographical Sketches

Janet Walberg Rankin is a Professor in the Department of Human Nutrition, Foods, and Exercise. She has been on the faculty at Virginia Tech since 1982. She earned her B.S. in Zoology from Duke University in 1977 and her Ph.D. in Nutrition from the University of California at Davis in 1982. Her research is primarily in sports nutrition and weight control for athletes and obese individuals. Publications of her research has appeared in journals such as *International Journal of Sport Nutrition, Medicine and Science in Sports and Exercise, International Journal of Sports Medicine, and Journal of Nutrition Education.*

Korinn Saker is an Assistant Professor in the Department of Large Animal Clinical Sciences at the Virginia/Maryland College of Veterinary Medicine. Her research specialty is the influence of nutrition on immunity.

Michael Houston is Professor and Department Head of Human Nutrition, Foods, and Exercise. He is an exercise biochemist with a research focus is role of antioxidants in exercise. He has written a textbook, Biochemistry Primer for Exercise Sciences, as well as numerous research articles in journals such as *Journal Applied Physiology, Canadian Journal of Applied Physiology, and Journal of Nutrition.*

Max Shute is a graduate student/teaching assistant in the Department of Human Nutrition, Foods, and Exercise. He earned his B.S. in Nutrition from Western Illinois University in 1993 and a Masters in Exercise Science from Appalachian State University in 2000. His research involvement during his Master's program included studies of the immune response to exercise, weight control for the obese, equipment validation. He has just completed a study using most of the equipment planned for use in this study-- effect of conjugated linoleic acid on metabolic rate and fuel use at rest and during exercise.

Sean Heffron is a graduate student/teaching assistant in the Department of Human Nutrition, Foods, and Exercise. He earned his A.B. in Biology from Duke Univeristy in 1998. He has been employed by the Nutrition component of the Duke University Diet and Fitness Center in Durham, North Carolina and by the Yokohama City Board of Education in Yokohama, Japan. He has been an assistant for several studies performed in Dr. Rankin's laboratory over the last year.

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Appendix D

Informed Consent Form

Informed Consent for Participants of Investigative Projects
Department of Human Nutrition, Foods, and Exercise
Virginia Tech

TITLE: Value of Whey Protein Concentrate on Glutathione Status, Exercise Performance, and Immunity in Men During Energy Balance and Restriction.

PRINCIPAL INVESTIGATORS: Janet Walberg Rankin, Ph.D., Korinn Saker, Ph.D. D.V.M., Michael Houston Ph.D., Max Shute, MS., Sean Heffron, AB.

PURPOSE: Cysteine is a naturally occurring amino acid found in large amounts in the whey portion of milk. Cysteine is the limiting factor in the body's synthesis of glutathione, a natural antioxidant n the body. Several studies have shown that consumption of whey protein increases blood levels of glutathione, a natural compound our body uses to protect itself from damage due to oxygen. Oxygen can damage cells in our bodies (oxidative stress) and the resulting damage has been implicated in a variety of conditions such as cancer, immunity, and heart disease. As oxygen damage can be elevated with increased use of oxygen, exercise increases the concentration of these molecules in the body. High oxidative stress has been hypothesized to contribute to muscle fatigue. Thus, it is possible that increasing concentration of natural body antioxidants such as glutathione will improve exercise performance.

Low levels of cysteine in the blood have been linked to loss of muscle mass in individuals that have poor immunity (e.g. HIV) or in those undergoing strenuous physical activity. Also, Lands et al (1999) reported increased muscle mass with three months of whey protein supplementation. However, it is hard to be sure that this was due to the whey protein because the subjects also increased their physical activity during the experimental period. So, more research must be done to determine if consumption of whey protein isolate improves body composition.

Finally, strenuous exercise has been shown to reduce immunity and increase susceptibility to illness in humans. Whey protein isolate has been used in clinical situations to boost glutathione in patients in order to improve immunity. It has yet to be determined, however, whether whey protein can reduce the depression in immunity resulting from hard exercise. Thus, this study will examine the value of whey protein concentrate on glutathione status, exercise performance, and immunity while maintaining your weight and after one week of weight loss.

Experimental Study

General Design

We will first ask you to complete a medical screening form to make sure that you would not be at elevated risk as a participant in the study. If you are chosen and agree to participate in the study, we will do some baseline measurements that include your body fat (measure the thickness of fat folds at three areas of your body: chest, abdomen, and thigh) and aerobic fitness (see below). Within one week of the completion of these measurements, you will return to the lab for a baseline submaximal exercise test (see below). You will then consume 40 grams of either Immunocal (whey protein isolate) or placebo (casein, another protein found in milk) daily (you will not know which treatment

you receive) for three weeks. One week prior to the completion of supplementation period, you will begin a formula diet that we will provide. This diet will consist of 20kcal/kg Ensure High Protein® (a meal replacement found at grocery stores) daily. During the week you consume this diet, you will collect all of your urine in containers provided by us. At both the beginning and end of this 7 day controlled diet period you will return to the lab and again complete the submaximal exercise test. At each of the submaximal exercise trials we will take 4 blood samples over approximately 3 hours, and make multiple measures of your exercise intensity with a machine that measures your expired air. In addition, on the first and last days of the controlled diet period you will report to the lab early in the morning in a fasted state for one blood sample. Following this sample, you will consume 20 grams of supplement and remain in the lab for 90 minutes until a second blood sample is taken.

Maximum oxygen consumption testing: This test will measure your aerobic fitness. You will cycle at a desired rpm on a stationary bike starting at a comfortable resistance (75 watts) for 2 minutes. The resistance will increase 25 watts every 2 minutes until exhaustion or you can no longer maintain 60rpm. During this test you will be breathing room air through a mouth-piece similar in nature to a snorkel.

Blood collection: A blood sample will be taken prior to the submaximal exercise tests, after 45 minutes of exercise, within 5 minutes of exhaustion, and 1.5 hours after the completion of exercise. Blood samples will also be taken prior to and following the controlled diet period, during the morning hours, in a fasted state and 90 minutes following consumption of 20 g supplement. The amount in each sample is about two teaspoons. All blood sampling will be performed by a Certified Medical Laboratory Technician, Janet Rinehart, or an experimenter trained by Ms. Rinehart (M. Shute) experienced in the procedure. Your blood will be measured for indicators of oxidative stress (i.e. amount of one of the body's antioxidants, glutathione, that has been modified by oxygen; lipids damaged by oxygen), immunity (e.g. ability of white blood cells to respond to an infectious protein, numbers of specific types of white blood cells), and levels of amino acids. Your blood sample will be tested for HIV if an experimenter becomes exposed to your blood during collection or later during analysis.

Submaximal exercise test: You will pedal on a stationary cycle for 45 minutes at a moderate-high intensity (about 70% of your maximum fitness level). You will then cycle at a high intensity (90% of your maximum fitness level) until you are exhausted and no longer able to maintain the set intensity. As described above, blood and expired air samples will be conducted several times during the exercise test.

Diet: During the formula diet period, we ask that you do not consume any other foods or beverages that contain calories. You may drink as much water or noncaloric beverages (i.e. diet soda, black coffee, unsweetened tea) as you like.

SUMMARY OF SUBJECT RESPONSIBILITIES

1. Refrain from taking any other nutritional supplements without checking first with the experimenters.
2. Give maximal effort on performance tests.
3. Inform the experimenters if you experience any unusual symptoms from any of the testing, or supplements.
4. Inform the researchers of any known medical conditions or allergies you are aware of prior to the study as well as any transmittable diseases acquired during the study.
5. Refrain from eating and/or drinking any caloric foods and/or beverages during the 7-day formula diet period.
6. Collect all urine produced for each of the 7 days of the formula diet period.

RISKS OF PARTICIPATION:

1. Studies in which subjects consumed 20 g/d whey protein for 3 months and 45 g/d for 6 months reported no severe adverse side effects of consumption. The most common mild side effect was gastrointestinal disturbance.
2. Consumption of a 20 kcal/kg/d formula diet, as will be used in this study, has been used by our group multiple times in the past for from 3 to 10 days. We expect that you will lose 2-4 kg over the week, although some of this is fluid weight. You may be tired as a result of the weight loss. As there is little fiber in the formula diet, you may experience some constipation during the weight loss week. We can recommend an appropriate laxative if you experience this.
3. Fatigue, muscle soreness, muscle strains or pulls may result from the aerobic fitness and sub-maximal exercise testing.
4. You may experience some bruising at the site of the blood withdrawal. There is a remote chance of infection as a result of the needle stick, but this is very unlikely due to the use of sterile supplies.
5. There is a remote risk of cardiovascular complications from maximal exercise testing. The American College of Sports Medicine states that the risk of death during or immediately after a maximal exercise test is less than 0.01% and that the risk of myocardial infarction (heart attack) is less than 0.04% (American College of Sports Medicine Guidelines 2000). As most of these studies that contribute to these statistics have involved testing of individuals at risk of disease, it is likely that testing of the young, healthy subjects in our study is of even lower risk.
6. The University will not be responsible for any medical expenses you may have unless the University has been negligent.

