THE EFFECTS OF WHEY PROTEIN AND SOY PROTEIN IN THE PREVENTION OF EXERCISE-INDUCED OXIDATIVE STRESS AND INFLAMMATORY DAMAGE IN HUMAN ATHLETES

By

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To the Faculty of Washington State University:

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Abstract

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Back ground: During endurance athletic events, metabolic changes occur that are detrimental to physiological homeostasis. Inflammatory response during exercise occurs, potentially resulting in increased oxidative damage. Protein supplements from whey and soy may therefore play a role in alleviating oxidative damage and improve body composition and immune support.

Objective: The objective of this study was to evaluate the effects of whey and soy protein isolate on oxidative stress, inflammatory damage, immune biomarkers, and body composition in female endurance athletes.

Design: Healthy female endurance athletes (18-25 yr; n =33) were fed 40 g of whey or soy protein daily for 6 wk. Blood samples were collected on wk 0 and 6 for analysis of oxidative, inflammatory, and immune biomarkers. DXA scans were performed to determine body composition.

Results: Whey protein intervention lowered ($P<0.05$) plasma CRP concentrations after 6 wk of supplementation in female athletes compared to those who received soy protein. There was no corresponding change in circulating IL-1 and IL-6, suggesting that the anti-inflammatory action of whey protein is unlikely due to changes in these cytokines. In addition, whey but not soy protein decreased ($P<0.01$) lipid peroxidation. In contrast, athletes given soy protein had lower plasma 8-OHdG ($P<0.07$). Total bone mineral density ($P<0.05$) and bone mineral composition ($P<0.06$) were higher ($P<0.06$) in subjects fed whey protein than those given soy protein. However, total bone mass ($P<0.06$) and lean mass ($P<0.05$) were higher in subjects given soy protein than those given whey protein.

Conclusions: Both whey and soy protein supplements can be beneficial to athletes. Both protein supplements provide anti-oxidative action. Specifically, WPI increased bone mineral, decreased inflammation, lipid peroxidation, and body fat, while SPI decreased DNA damage and increased total and lean body mass.

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REVIEW OF LITERATURE

Introduction

Competition in sport has increased the demand for elite athletes to reach above and beyond natural talent and hard work. Consequently, athletes are pursuing options such as nutrition supplement products to enhance their performance, fueling the competitive edge. Nutrition has come to the forefront as a key component for most successful athletes (Ray et al. 2004). Up to 89% of surveyed college athletes stated they have or currently use nutritional supplements (Froiland et al. 2004, Neiper et al. 2005). Athletes consider nutritional supplements critical for supporting current performance and promoting longevity in sport participation and physical wellbeing.

Exercise has undeniable health benefits; however, not all side effects of exercise are positive. Undesirable physiological changes, if not alleviated, are harmful to the human body including inflammation, increased blood pressure, DNA damage, and increased cytokine production.

Increased levels of competition lead to an increase in exercise time and intensity. Athletes are running, cycling, and swimming for longer periods of time, and at a higher level of intensity, creating an increase in aerobic metabolism. This results in a high oxygen demand necessary for increased ATP production to provide energy, with an unfortunate consequence of reactive oxygen species (ROS) and other metabolic byproducts. The ensuing cascade effect from these free radicals results in cell damage including apoptosis, lipid peroxidation, protein oxidation, and DNA modification.

During normal homeostasis, ROS are also produced, but within the capacity of the innate antioxidant system to effectively control and neutralize. However, endurance athletes place a

higher demand on these systems, leading to excessive ROS accumulation. This oxidative stress creates an imbalance between antioxidant defenses and the ROS, and a subsequent exercise-induced inflammation.

Endurance athletes can be broadly defined as those who use oxidative phosphorylation as their main energy source (Tarnopolosky, 2004). It is clear that carbohydrates and fats are the primary fuel source during endurance events. However, proteins are essential for recovery, rebuilding, and as a secondary energy source.

Inflammation and oxidative damage have been linked to muscle damage (Dekkers et al. 1996). Muscle damage requires increased recovery time to an athlete. The potential for a protein supplement with antioxidant capabilities to minimize muscle damage would then allow athletes more time to spend in practice and competition, and thus less recovery time.

Exercise and Body Composition

The health and success of an athlete is directly related to his/her overall body composition. Active people, more specifically athletes, require more energy to maintain lean muscle mass, immune function, reproductive health, and optimal performance. Adequate caloric and nutrient intake is critical to an athlete's performance. Various activities require different physiological demands. Power-strength exercise utilizes ATP-CP exclusively derived from glycolysis through anaerobic metabolism. Aerobic metabolism utilizes two systems to provide the ATP during endurance events: the Krebs cycle and oxidative phosphorylation pathways. Aerobic exercise results in reduced body fat, increased maximal oxygen uptake, increased respiratory capacity, lower blood lactate concentrations, increased mitochondrial and capillary densities, and improved enzyme activity (Campbell et al 2007, Haus et al 2009, Hamer and

Stamatakis 2009). During endurance exercise, the predominant fuels are carbohydrates and fats, although skeletal muscle utilizes approximately 1 -6% of total energy from amino acids. Physiological adaptations to aerobic training vary according to gender and age (McKenzie et al. 2000, Tarnoposky et al. 1990, Phillips et al.1993).

Muscle Development

Muscle is composed of 75% water, 20% protein, and 5% inorganic salts and other substances such as phosphates, urea, lactic acid, minerals, enzymes, ions, carbohydrates, and lipids. Muscle hypertrophy occurs when there is a change in normal balance between muscle protein synthesis and protein catabolism.

Proteins are comprised of amino acids. Of the 20 amino acids, 9 are considered essential: histidine, isoleuicine, leuicine, lysine, methionine, phenylalanine, theronine, tryptophan, and valine (Smith et al. 1996). Protein synthesis is a step-wise process requiring transcription and translation. Russell et al. (2003) established that protein synthesis is dependent on exercise intensity. After endurance exercise, it is proposed that the higher need for amino acids is determined by increased concentrations of enzymes, red blood cells, hemoglobin, and myoglobin. Bohe et al. (2003) found that muscle protein synthesis is modulated by extracellular amino acid availability. Consumption of protein supplements could increase the potential efficacy of amino acid use (Pilegaard et al. 2003, Tipton et al. 1999) and can therefore improve the metabolic function of muscle.