BENEFITS OF PARTICIPATION

Your participation will provide you with:

1. Data on your body composition and aerobic fitness.

COMPENSATION

We will pay you $20 for completion of all baseline testing and $80 additional for completion of the experimental study.

ANOYNMITY AND CONFIDENTIALITY

The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by subject numbers, without anything to identify subjects by name.

FREEDOM TO WITHDRAW

You are free to withdraw at any time from the study for any reason. Circumstances may come up that the researcher will determine that you should not continue as a subject in the study. For example, lack of compliance to diet or exercise, failure to attend testing sessions and illness could be reasons to have the researchers stop your participation in the study.

APPROVAL OF RESEARCH

This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech, and by the Department of Human Nutrition, Foods, and Exercise. You will receive a copy of this form to take with you.

SUBJECT PERMISSION

I have read the informed consent and fully understand the procedures and conditions of the project. I have had all my questions answered, and I hereby give my voluntary consent to be a participant in this research study. I agree to abide by the rules of the project. I understand that I may withdraw from the study at any time.

If I have questions, I will contact:

- Principal Investigator: Janet Walberg Rankin, Professor, Department of Human Nutrition, Foods, and Exercise. 231-6355
- Chairman, Institutional Review Board for Research Involving Human Subjects: David Moore, 231-4991
- Department reviewer: Robert Grange, 231-2725

Name of Subject (please print)_____

Signature of Subject_____ Date_____

Appendix E

Medical and Health History Form

VIRGINIA TECH LABORATORY FOR HEALTH AND EXERCISE SCIENCE

MEDICAL AND HEALTH HISTORY

Name: _____ Age: _____ Birth Date: _____

Address: _____ e-mail: _____

Phone Numbers: Home: _____ Work :_____

Summer Address:

_____

_____

Phone Number (during Winter Break): _____

Person to Contact in Case of an Emergency:

_____

Relationship: _____Phone: _____

Primary Care Physician: _____Phone: _____

Medical Insurance Carrier: _____

Are you employed by Virginia Tech? _____

Current Body Weight:_____

MEDICAL HISTORY

Please indicate any current or previous conditions or problems you have experienced or have been told by a physician you have had:

	Yes	**No**	
Heart disease or any heart problems:	_____		_____
Rheumatic Fever:	_____		_____
Respiratory disease or breathing problems (e.g. asthma):	_____		_____
Circulation problems:	_____		_____
Kidney disease or problems:	_____		_____
Urinary problems:	_____		_____
Musculoskeletal problems:	_____		_____

(i.e. Orthopedic injuries, osteoporosis)
Fainting and Dizziness: _____ _____
High Cholesterol: _____ _____
Diabetes: _____ _____
Thyroid problems: _____ _____
Mental illness: _____ _____
Hypoglycemia:(i.e. low blood sugar) _____ _____
Epilepsy or seizures: _____ _____
Blood clotting problems (e.g. hemophilia): _____ _____

Liver disorders (e.g. hepatitis B*)* _____ _____

If you answered "yes" to any of the previous questions, please indicate the date and describe:

_____
_____
_____
_____

Please list any hospitalizations/operations/recent illnesses (type/date):

_____
_____
_____
_____
_____

	Yes	**No**
Have you ever been diagnosed as having high blood pressure?	_____	_____
Are you currently being treated for high blood pressure?	_____	_____

If "yes", please explain:

_____
_____
_____

Please list all medications (prescription and over-the-counter) you are currently taking or have taken in the past week:

_____
_____
_____

For what reason(s) are you taking this medication?

_____

_____
_____

Health Habits

	Yes	**No**
Do you drink alcoholic beverages?	_____	_____
How many drinks per week? _____		
Do you smoke cigarettes?	_____	_____
Packs per day: _____		

	Yes	**No**
Do you engage in regular exercise?	_____	_____

If "yes", please list:

Activity	**Frequency (times per week)**	**Duration (minutes)**
_____	_____	_____
_____	_____	_____
_____	_____	_____

Do you ever faint, experience shortness of breath or chest discomfort with exertion?
_____

If "yes", please explain: _____
_____

Are there any orthopedic limitations you have that may restrict your ability to perform exercise and if "yes", please explain:
_____
_____
_____

Family History

Has anyone in your family been diagnosed or treated for any of the following?

	Yes	**No**	**Relationship**	**Age**
Heart attack	____	____	_____	____
Heart disease	____	____	_____	____
High blood pressure	____	____	_____	____
Stroke	____	____	_____	____
Kidney disease	____	____	_____	____
Diabetes	____	____	_____	____

Schedule Spring 2001 semester (indicate those times you have classes, work etc that you CANNOT be involved in testing or exercise training):

	Mon	*Tue*	*Wed*	*Thursday*	*Fri*
6:00-7:00am					
7:00-8:00					
8:00-9:00					
9:00-10:00					
10:00-11:00					
11:00-12:00					
12:00-1:00					
1:00-2:00					
2:00-3:00					
3:00-4:00					
4:00-5:00					
5:00-6:00					
6:00-7:00					
7:00-8:00					

Any explanation required for above

_____

_____

_____

Please sign to indicate that the above information is correct:

_____ _____ _____

Print name Signature Date

SUPPLEMENTARY QUESTIONS

Food Habits and Allergies

1. Are you allergic to any foods? ____ If yes, which ones?

2. Are you on any kind of special diet? ____ If so, what kind?

1. Do you take any dietary supplements?_____ If so, what kind and how often?

2. Has your weight been stable over the past year?____ If not, how has it changed?

Drug Allergies

3. Are you aware of any allergies you have to any drugs? ____ If yes, which ones?

4. Have you ever received Novocaine at the dentist's office or other local (injected into skin) anesthetic? _____.

If yes, did you have any allergic reaction to this?_____

Comfort with procedures

5. Do you have a fear of needles or having blood withdrawn

Appendix F

Immunocal® Study Exit Survey

Exit Survey

Name: _____ Subject #_____

Congratulations! You have completed the study. Before you leave we would like you to answer a few questions. You cannot be penalized for your responses so please be COMPLETELY honest. We enjoyed your participation and hope you will consider other study opportunities that may come along from the HNFE Dept.

1. During the 4 days of the Ensure diet, did you eat any other foods which contained calories? If so, please indicate when you ate, what items, and how much.

2. Did you consume 100% of your supplement throughout the study? If not, please indicate how much supplement was not taken and when.

3. Which supplement do you think you had, Immunocal or Placebo?

4. On a scale of 1-10 (10 being extremely difficult), how difficult was the energy restriction period?

5. Do you think the supplement had any positive or negative effects on you mentally and/or physically? If so, briefly describe.

6. Please provide any comments on how the study was run and any ways we could have been more helpful or done a better job.

7. Do you want your body composition, fitness, and performance information from the study? (please provide your summer address for us to send this information at the end of the study)

Your $100 check should arrive after the next VT pay period. Please contact Max Shute (mshute@vt.edu) or Dr. Rankin (jrankin@vt.edu) if you do not receive the check within 4-5 weeks. Thanks and good luck in the future!

Appendix G

Oxygen Consumption Data Collection Sheet

Immunocal Study Peak Oxygen Consumption Testing
Data collection sheet

Subject name: _____ ID#_____ Date:__ - ___ - ___

Age: _____ Ht: _____cm Wt: _____kg Ambient Temp: _____

Minute	watts	RPE	VO_2
1	100		
1:30	115		
2	130		
2:30	145		
3	160		
3:30	175		
4	190		
4:30	205		
5	220		
5:30	235		
6	250		
6:30	265		
7	280		
7:30	295		
8	310		
8:30	325		
9	340		
9:30	355		
10	370		
10:30	385		
11	400		
11:30	415		
12	430		
12:30	445		
13:00	460		
13:30	475		
14	490		

RelativeVO_{2pk}: _____ml/kg/min
70% VO_{2pk}: _____, watts = _____
90% VO_{2pk}: _____, watts = _____

Appendix

Appendix H

Test day data collection sheet

Subject #_____ Wt:_____kg Date:___/___/___

Test #_____

Time of start of exercise:_____

70% = _____watts 70% = _____ml/kg/min

90% = _____watts 90% = _____ml/kg/min

VO_2 check 1:_____

VO_2 check 2:_____

VO_2 check 3:_____

90% VO_2 time to exhaustion:_____

Wingate Test

Resistance: .09kg per kg body wt. = _____

Watts = load (kg) x revolutions x 11.765

5-second rpm counts: _____ _____ _____ _____ _____ _____

Peak power (greatest power in 5-sec period, usually the 1st 5-sec) = _____w

Avg. power for the six 5-second periods = _____w

Fatigue rate: 100 x (peak power – lowest power)/peak power = _____%

Appendix I

Data Collection Sheet for White Blood Cell Counting

Data Collection Sheet for White Blood Cell Counting

Subject #_____ Supplement g: _____ Visit#: _____ Body wt:_____

Date: ___/___/___

Time of BD: ___:___

Suspension = 200 ul

Dilution = 10 ul of susp. + 490 ul PBS = 500 ul (1 to 50)
take 100 ul of the 500 ul + 100 ul dye (1 to 2)
final DF = 1 to 100

Cell Count #1:	Cell Count #2

Avg = _____ Avg = _____

Avg x DF x10^4 = _____cell/ml

Cell/ml x susp = _____ (actual cells in CPT tube)

Calculations:

Appendix J

Summaries of Statistical Analyses

Table 13.

Repeated Measures Analysis of Variance for tGSH.

Effect	df	F	Sig
TRIAL	2	.719	.503
TRIAL X GRP	2	.741	.493
EXER	2	4.27	.034
EXER X GRP	2	.229	.798
TRIAL X EXER	4	1.58	.238
TRIAL X EXER X GRP	4	.099	.981

Table 14.

Repeated Measures Analysis of Variance for GPx.

Effect	df	F	Sig
TRIAL	2	20.8	.000
TRIAL X GRP	2	1.14	.344
EXER	2	8.13	.003
EXER X GRP	2	2.17	.145
TRIAL X EXER	4	.713	.596
TRIAL X EXER X GRP	4	2.82	.062

Table 15.

Repeated Measures Analysis of Variance for GSSG.

Effect	df	F	Sig
TRIAL	2	41.3	.000
TRIAL X GRP	2	.659	.530
EXER	2	2.36	.124
EXER X GRP	2	1.86	.185
TRIAL X EXER	4	.518	.724
TRIAL X EXER X GRP	4	.216	.925

Table 16.

Repeated Measures Analysis of Variance for GSH/GSSG ratio.

Effect	df	F	Sig
TRIAL	2	53.6	.000
TRIAL X GRP	2	.564	.581
EXER	2	.845	.449
EXER X GRP	2	.336	.720
TRIAL X EXER	4	.184	.943
TRIAL X EXER X GRP	4	.115	.975

Table 17.

Repeated Measures Analysis of Variance for LPO.

Effect	df	F	Sig
TRIAL	2	9.36	.002
TRIAL X GRP	2	.487	.623
EXER	2	.451	.644
EXER X GRP	2	1.66	.220
TRIAL X EXER	4	1.43	.271
TRIAL X EXER X GRP	4	.875	.502

Table 18.

Repeated Measures Analysis of Variance for WBCGSH.

Effect	df	F	Sig
TRIAL	2	6.38	.011
TRIAL X GRP	2	1.00	.390
EXER	2	4.45	.032
EXER X GRP	2	.733	.498
TRIAL X EXER	4	1.06	.416
TRIAL X EXER X GRP	4	.300	.872

Table 19.

Repeated Measures Analysis of Variance for CD3+ T-Lymphocytes.

Effect	df	F	Sig
TRIAL	2	.310	.738
TRIAL X GRP	2	.58	.571
EXER	2	51.1	.000
EXER X GRP	2	.212	.811
TRIAL X EXER	4	1.24	.336
TRIAL X EXER X GRP	4	1.75	.191

Table 20.

Repeated Measures Analysis of Variance for CD4+ T-Lymphocytes.

Effect	df	F	Sig
TRIAL	2	.386	.685
TRIAL X GRP	2	.044	.957
EXER	2	181	.000
EXER X GRP	2	.122	.886
TRIAL X EXER	4	1.79	.183
TRIAL X EXER X GRP	4	3.11	.047

Table 21.

Repeated Measures Analysis of Variance for CD8+ T-Lymphocytes.

Effect	df	F	Sig
TRIAL	2	1.51	.245
TRIAL X GRP	2	1.32	.292
EXER	2	51.8	.000
EXER X GRP	2	1.24	.312
TRIAL X EXER	4	.799	.544
TRIAL X EXER X GRP	4	.511	.729

Table 22.

Repeated Measures Analysis of Variance for CD4/CD8+ ratio.

Effect	df	F	Sig
TRIAL	2	.526	.600
TRIAL X GRP	2	.374	.693
EXER	2	98.7	.000
EXER X GRP	2	.773	.477
TRIAL X EXER	4	1.39	.284
TRIAL X EXER X GRP	4	1.39	.284

Table 23.

Repeated Measures Analysis of Variance for Phagocytosis.

Effect	df	F	Sig
TRIAL	2	7.30	.005
TRIAL X GRP	2	.698	.511
EXER	2	10.9	.001
EXER X GRP	2	.224	.802
TRIAL X EXER	4	.416	.795
TRIAL X EXER X GRP	4	1.38	.286

Table 24.

Repeated Measures Analysis of Variance for Oxidative Burst.

Effect	df	F	Sig
TRIAL	2	1.76	.204
TRIAL X GRP	2	.548	.589
EXER	2	5.34	.017
EXER X GRP	2	.235	.793
TRIAL X EXER	4	1.19	.357
TRIAL X EXER X GRP	4	1.84	.176

Table 25.

Repeated Measures Analysis of Variance for Performance.

Effect	df	F	Sig
TRIAL	2	3.21	.073
TRIAL X GRP	2	3.23	.072

Table 26.

Repeated Measures Analysis of Variance for Total Work.

Effect	df	F	Sig
TRIAL	2	.975	.397
TRIAL X GRP	2	.414	.668

Table 27.

Repeated Measures Analysis of Variance for Fatigue Rate.

Effect	df	F	Sig
TRIAL	2	.382	.688
TRIAL X GRP	2	1.54	.241

Table 28.

Repeated Measures Analysis of Variance for Weight Change.

Effect	df	F	Sig
TRIAL	2	69.0	.000
TRIAL X GRP	2	1.15	.339

Appendix K

Raw Data

Table 29.

Subject Characteristics

Group	Subject	Age	Ht (cm)	VO_{2peak}	Body Fat (%)*
WPI	1	20	170.2	63.5	9.1
	3	21	175.3	49.4	8.3
	4	20	192.4	54.1	18.4
	9	25	174	73.6	4.9
	11	20	162.5	56.4	10.4
	12	21	172.7	66	14.7
	13	19	166.4	68.1	6
	15	23	180.3	60	8
	18	24	184.2	58.7	11.8
Avg.		21.7±0.6	175.3±2.7	60.7±2.2	10.0±1.3
P	20	24	175.3	57.2	8.4
	2	23	176.5	48.6	16
	5	19	177.8	53.7	9.6
	6	29	167.6	55.7	10
	7	23	184.2	62.7	7.9
	8	25	187.9	68.5	5.3
	10	23	175.3	57.5	8.9
	16	28	186.7	51.4	11.6
	17	22	164.5	63.9	9.8
	21	19	185.4	57.1	11.2
Avg.		23.4±1.0	178.6±2.5	56.3±2.3	11.5±1.2

VO_{2peak} = ml/kg/min
* as assessed by 3-site skinfold

Table 30.

Body Weights (kg)

Group	Subject	Trial1	Trial2	Trial3
WPI	1	65.1	65	62.5
	3	81.4	82.5	80
	4	84.3	84.5	81.5
	9	66	65.9	64
	11	59.9	60	58.5
	12	69.7	68.2	66.3
	13	65.2	64.5	61.7
	15	76.7	73.7	73.6
	18	83.1	85.4	80
	20	68.5	69.6	66.6
Avg.		71.9±2.3	71.9±2.2	69.4±2.7
P	2	76.3	76.1	74.2
	5	68	69.1	64.1
	6	58.2	59.2	57.4
	7	75.2	76.5	72.2
	8	79.7	79.6	77.5
	10	85.6	88.6	85.7
	14	67.5	67.7	64.8
	16	76.7	76	73.6
	17	67.5	69	66.6
	21	75.5	76.6	73.5
Avg.		73±2.3	73.8±2.5	70.9±2.5

Table 31.