Muscle is comprised of three types: Type I (slow twitch), Type II (fast twitch), and Type III (involuntary contraction). Type II muscles use anaerobic metabolism to produce energy and therefore have a high electrochemical transmission of action potential, whereas Type I muscles

characteristically have a high activity level of myosin ATPase and a rapid release of calcium. Energy transfer in Type II utilizes well-developed, short-term glycolytic systems. Uptake by a highly developed sarcoplasmic reticulum and a high rate of cross-bridge turnover enable fast twitch muscle to generate quick and powerful contractions. Conversely, Type I is distinguished by low activity of myosin ATPase, slower calcium release, and a glycolytic capacity less developed than fast twitch. These defining characteristics result in slower muscle contraction, thus slow twitch. However, Type I contain high concentrations of myoglobin and mitochondria as seen in the characteristic red pigment. Competitive endurance athletes tend to have higher percentages of Type 1 fibers as opposed to sprinters with a higher percentage of Type II fibers (Gollnick et al. 1972). There is no evidence that fast-twitch can convert to slow-twitch muscles, or vice versa, through a normal training regime. Research in male athletes has shown, adaptation due to strenuous endurance training include increased mitochondrial content in Type II, but not necessarily Type I fibers. At the mitochondrial enzyme level, the difference between Type I and Type II fibers is mostly or completely eliminated in highly trained endurance athletes (Jansson and Kaijser 1977, Chi et al. 1983). Oxidative enzyme capacity in trained endurance athletes is increased. However, the amount of oxygen circulating in the muscle is also increased. Resulting in the production of ROS, this may in turn increase fatigue and muscle cell membrane damage.

Bone Composition

Classified as a connective tissue, bone is unique. It becomes mineralized and thereby able to provide a rigid structure. Bone is very sensitive to external forces, has the capacity to grow, and ability to regenerate if damaged. External forces such as bending, compressive, torsion, and internal forces created by muscle contraction at the tendon attachment site influence bone health.

The strongest determinants of maximal bone mass are speculated to be genetic factors; however, nutrition and mechanical loading play a significant role (Krall et al. 1993, Slemeda et al. 1991).

General nutrition is very important in maximizing bone health. Calcium (Cashman, 2002), magnesium (Toba et al. 2000), vitamin D (Nieves, 2005), vitamin K (Bugel, 2003), casein phosphopeptides (Saito et al.1998), and isoflavones (Ishimi, et al. 1999) are some of the supportive nutrients to essential bone mass. Calcium and magnesium are major constituents of bone. Vitamin D, casein phosphopeptides, vitamin K and isoflavones also directly affect bone health and metabolism. Bovine milk is an excellent source of bio-available calcium. More specifically, current research has demonstrated that whey protein has a functional role in bone remodeling (Takada et al. 1996, Takada et al. 1997).

Remodeling is the ongoing replacement of old bone tissue with new bone tissue. Bone, just like skin, forms *in utero* but continually renews itself during life. Bone formation occurs when a force reaches or exceeds the threshold stimulus; this is termed minimal essential strain (MES). This signals osteoblastic migration to the area. Matrix proteins are laid to reinforce bone strength in the area. The MES will vary with age and gender (Frost, 1987). Although bone loss is seen as a common symptom of aging and menopause, it is difficult to prevent. Maximizing peak bone mass in early adulthood is the most successful strategy for preventing osteoporosis, a reduction in bone mineral density (BMD) (Moreira-Kulak and Bilezikian, 1998).

A delicate homeostasis exists between the actions of the osteoclasts, which function to reabsorb previously made bone, and the osteoblasts. The osteoclasts are capable of breaking down bone protein and matrix, whereas osteoblasts function primarily in bone construction, and are regulated by numerous hormones, including estrogen.

The hormones estrogen, insulin, and leptin have been linked to increased bone formation markers (Cornish et al. 2002, Hamrick et al. 2005, Ducy et al. 2000). However, excess adipose tissue has been shown to hinder bone growth by inhibiting osteoblastic differentiation. In conditions of bone loss, such as estrogen depletion, bone marrow adipogenesis increases (Klein et al. 1998). The mechanisms by which bone and fat are regulated are closely related as both adipocytes and osteoblasts originate from mesenchymal stem cells in bone marrow (Turner et al. 1994).

In cross-sectional studies, athletes have been shown to have significantly higher bone mineral density compared to non-athletes (Heinonen et al. 1993, Slemenda and Johnston 1993). However, in athletes who participate in non-weight-bearing or partial weight-bearing activity such as swimming or cycling, BMD was not significantly different (Risser et al. 1990). The two groups are not necessarily discrete in terms of BMD, since it has also been found that female runners did not have higher bone mineral content in the spine and femoral neck (Heinrich et al 1990). This is contrary to the impression that running is beneficial to BMD due to the lower leg impact with the running surface. Robinson et al (1995) determined that female runners had a lower BMD than gymnasts, despite amenorrhea and oligomenorrhea. It has been hypothesized that a direct correlation exists between endurance trained female athletes, unstable menstrual function, and restricted dietary intake resulting in a deleterious effect on BMD (Myburg et al. 1993, Drinkwater et al. 1990, Cann et al. 1984).

Body Fat Composition

Body fat is an essential component of human body composition. The body is mainly comprised of water, lean muscle, bone, and fat. An ideal, healthy body composition is a delicate

balance of these components. Body fat is essential in protecting vital organs, reproduction health, maintaining body temperature, and as a source of stored energy. The upper limit threshold for fat storage is infinite. The American Council on Exercise recommends a minimum essential body fat of 12-15% for young females and 2-5% for young males. Most elite athletes maintain a body fat percentage within or below the recommendations.

Body fat percentage is a more accurate indicator of health in athletes than body mass index (BMI). BMI is less exact because it does not differentiate the components of total weight (Ode et al. 2007). Calipers, hydrostatic weighing, and electronic impedance systems can all measure body fat percentage; however, measurement with a DXAA scan is the most accurate (Pineau et al. 2007, Flakol et al. 2004).