Raw Data Lipid Hydroperoxides (μmol) Trial 1

Group	Subject	Ex1	Ex2	Ex3
WPI	1	15.0	4.4	13.5
	3	18.4	14.5	15.3
	4	9.1	11.6	16.3
	9	4.4	11.9	1.8
	11	11.4	14.2	9.3
	12	8.5	6.2	13.5
	13	11.1	3.6	9.3
	15	14.8	12.7	9.8
	18	7.0	11.9	28.0
	20	3.1	11.1	12.4
Avg.		10.3±1.4	10.2±1.4	12.9±1.6
P	2	8.0	14.8	7.0
	5	4.1	20.0	5.7
	6	14.0	14.8	11.1
	7	8.0	10.1	14.8
	8	16.3	11.6	7.0
	10	13.2	18.7	5.2
	14	1.8	6.5	9.8
	16	9.3	9.1	9.1
	17	10.9	3.3	12.9
	21	10.3	9.1	9.1
Avg.		9.6±1.4	11.8±1.4	9.1±1.6

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 32.

Raw Data Lipid Hydroperoxides (μmol) Trial 2

Group	Subject	Ex1	Ex2	Ex3
WPI	1	4.4	3.6	8.5
	3	13.2	9.8	5.7
	4	8.3	7.0	4.4
	9	9.8	11.9	8.5
	11	7.8	9.6	32.7
	12	1.8	9.1	5.9
	13	16.3	3.9	16.1
	15	6.7	5.9	7.8
	18	8.8	1.3	8.3
	20	4.9	9.6	4.1
Avg.		8.2±1.1	7.1±1.1	10.2±2.1
P	2	5.4	6.7	5.9
	5	7.5	9.6	12.7
	6	7.5	7.5	7.8
	7	10.9	12.7	10.1
	8	11.9	14.8	17.4
	10	12.7	6.7	5.4
	14	9.3	1.0	16.8
	16	11.1	5.7	11.6
	17	8.8	10.6	14.8
	21	3.9	5.9	11.9
Avg.		8.9±1.1	8.1±1.1	11.4±2.1

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 33.

Raw Data Lipid Hydroperoxides (μmol) Trial 3

Group	Subject	Ex1	Ex2	Ex3
WPI	1	7.5	8.5	10.6
	3	11.6	0.2	7.8
	4	14.5	11.4	7.0
	9	4.1	4.6	4.6
	11	11.9	8.8	8.3
	12	2.8	2.0	4.1
	13	10.6	6.2	8.3
	15	9.3	10.6	11.9
	18	12.4	10.1	10.3
	20	8.0	2.8	1.0
Avg.		9.3±1.0	6.5±1.2	7.4±1.0
P	2	10.9	12.4	13.2
	5	9.6	5.9	6.2
	6	10.1	7.8	5.9
	7	5.7	7.5	4.6
	8	1.5	9.1	4.1
	10	6.7	4.9	5.9
	14	6.5	4.4	4.9
	16	8.0	5.9	1.8
	17	4.4	18.1	11.1
	21	10.9	10.3	2.6
Avg.		7.4±1.0	8.6±1.2	6.0±1.0

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 34.

Raw Data GPx (μmol/ml) Trial 1

Group	Subject	Ex1	Ex2	Ex3
WPI	1	3.8	10.9	5.1
	3	6.5	5	6.1
	4	6	7.1	5.6
	9	9.1	8.1	6.9
	11	6.3	6.8	6.9
	12	5.4	9.1	4.1
	13	4.2	11.1	6
	15	8	6	5.1
	18	10.4	8.4	4.6
	20	5.8	10.5	5
Avg.		6.5±0.8	8.3±0.6	5.5±0.4
P	2	4	7.4	6.8
	5	3.8	9.9	8.7
	6	6.4	5.9	8.6
	7	7.5	9.8	7.9
	8	4.9	6.8	7.8
	10	6.3	5.7	8.5
	14	6.1	9	8.5
	16	6.7	5.5	8.5
	17	7	8.4	7.7
	21	5.8	9.2	4.8
Avg.		5.8±0.8	7.7±0.5	7.7±0.7

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 35.

Raw Data GPx (μmol/ml) Trial 2

Group	Subject	Ex1	Ex2	Ex3
WPI	1	6.4	10.4	8.1
	3	3.6	6.9	7.1
	4	3.1	7.2	6.2
	9	8.1	8.2	6.1
	11	7.3	7	4.1
	12	6.5	6.7	5.4
	13	7.6	6.4	2.9
	15	6.2	7.2	1.6
	18	3.7	7.4	9.9
	20	8.1	7.6	8.4
Avg.		6.0±0.6	7.5±0.6	5.9±.05
P	2	4.3	6.8	7.3
	5	4.1	8	6.6
	6	5.2	8	6.4
	7	2.2	7.4	6.6
	8	7.6	7.1	6
	10	4.9	6.2	5.7
	14	6.8	7.5	2.7
	16	4.1	6.6	7.9
	17	1.8	7.3	9.9
	21	7.3	6.5	7.6
Avg.		4.8±0.5	7.1±0.6	6.6±0.6

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 36.

Raw Data GPx (μmol/ml) Trial 3

Group	Subject	Ex1	Ex2	Ex3
WPI	1	6.6	9	7.7
	3	1.5	5.3	5.9
	4	6.7	8	8.5
	9	12.5	7	10.2
	11	8.9	10.7	8.5
	12	8.7	11.8	10.2
	13	9.4	8.6	10.6
	15	7.5	9.8	8.9
	18	9.6	8.4	8.4
	20	7.9	11.3	11.9
Avg.		7.9±0.4	8.9±0.7	9.0±.08
P	2	4.2	7.3	8
	5	8.5	8.1	9.7
	6	9.9	11.4	7.5
	7	11.7	10.8	6.1
	8	9.1	5.5	10
	10	8	6.8	9.2
	14	7.5	10.5	8.8
	16	5.8	13.2	7.2
	17	4.2	9.2	9.6
	21	12.3	6.5	8.1
Avg.		8.1±0.6	8.9±0.7	8.4±0.6

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 37.

Raw Data For tGSH/mg protein Trial 1

Group	Subject	Ex1	Ex2	Ex3
WPI	1	1460.36	1304.83	1480.22
	3	1337.90		1279.76
	4	1401.35	1725.68	
	9	1312.46	1226.04	1578.59
	11	1268.61	1617.28	1564.36
	12	1333.38	1666.76	1641.86
	13	1119.55	1684.47	1221.53
	15	1238.58	1794.32	1433.94
	18	1123.43	1281.08	1464.84
	20	1450.77	1279.8	1552.08
Avg.		1288±39	1482±94	1492±134
P	2	1389.65	2221.8	2529.82
	5	1259.39	1657.64	1256.72
	6	1485.95	1829.37	1372.75
	7	1275.32	1689.12	1490.96
	8	1458.46	1456.02	1664.31
	10	1415.23	1304.96	1656.25
	14	1439.21	1466.86	1560.49
	16	1221.27	1318.67	1228.56
	17	1277.41	1349.81	1577.02
	21	1369.56	1335.53	1809.47
Avg.		1359±35	1562±84	1514±120

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 38.

Raw Data For tGSH/mg protein Trial 2

Group	Subject	Ex1	Ex2	Ex3
WPI	1	1289.56	1284.7	1278.05
	3	1414.41	1145.73	1173.5
	4	1469.26	1357.66	1341.84
	9	1414.09	1713.34	2039.99
	11	1315.31	1357.48	1259.96
	12	1460.43	1622.6	1464.63
	13	1597.82	1233.19	1351.09
	15	1369.40	1525.16	1918.16
	18	1468.25	1099.44	1343.55
	20	1365.53	1236.40	1564.10
Avg.		1416±54	1357±58	1473±89
P	2	1438.13	1115.29	1226.29
	5	1344.91	1460.82	1405.94
	6	1259.04	1259.94	1249.13
	7	1439.14	1312.06	2133.08
	8	1306.47	1527.56	1301.25
	10	2014.95	1497.13	1609.23
	14	1320.88	1098.52	1514.56
	16	1257.36	1226.36	1272.78
	17	1329.62	1186.08	1213.30
	21	1283.39	1455.78	1322.34
Avg.		1399±54	1313±58	1424±89

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 39.

Raw Data For tGSH/mg protein Trial 3

Group	Subject	Ex1	Ex2	Ex3
WPI	1	1365.36	1179.12	1360.05
	3	1477.99	1191.64	1094.43
	4	1365.85	1306.45	1239.87
	9	1322.84	1551.68	1428.60
	11	1329.40	1393.75	1359.64
	12	1376.57	1322.74	1435.58
	13	1347.29	1075.16	1395.91
	15	1333.56	1568.83	1687.34
	18	1208.96	1294.23	1606.94
	20	1346.41	1464.64	1502.42
Avg.		1347±30	1334±70	1411±62
P	2	1537.10	1119.39	1755.49
	5	1358.49	1500.59	1482.87
	6	1457.87	1365.47	1375.70
	7	1482.37	1257.41	1484.72
	8	1365.54	1832.2	1971.89
	10	1506.92	1709.76	1571.40
	14	1346.35	1286.94	1338.48
	16	1362.35	1019.95	1539.04
	17	1281.66	1052.19	1210.57
	21	1139.62	1304.23	1347.70
Avg.		1383±30	1344±70	1507±62

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 40.

Raw Data For GSSG (µmol) Trial 1

Group	Subject	Ex1	Ex2	Ex3
WPI	1	7.01	9.34	6.79
	3	8.09	13.68	10.07
	4	11.02	20.81	12.20
	9	8.01	8.91	7.35
	11	14.06	15.97	10.91
	12	12.69	12.62	12.82
	13	6.33	9.10	10.94
	15	7.29	25.69	9.02
	18	15.95	11.12	23.78
	20	10.05	12.18	28.82
Avg.		10.0±1.1	13.9±0.6	13.2±1.3
P	2	7.97	11.73	11.61
	5	14.28	13.86	15.81
	6	11.02	20.81	20.81
	7	15.66	14.31	17.17
	8	5.45	11.55	8.01
	10	9.05	12.49	10.18
	14	7.08	11.60	7.97
	16	6.52	6.85	8.15
	17	9.08	9.23	8.89
	21	12.07	13.21	16.05
Avg.		12.8±1.4	12.5±0.8	12.4±0.8

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 41.

Raw Data For GSSG (µmol) Trial 2

Group	Subject	Ex1	Ex2	Ex3
WPI	1	4.12	3.89	5.48
	3	5.72	6.60	7.48
	4	12.71	13.88	15.92
	9	8.64	9.36	7.70
	11	8.43	8.97	7.99
	12	11.81	27.84	13.94
	13	9.07	12.79	10.19
	15	16.20	27.40	25.30
	18	9.88	9.02	11.19
	20	12.36	11.91	12.84
Avg.		11.8±0.9	13.1±1.1	11.8±0.7
P	2	16.15	5.69	10.31
	5	10.37	2.43	18.10
	6	13.67	18.92	12.25
	7	5.28	7.57	5.51
	8	6.20	10.83	8.48
	10	11.02	11.33	19.31
	14	12.72	20.73	13.67
	16	13.28	7.48	7.37
	17	7.48	9.07	10.50
	21	11.46	13.54	14.14
Avg.		10.7±0.7	10.8±0.8	11.9±0.6

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 42.

Raw Data For GSSG (µmol) Trial 3

Group	Subject	Ex1	Ex2	Ex3
WPI	1	5.84	10.68	7.45
	3	20.17	22.53	22.31
	4	19.47	36.26	34.71
	9	17.48	16.85	23.42
	11	26.62	19.82	17.4
	12	16.77	21.14	15.93
	13	20.19	22.18	25.80
	15	27.40	27.40	33.34
	18	18.38	26.78	21.53
	20	17.84	22.04	20.78
Avg.		19.0±1.2	22.5±1.4	22.2±1.7
P	2	11.73	13.40	12.17
	5	22.53	30.40	29.64
	6	23.36	24.68	27.66
	7	13.24	17.76	22.04
	8	27.03	22.66	26.72
	10	39.39	33.78	40.88
	14	22.07	20.71	27.62
	16	16.59	15.15	18.99
	17	21.37	17.62	22.44
	21	26.02	22.31	21.71
Avg.		22.3±1.5	21.8±1.1	24.9±1.3

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 43.

Raw Data For GSH/GSSG ratio Trial 1

Group	Subject	Ex1	Ex2	Ex3
WPI	1	225.81	237.54	352.64
	3	167.06		143.39
	4	110.47	83.15	
	9	173.51	135.71	200.86
	11	109.81	94.93	143.82
	12	121.68	130.30	115.83
	13	181.34	159.42	114.32
	15	193.56	58.21	159.87
	18	76.67	112.72	62.00
	20	154.69	109.04	47.16
Avg.		154±20	129±14	149±26
P	2	167.32	110.28	137.71
	5	88.32	119.96	89.95
	6	140.47	81.59	70.16
	7	83.93	127.36	84.46
	8	251.00	121.74	223.73
	10	38.00	98.80	167.59
	14	199.24	115.27	192.72
	16	190.69	193.26	155.62
	17	139.48	145.27	70.07
	21	108.19	102.33	88.76
Avg.		140±18	121±13	128±23

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 44.

Raw Data For GSH/GSSG ratio Trial 2

Group	Subject	Ex1	Ex2	Ex3
WPI	1	301.90	280.04	230.15
	3	198.57	179.83	166.08
	4	32.45	101.79	83.05
	9	155.89	167.31	258.02
	11	161.39	149.96	162.16
	12	123.79	45.38	36.71
	13	128.11	84.80	107.10
	15	80.74	54.16	60.25
	18	133.94	123.21	120.61
	20	110.67	107.25	121.81
Avg.		142±20	129±40	134±26
P	2	83.45	210.24	126.74
	5	142.19	610.11	74.37
	6	94.91	71.65	105.29
	7	238.31	15.52	395.17
	8	236.39	151.34	166.50
	10	174.56	117.94	74.58
	14	99.65	54.75	101.83
	16	86.21	157.60	170.35
	17	154.95	131.69	113.30
	21	120.35	102.32	93.67
Avg.		143±20	162±40	142±26

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 45.

Raw Data For GSH/GSSG ratio Trial 3

Group	Subject	Ex1	Ex2	Ex3
WPI	1	191.43	105.2	216.15
	3	51.15	44.66	43.52
	4	56.95	30.82	34.68
	9	73.43	84.57	60.76
	11	46.64	66.64	69.49
	12	82.45	67.77	80.88
	13	56.67	45.84	41.82
	15	58.83	50.90	41.02
	18	67.99	45.02	57.34
	20	64.99	60.88	58.59
Avg.		75±10	60±5	70±12
P	2	69.83	84.34	101.29
	5	62.89	45.24	48.33
	6	62.98	51.89	47.88
	7	97.06	67.45	58.77
	8	57.62	64.64	63.09
	10	36.29	45.73	40.28
	14	53.88	58.70	43.32
	16	70.43	67.47	64.88
	17	52.46	61.38	44.98
	21	47.45	53.06	49.34
Avg.		61±10	59±5	56±12

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 46.

Raw Data For WBCGSH (µmol/10^6cells) Trial 1

Group	Subject	Ex1	Ex2	Ex3
WPI	1	1.33	1.31	
	3	.66	.63	.74
	4	.71	.69	.58
	9	.71	1.02	.62
	11	.47	.46	.47
	12	1.08	.73	.91
	13	1.28	.51	.57
	15	.66	.73	.42
	18	.87	.49	.64
	20	.67	.57	.64
Avg.		.84±.09	.72±.11	.62±.10
P	2	1.02	.60	1.08
	5	1.04	.47	.85
	6	1.56	1.62	1.85
	7	1.04	1.27	.41
	8	.73	.54	.56
	10	.87	.56	.38
	14	.61	.55	.63
	16	.51	.54	.81
	17	.63	.54	.61
	21	.74	.63	.59
Avg.		.87±.09	.73±.10	.82±.10

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 47.

Raw Data For WBCGSH (µmol/10^6cells) Trial 2

Group	Subject	Ex1	Ex2	Ex3
WPI	1	1.62	1.53	1.16
	3	.88	.68	.51
	4	.91	.68	.83
	9	.81	.64	.75
	11	.66	.59	.58
	12	1.55	1.51	1.10
	13	.73	.65	.59
	15	.91	.89	.82
	18	.81	.53	.79
	20	.79	.53	.77
Avg.		.96±.10	.84±.13	.74±.05
P	2	1.01	.63	.59
	5	.91	.94	.77
	6	.78	1.70	.75
	7	.67	1.22	1.79
	8	1.59	.48	.72
	10	.53	.66	.84
	14	.81	.56	.92
	16	.30	.64	.62
	17	.54	2.30	.98
	21	6.27	5.93	5.07
Avg.		.77±.1	.77±.13	.74±.05

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 48.