Fatty acid (FA) oxidation provides the majority of energy during low intensity prolonged exercise. Whole body lipid oxidation increases and lactate formation is reduced progressively during prolonged exercise. Plasma concentrations of FA increase the availability of FA, which in turn increases lipid oxidation. The control of lipid oxidation during exercise is not completely understood (Spriet et al. 2002). During prolonged exercise, performance is dependent upon the efficiency in using FA as a fuel. An increased capacity of mitochondria to oxidize FA would therefore be advantageous to an endurance athlete. Fernstrom et al. (2007) showed that mitochondrial efficiency is reduced with endurance exercise. This decrease creates a cascade of increased oxygen consumption, increased oxidative stress, increased lipid peroxidation, and tissue damage (Davies et al. 1982, Mastaloudis et al. 2001).

Exercise, Immune Response, and Oxidative Damage

General Immune Response

The immune system is composed of two branches of immunity: innate immunity, also known as natural immunity, and adaptive (acquired) immunity. Innate immunity mechanisms are the human body's first line of defense. Adaptive immunity occurs in response to specific antigens. The epithelial surfaces of the body are a barrier against most microorganisms, but the surface epithelium is more than a physical barrier. It also produces antimicrobial enzymes and peptides. If a microorganism is able to cross the barrier, macrophages are the first to encounter the pathogens. Macrophages release cytokines, chemokines, and other mediators that cause a state of inflammation in the tissue. Inflammation brings neutrophils and plasma proteins to the site of an infection. An inflammatory response includes symptoms of pain, swelling, redness, and heat.

It is well known that exercise is beneficial to health. Regular exercise decreases the risk of developing cardiovascular disease (Fletcher et al. 2001) and produces anti-inflammatory effects; these are, as yet, unconfirmed. However, it is speculated that the anti-inflammatory effects are due to a transient increase in circulating anti-inflammatory cytokines and decrease in basal pro-inflammatory cytokines (Wilund et al. 2007, Goldhammer et al. 2005, Kasapis et al. 2005). Wilson et al. (1990) and Mundal et al. (1994) both demonstrated that exercise could increase cardiovascular mortality in men due to an abnormal increase in systolic blood pressure during exercise. It is not completely understood why this is correlated to an increased risk for hypertension and cardiovascular incidences. It has been found that the increased blood pressure is related to impaired endothelial vasodilator function (Stewart and Hoskin 2004). The endothelium plays a vital role in blood flow regulation, coagulation, fibrinolysis, and

inflammation between blood and tissue. During exercise, increased blood flow creates increased stress on the blood vessels.

Although inflammation during exercise is a normal physiological response, it could exceed the body's capacity to control inflammation during intense exercise. The incidence of upper respiratory tract infections (URTI) is more prevalent following endurance exercise; it was therefore proposed that the body is taxed from the exercise that the immune system becomes vulnerable (Bermon et al. 2007, Nieman et al. 2003).

Humans have an elaborate antioxidant defense system to protect cells against carbon-centered free radicals. During the resting state, a healthy human body is capable of defending against ROS that are produced as a result of improved cellular metabolism. However, exercise disrupts the homeostasis of the defense system (Alessio 1993, Kanter et al. 1993, Jenkins et al. 1993, Robertson et al. 1991). During aerobic exercise, increased oxygen consumption causes a greater rate of flow of electrons through the mitochondria electron transport chain. This amplifies free radical production.

A "free radical" can be defined as any species that contains one or more unpaired electrons, an unpaired electron being one that is alone in orbital. Most biological molecules contain only one-paired electrons. A radical might donate its unpaired electron to another molecule or it may take an electron from another molecule in order to pair the electrons. Most of the free radicals originate during the final stages of cell respiration.. When the mitochondria are functioning, an electron may leak directly onto an oxygen molecule resulting in a superoxide radical:

$$O_2 + e^- \rightarrow O_2^-$$

Electrons added to molecular oxygen must be transferred one at a time during reduction (Sen, 1995). This transfer results in several highly reactive intermediates (Yu, 1994). Four steps are required to complete the reduction of oxygen to water which generates several free radicals and H_20_2 (hydrogen peroxide). As an ROS, H_20_2, can generate highly reactive free radicals through their interaction with reactive transition metals (Aruoma et al. 1987, Aruoma et al. 1991).

Lipid peroxidation destroys cell membrane polyunsaturated fatty acids (PUFA), and is one of the well-described consequences of the destructive nature of ROS and free radicals. However, the univalent reduction of oxygen produces a series of free radicals and ROS that interact not only with lipids, but also with DNA and proteins. When DNA and protein interact with the free radicals and ROS, there is subsequent degradation of proteins, DNA-strand breaks, and damage to other genomic structures (Clarkson and Thompson 2000).

Cells have their own protective mechanisms against free radical and ROS attacks, the endogenous antioxidant. Glutathione (GSH), as a substrate for glutathione peroxidase, can deliver the H necessary for the removal of H_20_2. When GSH is acted on by glutathione peroxidase, the reduced form of glutathione (GSSG) is produced. Selenium, a mineral, is an essential component of glutathione peroxidase. These antioxidant enzymes function to reduce lipid peroxidation (Bounous et al. 2003, Clarkson et al. 2000). In the plasma, the active SH (thiol) group of cysteine can deliver the necessary H (Bounous et al. 2003). Not only do these endogenous antioxidants work to combat ROS and free radicals but, they also nutrients.

Vitamin E is the major lipid-soluble antioxidant in cell membranes, and protects against lipid peroxidation (Bieri, 1990). Vitamin C can interact with tocopherol (vitamin E) radicals to regenerate reduced tocopherol. Vitamin C is water-soluble and can react with superoxide,

hydroxyl radicals and singlet oxygen (Sauberlich, 1990). Beta-carotene is considered the most efficient “quencher” of singlet oxygen. It is the major precursor to vitamin A (Olson, 1990).

Whey and soy protein have been shown to exhibit immunological properties similar to that of antioxidants. In whey, lactoferrin and its peptide product, lactoferricin, possess free metal ion (iron and copper) sequestering activity in tissue culture and animal studies (Stocker et al. 1991). Beta-lactalbumin, beta-lactoglobulin, and whey proteins also have immune-modulation activity. Whey contains cysteine-rich proteins that are required for proteins involved in an acute phase response.

Glutamine, an amino acid, is highly concentrated in whey protein (Bucci and Unlu 2000). Glutamine is a fuel for rapidly dividing cells during times of stress or illness. In ‘over-trained athletes’ and following a period of intense exercise, endogenous glutamine concentrations are depressed. This decrease in plasma glutamine may cause immunosuppression, resulting in a higher incidence of infection and slower wound healing (Hiscock and MacKinnon 1998). It has been suggested that supplemental glutamine could enhance immune response during times of stress and illness. Whey protein isolate contains the highest concentration of essential amino acids, including the branched-chain amino acids (BCAA) (Butterfield and Calloway 1984). The BCAA are the precursors to glutamine synthesis. Supplementation with whey protein may attenuate the decline in plasma glutamine in endurance athletes; thereby maintain normal immune response (Bassit et al. 2002).