Raw Data For WBCGSH (µmol/10^6cells) Trial 3

Group	Subject	Ex1	Ex2	Ex3
WPI	1	.89	.49	.41
	3	1.08		1.36
	4	1.37	1.53	1.15
	9	1.73	1.47	1.41
	11	.73	.36	.45
	12	1.26	1.3	.23
	13	1.34	.42	1.21
	15	1.37	1.40	.86
	18	.99	.45	.97
	20	1.63	1.42	1.65
Avg.		1.23±.11	.98±.13	1.03±.13
P	2	1.07	.76	.71
	5	1.69	1.02	1.07
	6	.78	.82	1.06
	7	.59	.78	
	8	1.59	1.46	.55
	10	1.29	1.08	1.51
	14	1.66	1.31	1.39
	16	.95	.52	.85
	17	.94	1.00	1.44
	21	.95	.93	1.28
Avg.		1.15±.11	.96±.13	1.09±.13

EX1=baseline
EX2=immediate post submaximal exercise
EX3=immediate post exhaustion
WPI=whey protein isolate
P=placebo

Table 49.

Raw Data For CD3+ T-lymphocytes (%) Trial 1

Group	Subject	Ex1	Ex3	Ex4
WPI	1	69.9	69.5	71.5
	3	68.7	56.0	56.2
	4	73.4	68.0	75.1
	9	46.2	43.0	62.2
	11	79.4	66.7	72.8
	12	71.1	63.4	69.3
	13	79.5	66.7	71.0
	15	52.0	44.5	63.2
	18	65.7	47.4	67.3
	20	66.6	54.6	67.0
Avg.		67.3± 2.6	57.9±3.2	67.6±2.3
P	2	74.6	65.3	77.2
	5	44.0	30.3	60.1
	6	56.5	42.0	58.8
	7	72.6	58.8	71.1
	8	68.2	61.0	69.0
	10	62.8	50.7	62.1
	14	72.5	55.2	76.0
	16	71.0	67.7	79.7
	17	61.5	49.2	66.8
	21	67.7	56.0	60.5
Avg.		65.1±2.1	53.6±3.3	68.1±2.1

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 50.

Raw Data For CD3+ T-lymphocytes (%) Trial 2

Group	Subject	Ex1	Ex3	Ex4
WPI	1	71.9	67.0	69.5
	3	65.2	55.5	62.2
	4	73.7	76.3	73.2
	9	60.2	37.2	55.1
	11	79.9	65.0	73.0
	12	73.4	63.3	70.6
	13	70.8	66.6	78.9
	15	55.3	46.5	63.8
	18	62.4	52.3	72.3
	20	66.3	52.9	69.0
Avg.		67.9±2.4	58.2±3.2	68.8±2.6
P	2	69.1	62.1	77.0
	5	44.5	34.5	62.3
	6	48.2	44.3	59.4
	7	57.6	55.4	59.7
	8	75.2	68.4	72.5
	10	62.3	46.1	60.3
	14	72.9	51.4	74.2
	16	76.5	65.8	78.0
	17	52.4	42.2	56.2
	21	73.1	59.9	70.7
Avg.		63.2± 2.6	53.0±3.2	67.0±2.8

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 51.

Raw Data For CD3+ T-lymphocytes (%) Trial 3

Group	Subject	Ex1	Ex3	Ex4
WPI	1	66.6	56.3	68.1
	3	59.8	55.9	57.2
	4	71.8	76.2	76.7
	9	50.4	43.6	50.8
	11	81.8	62.0	73.0
	12	77.6	63.0	77.1
	13	77.9	72.8	80.2
	15	60.3	44.4	63.2
	18	53.0	39.6	61.3
	20	71.9	56.8	69.0
Avg.		67.1±2.3	57.0±3.4	67.7±3.2
P	2	69.5	59.9	71.8
	5	38.7	27.6	44.8
	6	56.2	46.7	49.5
	7	61.0	59.3	58.1
	8	71.6	66.8	74.7
	10	64.0	46.4	44.0
	14	71.1	58.2	72.2
	16	74.2	73.2	79.4
	17	59.8	54.7	68.8
	21	73.5	61.0	67.2
Avg.		63.9±2.5	55.4±3.5	63.1±3.8

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 52.

Raw Data For CD4+ T-lymphocytes (%) Trial 1

Group	Subject	Ex1	Ex3	Ex4
WPI	1	38.0	38.0	45.0
	3	25.9	14.0	24.5
	4	45.6	38.3	47.0
	9	31.2	27.1	42.5
	11	42.2	26.7	35.5
	12	34.5	25.2	37.1
	13	41.6	24.1	38.0
	15	34.4	25.2	45.0
	18	44.4	23.7	44.2
	20	34.7	18.8	41.0
Avg.		37.3±2.1	26.1±2.3	39.9±1.8
P	2	30.0	17.3	35.0
	5	24.3	11.4	37.6
	6	37.6	23.2	40.6
	7	41.2	21.0	42.7
	8	34.6	21.2	39.7
	10	44.7	31.5	42.7
	14	49.5	31.8	53.0
	16	43.0	37.3	52.6
	17	36.5	23.4	40.2
	21	46.0	29.6	41.2
Avg.		38.7±2.3	24.7±2.5	42.5±1.7

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 53.

Raw Data For CD4+ T-lymphocytes (%) Trial 2

Group	Subject	Ex1	Ex3	Ex4
WPI	1	43.3	32.3	41.8
	3	27.4	15.7	28.3
	4	31.0	24.5	37.0
	9	42.8	23.6	39.3
	11	41.0	26.5	38.1
	12	35.8	22.9	36.1
	13	35.1	23.7	44.0
	15	37.3	27.3	44.6
	18	42.0	27.5	49.0
	20	35.1	18.8	42.5
Avg.		37.8±2.0	24.3±1.8	40.0±1.8
P	2	30.3	20.4	36.4
	5	25.0	13.2	37.5
	6	31.4	25.0	43.2
	7	32.9	21.3	32.7
	8	38.9	23.2	38.4
	10	43.5	28.0	41.8
	14	52.9	32.4	53.6
	16	43.6	32.8	48.6
	17	27.4	17.3	30.6
	21	47.7	33.0	48.4
Avg.		24.7±2.1	24.6±2.1	41.1±2.2

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 54.

Raw Data For CD4+ T-lymphocytes (%) Trial 3

Group	Subject	Ex1	Ex3	Ex4
WPI	1	42.5	27.6	44.9
	3	28.6	22.1	29.9
	4	36.7	31.7	43.9
	9	35.7	27.2	36.3
	11	42.5	23.3	35.0
	12	38.7	21.7	38.8
	13	41.4	26.7	42.4
	15	40.0	25.2	44.4
	18	31.4	17.1	37.0
	20	37.3	20.9	44.0
Avg.		39.9±2.1	24.4±1.7	39.6±1.9
P	2	32.1	17.8	33.5
	5	21.1	10.0	26.9
	6	38.5	28.7	35.0
	7	36.3	24.7	33.6
	8	43.0	23.4	44.4
	10	44.7	29	29.8
	14	49.8	34.4	49.1
	16	44.5	40.5	48.6
	17	35.7	26.4	43.5
	21	49.1	32.5	44.0
Avg.		42.5±2.3	26.7±2.8	38.8±2.4

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 55.

Raw Data For CD8+ T-lymphocytes (%) Trial 1

Group	Subject	Ex1	Ex3	Ex4
WPI	1	30.0	28.1	22.4
	3	38.3	36.8	27.5
	4	20.2	24.5	23.3
	9	19.7	23.8	20.3
	11	30.8	31.1	31.0
	12	33.1	39.4	30.2
	13	29.6	32.2	26.9
	15	23.0	32.2	20.4
	18	25.3	35.9	27.1
	20	29.5	36.4	24.8
Avg.		27.9±2.0	32.0±2.0	25.4±1.2
P	2	40.5	43.5	37.2
	5	33.3	34.9	29.1
	6	23.4	32.0	18.6
	7	24.1	33.5	21.3
	8	34.1	44.6	29.0
	10	21.4	25.9	22.2
	14	25.1	38.0	24.7
	16	33.0	34.1	27.4
	17	27.3	35.9	27.2
	21	26.0	30.1	18.3
Avg.		28.8±2.1	35.2±2.2	25.5±2.1

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 56.

Raw Data For CD8+ T-lymphocytes (%) Trial 2

Group	Subject	Ex1	Ex3	Ex4
WPI	1	26.6	34.6	27.0
	3	36.0	39.6	31.9
	4	37.4	43.4	32.4
	9	21.0	22.6	17.4
	11	31.0	29.5	29.0
	12	37.4	40.5	32.2
	13	27.2	32.9	29.7
	15	24.8	32.6	22.1
	18	26.6	37.6	24.5
	20	28.7	36.5	25.0
Avg.		29.7±1.8	34.9±2.4	27.1±1.2
P	2	35.2	37.4	34.1
	5	28.0	35.5	26.0
	6	20.6	33.7	19.1
	7	20.6	33.2	23.7
	8	36.5	50.5	33.4
	10	22.8	26.4	20.8
	14	23.2	33.4	21.7
	16	33.4	36.8	28.5
	17	26.8	32.7	26.8
	21	26.5	32.1	22.4
Avg.		27.4±2.1	35.1±2.1	25.7±2.0

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 57.

Raw Data For CD8+ T-lymphocytes (%) Trial 3

Group	Subject	Ex1	Ex3	Ex4
WPI	1	25.4	32.7	21.6
	3	29.7	33.4	26.4
	4	27.7	32.4	26.8
	9	18.4	22.8	17.5
	11	31.5	28.3	29.6
	12	35.7	41.6	35.4
	13	27.3	33.4	29.5
	15	27.6	35.0	24.1
	18	26.0	35.7	25.5
	20	30.3	36.0	26.0
Avg.		27.9±2.4	33.1±1.8	26.2±1.4
P	2	33.3	38.8	33.4
	5	34.2	35.2	20.6
	6	21.2	33.4	18.5
	7	21.0	29.5	20.9
	8	28.3	49.1	29.4
	10	23.0	25.1	19.3
	14	25.0	33.1	25.4
	16	29.9	34.5	28.4
	17	28.7	37.0	26.2
	21	23.5	32.0	23.7
Avg.		26.8±1.6	34.7±2.4	24.6±1.4

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 58.

Raw Data For CD4/CD8+ T-lymphocyte ratio Trial 1

Group	Subject	Ex1	Ex3	Ex4
WPI	1	1.27	1.35	2.01
	3	0.68	0.38	0.89
	4	2.26	1.56	2.02
	9	1.58	1.14	2.09
	11	1.37	0.86	1.15
	12	1.04	0.64	1.23
	13	1.41	0.75	1.41
	15	1.50	0.78	2.21
	18	1.75	0.66	1.63
	20	1.18	0.52	1.65
Avg.		1.4±0.1	0.8±0.1	1.6±0.1
P	2	0.74	0.40	0.94
	5	0.73	0.33	1.29
	6	1.61	0.73	2.18
	7	1.71	0.63	2.00
	8	1.01	0.48	1.37
	10	2.09	1.22	1.92
	14	1.97	0.84	2.15
	16	1.30	1.09	1.92
	17	1.34	0.65	1.48
	21	1.77	0.98	0.25
Avg.		1.4±0.1	0.7±0.1	1.7±0.1

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 59.

Raw Data For CD4/CD8+ T-lymphocyte ratio Trial 2

Group	Subject	Ex1	Ex3	Ex4
WPI	1	1.63	0.93	1.55
	3	0.76	0.40	0.89
	4	0.83	0.56	1.14
	9	2.04	1.04	2.26
	11	1.32	0.90	1.31
	12	0.96	0.57	1.12
	13	1.29	0.72	1.48
	15	1.50	0.84	2.02
	18	1.58	0.73	2.00
	20	1.22	0.52	1.70
Avg.		1.3±0.1	0.7±0.07	1.5±0.1
P	2	0.86	0.55	1.07
	5	0.89	0.37	1.44
	6	1.52	0.74	2.26
	7	1.60	0.64	1.38
	8	1.07	0.46	1.15
	10	1.91	1.06	2.01
	14	2.28	0.97	2.47
	16	1.31	0.89	1.71
	17	1.02	0.53	1.14
	21	1.80	1.03	2.16
Avg.		1.4±0.1	0.7±0.07	1.7±0.1

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 60.

Raw Data For CD4/CD8+ T-lymphocyte ratio Trial 3

Group	Subject	Ex1	Ex3	Ex4
WPI	1	1.67	0.84	2.08
	3	0.96	0.66	1.13
	4	1.32	0.98	1.64
	9	1.94	1.19	2.07
	11	1.35	0.52	1.10
	13	1.52	0.80	1.44
	15	1.45	0.72	1.84
	18	1.21	0.48	1.45
	20	1.23	0.58	1.69
Avg.		1.3±0.1	0.7±0.08	1.5±0.1
P	2	0.96	0.46	1.00
	5	0.62	0.28	1.31
	6	1.82	0.86	1.89
	7	1.73	0.84	1.61
	8	1.52	0.48	1.51
	10	1.94	1.16	1.54
	14	1.99	1.04	1.93
	16	1.49	1.17	1.71
	17	1.24	0.71	1.66
	21	2.09	1.02	1.86
Avg.		1.5±0.1	0.8±0.08	1.6±0.1

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 61.

Raw Data For Oxidative Burst Activity (% cells) Trial 1

Group	Subject	Ex1	Ex3	Ex4
WPI	1	96.6	98.2	97.9
	3	97.9	96.8	98.9
	4	97.2	94.0	99.5
	9	94.4	87.5	
	11	94.3	87.3	94.1
	12	96.6	95.4	98.6
	13	95.5	94.4	98.8
	15	96.5	94.5	97.4
	18	94.7	70.6	91.9
	20	97.8	98.1	99.4
	2	93.2	96.5	99.0
Avg.		96.3± 1.9	92.1±2.3	97.3±1.3
P	5	72.3	89.4	83.5
	6	97.0	95.8	97.9
	7	95.1	95.9	97.3
	8	97.3	91.0	98.0
	10	88.8	95.8	98.2
	14	94.0	97.2	98.0
	16	98.9	98.1	98.4
	17	95.6	81.3	82.2
	21	96.9	98.5	97.7
Avg.		92.9±1.8	93.9±2.2	96.0±1.3

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 62.

Raw Data For Oxidative Burst Activity (% cells) Trial 2

Group	Subject	Ex1	Ex3	Ex4
WPI	1	97.7	98.6	98.3
	3	98.9	97.8	97.7
	4	98.6	98.5	97.2
	9	93.3	99.3	96.7
	11	96.3	94.0	95.6
	12	88.5	95.4	98.8
	13	68.2	55.1	87.5
	15	93.2	93.2	97.0
	18	87.9	84.6	93.5
	20	96.8	98.2	97.6
Avg.		91.9±3.1	91.4±5.4	95.9±3.1
P	2	96.0	97.9	97.7
	5	91.7	94.3	95.0
	6	93.4	97.6	97.0
	7	96.7	92.9	97.9
	8	98.5	98.0	99.0
	10	83.5	50.0	68.1
	14	95.1	97.4	96.6
	16	63.4	47.3	62.5
	17	95.4	98.2	96.6
	21	97.0	96.0	98.0
Avg.		91.0±3.1	86.9±5.4	90.8±3.1

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 63.

Raw Data For Oxidative Burst Activity (% cells) Trial 3

Group	Subject	Ex1	Ex3	Ex4
WPI	1	94.8	94.1	89.8
	3	98.8	99.2	99.6
	4	99.5	99.2	99.1
	9	67.4	80.4	78.6
	11	95.8	95.0	97.7
	12	95.6	95.2	92.6
	13	91.5	81.4	96.2
	15	93.3	97.3	94.4
	18	87.1	95.2	94.2
	20	96.0	95.0	98.1
Avg.		91.9±2.2	93.2±1.5	94.0±1.5
P	2	93.7	97.8	96.3
	5	93.8	93.9	97.0
	6	96.4	97.1	96.8
	7	97.5	92.4	99.3
	8	93.7	95.8	94.8
	10	87.5	95.2	89.0
	14	94.0	95.2	97.8
	16	90.9	90.2	95.0
	17	97.2	96.1	99.0
	21	96.7	93.8	98.0
Avg.		94.1±2.2	94.7±1.5	96.3±1.5

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 64.