Soy protein isolates, and more specifically isoflavone components of the soybean, have been shown to aid against oxidative stress. Oh et al. (2007) demonstrated that soy isoflavone supplementation improved the antioxidant status in Sprague- Dawley rats fed 2.39 mg/g protein for 12 days. More specifically, DNA damage can be inhibited through isoflavone intervention

(Ryan-Borchers et al. 2006). DNA damage is the culprit in many diseases and cancer. However, Vega-Lopez et al. (2005) reported that diets high in soy protein or soy-derived isoflavones had little effect on plasma antioxidant and biomarkers of oxidative stress.

Inflammatory Response

Physical exercise induces various changes in the human immune system. Strenuous exercise induces an increase while moderate walking reduces inflammation (Nieman et al. 1998). Cardinal symptoms of inflammation include redness, swelling, pain, and heat. Cytokines and natural killer (NK) cells are critical components of the defense immune system. Specialized proteins that stimulate or inhibit the differentiation, proliferation, or function of immune cells are categorized as cytokines. NK cells induce the production of the cytokine interferon-gamma (IFN-γ) to activate macrophages and T-cytotoxic cells. Tumor necrosis factor- alpha (TNF-α) and IFN-γ stimulate the synthesis of nitric oxide and other inflammatory mediators that promote the immune response. Specific cytokines function as proinflammatory or antinflammatory mediators.

Proinflammatory cytokines such as interleukin-6 (IL-6), interleukin 1-beta (IL-1β) and TNF-α normally respond in a localized and a low concentration manner (Beal, et al. 1994, Suzuki et al. 2002, Tilg et al. 1997). However, at times of endurance athletic events, these proinflammatory cytokines from a damaged site can elicit a systemic inflammatory response (Suzuki et al. 2002). Fever, inflammation, tissue destruction, and in some circumstances, shock and death are the potential results of proinflammatory cytokines.

In contrast to proinflammatory cytokines, anti-inflammatory cytokines such as interleukin-10 (IL-10) act to prevent inflammatory damage and ROS production through the activation of neutrophils (Kawai et al. 1998, Suzuki et al. 2002, Tilg et al. 1997). Anti-

inflammatory cytokines work to reduce inflammation and promote healing. Kulkarni and Karlsson (1993) demonstrated that neonatal mice genetically deficient in cytokine transforming growth factor beta (TGFβ), a potent immune suppressant, developed chronic inflammation in their lower intestines. These mice remained alive only as long as they received maternal milk containing an immunosuppressive TGFβ equivalent.

Cannon and Kluger (1983) were the first to report a relationship between exercise and cytokines. Recent studies have demonstrated that cytokines IL-6, IL-8, IL-15 are all released by contracting skeletal muscle. Pederson et al. (2000) proposed that such classes of cytokines be named “myokines”. IL-6 is the cytokine of interest to researchers due to its dual action as a proinflammatory cytokine, as well as having the ability upregulate the anti-inflammatory cytokines IL-1α and IL-10 (Pederson et al. 2005, Ostrowski et al. 2000, Ostrowski et al.2001). IL-6 also induces the secretion of the hormone cortisol (Bethin et al. 2000, Stouthard et al. 1995) and C-reactive protein (CPR) (Steensberg et al. 2003). CRP is an acute phase protein that responds as an inflammatory indicator since concentrations increase in an immune response. Starkie et al. (2003) determined that, with exercise, concentrations of IL-6 suppressed TNF-α production in humans. It has been noted that regular exercise also appears to significantly lower circulating CRP (Goldhammer et al. 2005, Okita et al. 2004, Toft et al. 2002). However, in endurance events, CRP has been shown to increase (Taylor et al. 1987, Siegel et al. 2001, Strachan et al. 1984).

Immunoglobulins are B-cell antibody-recognition molecules, whose isotypes are selectively distributed in the body. Colostrum contains very high concentrations of immunoglobulins (40-200 mg/ml) (Kruse, 1970). IgG1 accounts for over 75% of the immunoglobulins in colostral whey followed by IgM, IgA, and IgG_2 (Korhonen et al. 1995,

Larson, 1992). All of these immunoglobulins decrease within a few days of birth to a total concentration of 0.7-1.0 mg/ml (Butler, 1994). Gleeson et al. (2000) reported that salivary immunoglobulin A (IgA) decreases after intense exercise and that lower concentrations of IgA after exercise is directly correlated with URTI. Minehira et al. (2000) reported that in young rats given soy protein, IgA concentrations were lower compared to those fed casein. However, in aged rats fed whey protein isolate, concentration was lower compared to those fed casein or soy protein. Soy isoflavones influence both non-specific and specific immune responses by influencing signal transduction of macrophages and other phagocytic cells and the activity of cytotoxic T lymphocytes (Rumsey et al.1994, Stewart and Hoskin 1997).

Oxidative Stress

Lipid Peroxidation

Performance during endurance exercise is dependent upon FA as a fuel. The oxidation of FA is augmented and lactate formation is reduced during endurance training. An increased mitochondrial density in skeletal muscle results in an increased activity of oxidative enzymes (Holloszy et al. 1984). During endurance exercise there is a 10- 20 fold increase of whole body oxygen consumption (Astrand et al. 1986). In the active skeletal muscle, there is an increase of 100–200 fold in oxygen consumption (Halliwell et al. 1999, Keul and Doll 1972). Whole body lipid oxidation during endurance training increases, thus increasing plasma concentrations of available FA (Jong-Yeon, et al. 2002). The increased oxidative stress associated with strenuous training results in lipid peroxidation and tissue damage (Davies et al.1982, Mastaloudis et al. 2001). Following exercise, some tissues may become transiently hypoxic during contraction; upon reperfusion during relaxation, these tissues become susceptible to peroxidation, and the release of oxygen-derived free radicals may produce for further damage. Lipid peroxidation of

cell membranes results in decreased membrane fluidity, the inability to maintain ionic gradient, and cellular swelling and tissue inflammation (Alessio, 1993).