Raw Data For Phagocytic Activity (% cells) Trial 1

Group	Subject	Ex1	Ex3	Ex4
WPI	1	16.4	17.2	24.3
	3	8.3	19.5	15.0
	4	14.8	16.4	12.8
	9	49.5	36.2	48.9
	11	38.1	38.6	36.2
	12	34.3	29.7	26.3
	13	74.8	62.0	59.8
	15	78.2	68.8	80.8
	18	29.1	26.0	35.0
	20	34.7	34.8	31.6
Avg.		37.8±7.6	34.9±6.4	37.0±6.9
P	2	10.4	12.0	6.1
	5	25.5	26.0	23.7
	6	12.0	12.1	12.0
	7	15.6	18.0	19.0
	8	62.5	39.5	53.7
	10	73.3	71.2	71.7
	14	45.6	65.2	38.8
	16	73.0	59.7	70.1
	17	25.1	21.7	39.3
	21	26.9	23.6	33.2
Avg.		36.9±7.6	34.9±6.4	36.7±6.9

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 65.

Raw Data For Phagocytic Activity (% cells) Trial 2

Group	Subject	Ex1	Ex3	Ex4
WPI	1	19.9	18.1	13.3
	3	32.0	25.1	24.9
	4	18.5	21.2	16.5
	9	20.2	17.7	16.3
	11	30.7	39.9	37.7
	13	16.7	17.5	17.3
	15	15.7	11.0	17.5
	18	39.1	30.1	46.6
	20	48.9	43.6	54.5
Avg.		27.0±4.6	24.8±4.7	29.0±5.9
P	2	17.1	20.3	10.0
	5	22.8	17.6	12.1
	6	28.1	12.1	8.8
	7	17.4	8.9	11.6
	8	19.5	16.8	21.6
	10	19.7	21.6	21.4
	14	24.4	18.0	17.7
	16	11.5	11.8	6.5
	17	74.0	73.9	80.3
	21	28.4	25.2	25.7
Avg.		26.2±4.6	22.6±4.7	21.5±5.9

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 66.

Raw Data For Phagocytic Activity (% cells) Trial 3

Group	Subject	Ex1	Ex3	Ex4
WPI	1	31.0	25.4	21.2
	3	41.6	39.7	36.8
	4	35.4	37.0	39.6
	9	70.9	70.0	49.5
	11	78.3	78.2	75.9
	12	71.1	57.7	65.0
	13	23.0	27.7	25.6
	15	33.0	28.3	21.5
	18	63.8	52.9	64.3
	20	61.2	50.5	58.4
Avg.		50.9±6.8	46.7±6.2	45.7±6.7
P	2	20.2	20.8	17.3
	5	18.9	18.1	19.5
	6	16.4	15.6	19.2
	7	11.8	9.4	11.0
	8	69.8	67.9	73.8
	10	34.0	37.1	29.1
	14	66.4	63.1	64.0
	16	17.8	19.2	15.3
	17	60.4	47.6	50.3
	21	54.9	50.3	51.3
Avg.		37.0±6.8	34.9±6.2	35.0±6.7

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 67.

Raw Data For Lymphocyte Proliferation Trial 1

Group	Subject	Ex1	Ex3
WPI	3	1077	1059
	4	939	942
	9	1045	1074
	11	848	851
	12	455	804
	13	1073	1007
	15	1049	1020
	18	934	1003
	20	876	632
Avg.		921±59	932±50
P	5	779	771
	6	648	808
	7	848	788
	8	1055	1075
	10	1003	1150
	14	974	935
	16	1111	1074
	17	1096	1170
	21	1037	970
Avg.		930±62	958±54

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 68.

Raw Data For Lymphocyte Proliferation Trial 2

Group	Subject	Ex1	Ex3
WPI	3	1866	2507
	4	1606	2757
	9	2671	2960
	11	2048	2933
	12	3074	2975
	13	1457	2422
	15	2322	2602
	18	2830	2650
	20	2645	2921
Avg.		2279±236	2747±200
P	5	1786	1111
	6	762	896
	7	1931	1669
	8	2411	2555
	10	2098	2236
	14	3236	3060
	16	2950	2610
	17	2686	2249
	21	3352	3232
Avg.		2282±250	2126±212

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo

Table 69.

Raw Data For Lymphocyte Proliferation Trial 3

Group	Subject	Ex1	Ex3
WPI	3	2601	3223
	4	2483	3331
	9	2257	3147
	11	2661	3554
	12	2277	2667
	13	1575	1353
	15	1942	2992
	18	2380	1725
	20	2612	2798
Avg.		2309±203	2754±277
P	5	1752	905
	6	2210	1797
	7	1007	885
	8	1233	609
	10	2379	1675
	14	3115	2899
	16	293*	123*
	17	3059	3001
	21	2851	1079
Avg.		2200±216	1606±294

EX1=baseline
EX3=immediate post exercise
EX4=1 h post exercise
WPI=whey protein isolate
P=placebo
*> 2 SD away from mean, thrown out

Table 70.

Performance Tests (seconds)

Group	Subject	Trial1	Trial2	Trial3
WPI	1		72	128
	3	248	276	98
	4	274	123	62
	9	309	258	151
	11	124	244	241
	12	370	271	360
	13	155	222	98
	15	219		251
	18	320	316	272
	20	212		272
Avg.		257±89	244±61	183±11
P	2	200	225	175
	5	128	301	422
	6			121
	7	105	331	128
	8	254	310	420
	10	124	200	129
	14	222	252	216
	16	246	363	146
	17	192	307	184
	21	146	161	106
Avg.		179±55	272±66	214±121

Table 71.

Peak Power (watts)

Group	Subject	Trial1	Trial2	Trial3
WPI	1	688	688	688
	3	944	858	858
	4	847	715	715
	9	564	635	635
	11	571	571	571
	12	667	667	815
	13	763	763	694
	15	649	730	892
	18	705	705	705
	20	717	789	652
Avg.		711.8±45	712.4±33	722.7±35
P	2	640	720	720
	5	510	510	510
	6	612	489	550
	7	1024	867	709
	8	847	847	847
	10	905	815	905
	14	635	776	705
	16	730	730	730
	17	635	706	777
	21	560	720	720
Avg.		709.8±45	718.1±34	718.1±34

Table 72.

Fatigue Rate (%)

Group	Subject	Trial1	Trial2	Trial3
WPI	1	40	30	30
	3	45	39	45
	4	38	41	42
	9	42	40	42
	11	11	11	11
	12	34	36	34
	13	42	42	42
	15	24	24	26
	18	40	41	40
	20	28	29	28
Avg.		34.4±3.3	33.3±3.1	34.0±3.3
P	2	25	44	25
	5	22	25	22
	6	40	42	42
	7	45	45	45
	8	36	36	36
	10	24	25	24
	14	14	16	16
	16	42	42	40
	17	36	38	36
	21	42	42	44
Avg.		32.6±3.3	35.5±3.1	33.0±3.3

Table 73.

Total Work (watts)

Group	Subject	Trial1	Trial2	Trial3
WPI	1	3441	3785	3647
	3	4465	4637	4465
	4	3845	3845	3765
	9	3664	3548	3664
	11	3176	3176	3096
	12	4128	4284	4128
	13	3874	3874	3874
	15	3447	3447	3223
	18	3664	3442	3664
	20	3541	3426	3541
Avg.		3724±135	3746±136	3706±141
P	2	3360	3440	3360
	5	3264	3389	3264
	6	3544	3544	3544
	7	4508	4508	4508
	8	3888	3888	3888
	10	3864	3864	3864
	14	3211	3446	3446
	16	4465	4465	4668
	17	3324	3894	3726
	21	3894	3894	3726
Avg.		3732±135	3789±136	3759±141

Vita

Max Shute

Max Shute, son of Dixie L. and David T. Shute, was born July 7, 1970, in Peoria, IL. He has always had an interest in nutrition, sport, and exercise. In 1992, he received a Bachelor of Science degree in Dietetics from Western Illinois State University. He then spent the next few years racing his bicycle and working as a bicycle mechanic in Washington, IL. There his interest grew in counseling both recreational and elite level cyclists in nutrition and training.

In 1998, Max had the opportunity to pursue graduate studies at Appalachian State University in Boone, NC. He received a Master of Science in Exercise Science in 2000. While a student at Appalachian State he enjoyed working as a teaching and research assistant. During this time he discovered a love for teaching. Motivated by a very supportive ASU faculty he decided to pursue further education at the doctoral level. In the Fall 2000 he began coursework in Sports Nutrition at Virginia Tech.

On a personal note, Max has one brother Truman, two nieces and one nephew residing in Peoria, IL close to his parents. Jen, his wife of 8 years, is finishing up her Ph.D. in Marriage and Family Therapy also from Virginia Tech. Jen and Max enjoy spending time with their two dogs, Solomon and George, and one cat, Herman. In his spare time Max enjoys road and mountain cycling and cooking.