Using male Long Evans rats, Davies et al. (1982) conducted one of the earliest studies which reported that exercise increased lipid peroxidation; the latter was measured using the thiobarbituric acid reactive species (TBARS) assay. Mastaloudis et al. (2001) studied the rate of plasma vitamin E disappearance in 11 ultramarathon runners and. The researchers hypothesized that if exercise increases lipid peroxidation, increased amounts of vitamin E should be oxidized and plasma α-tocopherol concentrations should diminish. α-Tocopherol acts to protect PUFA in biological membranes against lipid peroxidation (Cachia et al.1998). Mastaloudis et al. (2001) report that exercise increased the rate of vitamin E disappearance, indicating that during exercise there is an increase in lipid peroxidation.

Studies have associated the consumption of soy protein with a reduction in cardiovascular risk (Anderson, et al. 1995, Meeker and Kesten 1940, Sugano and Koba 1993;). Morifuji et al (2006) showed that soy protein supplementation and exercise led to increased activity and expression of enzymes involved in FA oxidation in the skeletal muscle. Box et al. (2005) compared soy and whey protein (40 g/day) in moderately exercised young adult women and the effects on serum concentrations of lipid peroxides. After 4 weeks, soy but not whey treatment decreased lipid peroxides. However, in a study on sedentary postmenopausal women, soy protein isolate did not decrease lipid peroxidation (Ryan-Borchers et al. 2006).

DNA Damage

The most significant target of oxidative damage is the DNA. Damage to nuclear DNA is considered a pathophysiological factor in the development in cancer. Oxidative modifications of mitochondrial DNA lead to an accumulating rate of mutations, which in turn result in deficient

mitochondrial respiratory function (Johns, 1995). The detection of elevated 8-hydroxy-deoxy-guanosine after endurance exercise confirms the presence of oxidative DNA damage due to physical exertion (Alessio, 1990; Okamura et al. 1997). However, DNA damage can be alleviated with antioxidant supplementation. Heaton et al. (2002) found a significant reduction in DNA damage in mixed adult population given an antioxidant supplement. Similarly, Chew et al. (2003) showed that BALB/c mice fed the antioxidant, lutein, inhibited tumor growth.

The mechanisms by which soy or whey act to reduce cancer incidence is not known; however, they have both been shown to aid in cancer prevention. The consumption of bovine whey milk proteins may contribute to cancer prevention (Bounous et al. 1991). In Sprague-Dawley rats, dietary whey protein hydrosylate and soy protein isolate fed throughout development prevented 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary cancers (Hakkak et al. 2000). It is possible that genistein in soy protein isolates can stimulate mammary gland differentiation and increase resistance to cancer (Murrill et al. 1996).

Protein Oxidation

Redox cycling cations such as Fe^{2+} or Cu^{2+} can bind to the cation binding sites on proteins, and along with further attack by H_2O_2 can transform side-chain amine groups into carbonyls.

A major mechanism of oxidative protein modification is the initial OH^- induced abstraction of hydrogen from amino acid residue, thus forming carbon-centered free radicals. Further reactions lead to the formation of radicals, which in turn may also abstract hydrogen from amino acid residues (Stadtman, 2001). The removal of the radicals by antioxidants will reduce the oxidative damage to amino acid residues.

Reznik et al. (1993) demonstrated that rats subjected to exhaustive exercise accumulated reactive carbonyl derivates in skeletal muscle. This indicates an increased rate of oxidized damage to proteins. Similarly, Witt et al. (1992) observed immobilization of the hind leg of a rat on a three-month endurance exercise regime, and increased protein oxidation.

Soy protein has been shown to prevent exercise-induced protein degradation of the skeletal muscle. It is hypothesized that this occurs possibly through the inhibition of the calpain-mediated proteolysis (Nikawa et al. 2002). Elia et al. (2006) showed that exhaustive exercise significantly increased free radical concentrations the muscle tissue of mice. However, in mice fed the whey protein isolate, oxidative stress in the muscle induced by heavy exercise was prevented. In the same study, the trained mice showed lower protein carbonyl concentrations than in the untrained mice regardless of whether they consumed soy or whey protein.

Antioxidants, Oxidative Stress and Immune Response

Antioxidants are substances that help reduce the severity of the oxygen stress created by the formation of free radicals and ROS. Dietary supplementation with antioxidants has been shown to have favorable effects on lipid peroxidation after exercise. Oxidative stress may contribute to fatigue, risk of muscle injury, and possibly high cancer risk in some types of athletes (Karlsson, 1997; Polednak, 1976). Exercise may stimulate production of lipid peroxides by initiating oxidant stress but may also promote their breakdown in part by increasing enzyme activity related to glutathione metabolism (Gonenc et al. 2000, Gurcan et al. 1998, Kedzior et al. 1995).

The major determinant of redox status is glutathione. It couples with its disulfide, oxidized form the GSSG. Whey protein has been shown to be a safe and effective cysteine donor

for glutathione (GSH) antioxidant system during GSH depletion in immune deficiency states. Lymphocytes' ability to offset oxidative damage is determined by the capacity of these cells to regenerate intracellular stores of GSH (Noelle and Lawrence 1981, Fidelus et al. 1987). GSH depletion has been linked to the pathophysiology of disease conditions (Cantin et al. 1989, Bunnell and Pacht 1993, Roum et al. 1993). As mediators of oxidative stress (Nussler et al. 1992), cytokines can alter redox equilibrium by affecting GSH/GSSG shuttling and recycling (Chen et al. 1998). Hydrogen peroxide is effectively reduced in the presence of GSH as a substrate. During this reaction, GSH is converted to GSSG (Hayes and McLellan 1999, Haddad 2002).

Exercise increases metabolic rate up to 100 times higher than during resting conditions. This causes a significant increase of oxygen flux through mitochondria, and is considered the major mechanism of exercise-induced formation of superoxide (Sjodin et al. 1990).

The total antioxidant activity of a system is represented by the sum of endogenous and food-derived antioxidants. A greater protection is provided against attack by ROS or reactive nitrogen species when there is cooperation among the different antioxidants. Therefore, the overall antioxidant capacity may provide more relevant information compared to measurements of individual components.

As a major antioxidant in human tissues, GSH provides reducing equivalents for the glutathione peroxidase catalyzed reduction of H_20_2 and lipid hydoperoxides to water and the respective alcohol. GSH, a tripeptide with a free thiol group, becomes oxidize glutathione (GSSG) which is then recycled into GSH by glutathione reductase (GR) and β-nicotinamide adenine dinucleotide phosphate (NADPH). When human tissues are exposed to increased oxidative stress, the ratio of GSH/ GSSG will decrease as a result of GSSG accumulation.

Therefore, the GSH/GSSG ratio is an indicator of oxidative stress and the effectiveness of antioxidant intervention.

Whey Protein

Milk is a nutritional influence capable of directing the physiological development of immunologically immature neonates through a period of development and intensive growth (Walzem et al. 2002). Major components of bovine milk can modulate physiological functions. These functions are modulated by more than 50 growth factors and hormones contained in milk (Guimont et al. 1997, Pakkanen et al. 1997). Whey protein is constitutes about 85-90% of cheese (Sinha et al. 2007). Due to its amino acid balance and relatively high proportion of BCAA, whey protein is a high quality protein (Aoyama et al. 2000, Bos et al. 2000). The most abundant whey proteins are β-lactoglobulin, α-lactalbumin and lactoferrin (Gauthier, 2006).

Whey protein has been show to increase lean body mass and increase resisted muscle strength (Anderson et al. 2005). In muscle, as a protein source, whey protein provides almost all of the amino acids in approximately the same proportion as found in muscle protein. Logically, this similarity makes whey protein an effective anabolic supplement (Bergstrom et al. 1974, Rasmussen et al. 2000).

Whey-derived bioactive components offer the potential to extend health benefits to active people beyond body composition. The functions associated with these amino acids and whey components can influence the immune response (Dillar et al. 2002, Shah, 2000). Whey protein contains numerous immune-modulatory peptides that are either naturally contained or part of a primary sequence of whey proteins that can be released during digestion. In comparison, whey protein has a significantly different rate of absorption than casein. Whey is more rapidly emptied

into the duodenum, which has a major affect on whole body protein anabolism (Boirie et al. 1997, Mahe et al. 1996). Mahe et al. (1996) compared the protein quality of β-lactoglobulin and casein on the jejunum of 35 volunteers. The jejunum digests proteins mix with many endogenous secretions. They demonstrated that β-lactoglobulin transits more rapidly into the jejunum and mostly in the form of intact protein. The intact proteins can maintain physiologic and immunologic responses (Bahna et al. 1985, Bland and Kambarage 1991). Recent studies have shown that whey protein compared to casein, decreased serum cholesterol, suggesting a difference in the metabolism of casein and whey protein (Nagaoka et al. 1991, Nagaoka et al. 1992, Nagaoka et al. 1996) However, further investigation is needed to determine the physiological utilization of the proteins in humans.

Soy Protein

Soy protein isolate is derived from soybeans. Soybeans and products derived from soybeans constitute the major source of dietary isoflavones. Although, many varieties of fruits, vegetables, grains, and legumes contain small amounts of isoflavones, the highest amount is found in soybeans (Bingham et al. 2003, Wang, et al. 1994). Isoflavones are a category of diphenol compound, phytoestrogens, a nonsteroidal estrogen-like plant compound. The isoflavones genistein, daidzein, and glycitein are most abundant in soy. Both are precursors to equol (Axelson et al. 1984). These isoflavones are structurally similar to 17β-estradiol, an endogenous estrogen, and have similar estrogen-like effects. Due to these characteristics, soy has been controversially suggested as a hormone-replacement therapy in menopausal women (Adlercreurtz et al. 1992, Anderson et al. 1995, Brandi, 1997, Messina, 2002). Estrogen

receptors are distributed on more than just reproductive organs. Immune tissues and cells also express estrogen receptors and respond to estrogen.

Soy protein supplementation has provided numerous health benefits including cancer prevention, increased cardiovascular health, and prevention of osteoporosis. Wu et al. (2004) demonstrated in ovariectomized mice that combined exercise along with soy isoflavone supplementation prevented body fat accumulation, increased lean body mass and restored bone mass. In a study comparing men and women, only the women showed higher serum concentrations of IL-6 indicating a possible estrogenic effect of soy isoflavones in enhancing the immune response (Jenkins et al. 2002).

Major antioxidants such as ascorbic acid, α-tocopherol, beta-carotene, and other carotenoids are present in relatively higher concentrations in the plasma compared to isoflavones (Nyyssonen et al. 1997, Aldini, et al. 2001). However, plasma isoflavones can reach comparable concentrations after the consumption of meals containing soy products high in isoflavones or isoflavone supplements (Teixeira et al. 2000, Upritchard et al. 2003, Wiseman et al. 2002, Yeum et al. 1996).

Similarly to whey protein, soy protein is digested mostly in the jejunum. It is there that the isoflavones are partially hydrolyzed to release aglycones, daidzein, genistein, and glycitein, which are then absorbed by the intestinal epithelium (Richelle et al. 2002, Zubik et al. 2003).

Summary

Exercise is beneficial to health; however, when it becomes an endurance event, metabolic changes occur that are detrimental to physiological homeostasis. The production of ROS and free

radicals are inevitable during endurance events. Dietary antioxidants work to alleviate any oxidants that endogenous antioxidants are unable to dispose.

Protein supplementation for athletes has been shown to be beneficial not only for muscle building but also for immune modulation. Soy protein is high in isoflavones, a phytoestrogen. Whey protein is considered a high quality protein because of the amino acid composition, more specifically cysteine because the biosynthesis of the major intracellular antioxidant, glutathione depends on the intracellular availability of cysteine.

Both whey and soy protein supplements possess specific characteristics that could and in some instances, has been shown to attenuate immune response before, during, and after exercise. This is the first research study in which soy and whey protein are compared in young elite endurance female athletes.

Research Purpose

The purpose of this research study is to assess the effects of whey protein and soy protein supplementation in alleviating exercise-induced oxidative damage and inflammation and on body composition in female athletes.

Research Objectives and Hypotheses

To compare the effects of soy and whey protein supplement on 1) immune function, 2) oxidative and inflammatory damage, and 3) body composition in elite endurance female athletes. We hypothesize that whey protein will improve immune function, decrease oxidative and inflammatory damage, and improve body composition.

SUBJECTS AND METHODS

Subjects

Thirty-three elite female endurance athletes between the ages of 18 and 25 were recruited from the Washington State University intercollegiate track & field/ cross country team and the surrounding Pullman/ Moscow community. Elite endurance status was defined as running a minimum of one hour per day, five days per week for at least the previous calendar year. Inclusion factors included non-smokers with no history of cancer, HIV, diabetes or other metabolic diseases. Consumption of dietary supplements and foods containing high amounts of antioxidants, whey protein, or soy protein during the study was discouraged; however subjects were instructed to not make distinctive changes to their normal dietary habits.

The head track & field coach, head cross-country coach, and the Washington State University athletic department approved the study procedures and participation of the intercollegiate athletes. The Institutional Review Board (IRB) of Washington State University approved all study procedures, and all participants provided informed consent.

Study Design

Subjects were assigned to two treatment groups consisting of whey or soy protein supplementation in a double-blind placebo-controlled study design. Baseline DXA scans were used to randomize participants with body fat percentage to the treatment groups. Body fat percentage was used rather than body mass index (BMI) because research has shown that body fat percentage more appropriately represents an athlete's true body composition (Mazess et al. 1990). Until recently, BMI has been the prevalent method for estimating weight composition,

including fat, muscle, and bone. However, this method provides only estimates of body fat whereas body fat percentage is accurately measured with DXA.

Prior to initiation of the study, all subjects completed a consent form, medical history questionnaire, and attended an information meeting with researchers. During the study period, subjects kept an exercise log, when supplements were consumed daily, and completed a three-day dietary intake record. Each subject kept a dietary diary for two weekdays and one weekend day.

Participants received either commercially available soy or whey isolate protein powders (NOW Foods, Surfside, FL). The composition of the supplements as presented in Table 1. The supplements did not contain any additional ingredients other than the isolate proteins (Table 1) and the daily amount was standardized to 40 g protein. The daily amount was weighted and sealed in plastic bags. Each participant received a weekly supply of their respective supplement and returned each week to allow communication with the investigator. A protein shaker and directions on how to mix the powder were given to each participant. A certified athletic trainer monitored the participants' health condition and exercise intensity.

Blood was collected from each subject within an hour of completion of the daily run, a baseline (week 0) and at the end of the dietary intervention week 6 and a DXA scan also was performed at those periods. Blood was collected from the antecubital vein into two 10 ml vacutainer tubes containing heparin as an anticoagulant. Blood was immediately centrifuged at 400 ×g for 30 minutes at 4ºC and plasma collected, aliquoted, topped with nitrogen gas and frozen at -80ºC. The buffy coat containing the leukocytes was collected into phosphate buffered saline (8 mM Na_2HPO_4, 2 mM NaH_2PO_4, 150 mM NaCl, pH 7.3) for further processing.

Table 1 Nutritional information and ingredient listing for Protein Supplements (NOW Foods)

	WHEY	SOY
Serving size, g protein	44.8	48.0
Calories, Kcal	168	171
Protein, g	40	40
Total fat, g	0.48	1.90
Cholesterol, mg	6.4	0
Sodium, mg	64	419
Total carbohydrate, g	1.6	----
Sugars, g	1.6	----
Calcium, mg	200	76
Magnesium, mg	35	114
Iron, mg	----	9
Ingredients	Microfiltered whey protein isolate	90% Soy protein isolate. Vegetarian Product.

Antioxidant Biomarker Assays

Antioxidant biomarkers measured included plasma total antioxidant capacity (TAC) and reduced glutathione (GSH) and oxidized glutathione (GSSH) activity in whole blood.

Total Antioxidant Capacity. The TAC assay measured both aqueous and lipid soluble antioxidants, including constitutive vitamins, proteins, lipids, glutathione, and uric acid in plasma. These antioxidants inhibit oxidation of substrate by metmyoglobin. Measurement of TAC was completed using a commercial kit (Antioxidant Assay kit, Cayman Chemical Company, Ann Arbor, MI). The assay measures the ability of antioxidants to inhibit the oxidation of 2,2-Azino-di-[3-ehtylbencthiazoline sulphonate] (ABTS) by metmyoglobin. Plasma was first diluted 1:20 with assay buffer, and 10 µl of the diluted sample or standards were mixed with the metmyoglobin and chromogen in a microwell plate. Hydrogen peroxide (441 µM) was added to initiate the reaction and the plate was covered, incubated, and absorbance read at 750 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). A standard curve was generated using Trolox, a water-soluble tocopherol analog. Antioxidant concentrations were expressed as millimolar Trolox equivalent. The sensitivity of the assay is 0.044 mM Trolox equivalent.

Glutathione assay. A commercial kit (Bioxytech GSH_t/GSSG-412 kit, OXIS Health Products, Inc., Portland, OR) was used to measure the GSH_t and GSSG concentrations in whole blood. To prevent oxidation of GSH_t to GSSG, the thiol-scavenging reagent, 1-methyl-2-vinylpyridium trifluoromethanesulfonate (M2VP) was added to an aliquot of the whole blood immediately following collection. The samples were stored at -80°C and assayed within 4 weeks

of collection. Samples were thawed, vortexed, incubated at room temperature for approximately 5 minutes, and 50 µl of a 5% metaphosphoric acid (MPA) were added. Following centrifugation at 2000×g for 10 minutes at 4ºC, the supernatant was diluted in the Assay Buffer ($Na{\cdot}PO_4$ with EDTA) and kept on ice. The chromogen (5,5'-dithiobis-2-nitrobenzoic acid), enzyme (glutathione reductase), and samples or standards were added. The plates were incubated at room temperature for 5 minutes after which NADPH was added to initiate the reduction of GSSG to GSH_t. The absorbance (412 nm) was immediately read and read again at one minute intervals for three minutes. The change in absorbance is a linear function of GSH concentration where the slope of the regression equation represents the rate of reaction. The slopes of the individual standards were used to generate a standard curve. The lower limit of detection is 0.009 µmol/L for the reaction mixture and 0.54 µmol/L for the original sample. The GSH_t/GSSG ratio was calculated as follows:

$$GSH_t/\ GSSG\ Ratio = (GSH_t - 2GSSG)/\ GSSG$$

Oxidation Biomarkers

Protein Carbonyl. Protein oxidation results in the modulation of protein carbonyls present are reacted with 2,4-dinitrophenylhydrazine (DNPH) and to modulate hydrazone products. Plasma protein carbonyl measured using a commercial kit (Protein Carbonyl Assay Kit, Cayman Chemical Company, Ann Arbor, MI). The DNPH dissolved in 2.5 M HCL was added to each sample to initiate the reaction, with a similar 2.5 M HCl sample without DNPH serving as control. After incubation at RT for one hour, 1 ml of 20% trichloroacetic acid (TCA) were added and incubated at 4ºC to precipitate the proteins. The tubes were centrifuged at 1000×g for 10 minutes at 4ºC, the supernatant discarded, and a second precipitation with 10%

TCA was performed. The protein pellet was washed three times with an ethanol/ethyl acetate (1:1) mixture. Following the final wash, the protein pellet was resuspended in guanidine hydrochloride, centrifuged and 220 µl of the supernatant transferred to a 96-well plate. Absorbance was measured at 385 nm using a plate reader and corrected absorbance (CA) calculated by subtracting the control absorbance from the sample absorbance. The concentration of carbonyl was calculated using the extinction coefficient in the following equation:

$$\text{Protein carbonyl (nmol/ml)} = [(CA)/(0.022\ \mu M^{-1})](500\ \mu l/220\ \mu l)$$

DNA Damage. During DNA repair, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is produced. Therefore, measurement of 8-OHdG is a useful indicator of oxidative DNA damage. Plasma 8-OHdG was measured in by ELISA (Bioxytech 8-OHdG-EIA kit, OXIS Health Products, Inc., Portland, OR). Plasma samples were centrifuged in a microcentrifuge set at maximum speed for 3 min at 4ºC. The 8-OHdG monoclonal antibody and the sample or standard were pipette into a microtiter plate pre-coated with 8-OHdG. In this competitive ELISA, plasma 8-OHdG competes with bound 8-OHdG for the 8-OHdG monoclonal antibody binding sites. After incubating for one hour at 37ºC, the plate was washed, HRP-conjugated antibody added and incubated for an additional hour, before being washed. The chromogen (3,3',5,5'-tetramethylbenzidine) was added and color allowed to develop for 15 minutes. The reaction was stopped with the addition of 1 M phosphoric acid, and absorbance read at 450 nm. Standards were run in duplicate. The average absorbance was used to calculate the calibration curve. The assay sensitivity is 0.5 ng/ml.

Lipid Peroxidation. During **l**ipid peroxidation polyunsaturated fatty acids are damaged by ROS react to form lipid peroxides. Further decomposition of these lipid peroxides leads to the formation of malonaldehyde. Thiobarbituric acid (TBA) reacts with malonaldehyde to form a complex that can be detected spectrophotometrically. Lipid peroxidation in plasma was measured by the TBA procedure as described by Uchimaya and Mihara (1978). A standard curve (0-48.8 mmol/L) was generated with tetramethoxypropane (TMP) by serial dilution of a stock solution (50mmol TMP/L H_20) ranging from. Standards and samples were assayed in duplicate. Plasma or standard (500 µl) was pipetted into duplicate tubes, 50 µl saturated KCl added, followed by the addition of 3 ml 1% phosphoric acid and 1 ml 0.6% TBA solution. The mixture was heated for 45 minutes in a 95ºC water bath, cooled on ice, 4 ml of n-butanol added and the mixture vortexed. The butanol phase was separated by centrifugation at 400×g for 10 minutes and absorbance measured at 532 nm with a reference OD at 520 nm. The TBA concentration was as µmol malondialdehyde/ L.

Inflammatory Biomarkers

C-Reactive protein (CRP) is a nonspecific but sensitive indicator of the inflammatory process. At the onset of inflammation, CRP concentrations increase and then they peak during the acute stage of inflammation.

Although many cytokines are involved in regulating the immune response, only a small subset is involved in the inflammatory response. These include: interferon- gamma (IFN -γ), tumor neurosis factor- alpha (TNF-α), and the interleukins IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10.

C-Reactive Protein. Plasma CRP was measured using a commercial sandwich ELISA kit (Human CRP ELISA kit, Alpha Diagnostic International, San Antonio, TX). Plasma samples were diluted 1:100 in a diluent and 10 µl pipetted into a microplate well coated with an anti-CRP-antibody; an anti-hCRP-HRP enzyme conjugate (100 µl) was then added. Following 30 min at RT, the wells were washed and100 µl of the HRP substrate solution added. The plate was covered, incubated for 10 min at RT and a stop solution (1M H_2SO_4) was added. Absorbance was read at 450 nm. The lower limit of detection for the CRP is 0.35 ng/ml.

Cytokine Production. Plasma IFN-γ was analyzed by ELISA (human IFN-γ, BD OptEIA set, BD Biosciences, San Diego, CA). Plasma IL1-α, IL-1β, IL-2, IL-4, IL-8, IL-10, and TNF-α were analyzed using a multiplex format ELISA (Quansys Q-Plex Human Cytokine Array, Logan, UT). In the assay, IFN-γ, 96 well plates were coated overnight with 100 µl of capture antibody (anti-human IFN-γ monoclonal) followed by blocking and washing. Plasma standards (100µl) were added and incubated for 2 hours at RT. Plates were washed, incubated for another hour in the presence of a detection antibody, and the substrate added. The mixture reacted 30 minutes at RT in the dark. After the addition of a stop solution, absorbance was read at 420 nm with a reference OD of 570 nm.

For the multiplex cytokine ELISA, 30 µl of the plasma or standard were pipette into the plates and incubated at RT on an orbital shaker for one hour. Plates were washed, detection antibody added, and incubated at RT for one hour. Following washing, plates were washed, of streptavidin HRP (30 µl) added to each well, incubated for 15 min at RT, washed, and substrate added. The plates were immediately imaged in an imaging cabinet, and digital images acquired and analyzed using the Quansys Q-View 2.5.2 software. The lower limit of detection was 4.10,

1.10, 1.36, ≤ 1.0, 1.36, ≤ 1.0, ≤ 1.0 and 2.11 pg/mL for IL1-α, IL-1β, IL-2, IL-4, IL-8, IL-10, IFN-γ and TNF-α, respectively.

Iron Analysis

Serum samples collected on week 6 were analyzed for iron, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC) and transferrin saturation (Pathologists Regional Laboratories, Moscow, ID).

DXA Scans

Dual x-ray energy absorptiometry (DXA) scans were performed on all participants at baseline and week 6. A registered radiographic technologist performed the scans on each participant utilizing a central DXA machine (Hologic, INC., Bedford, MA) at the Palouse Medical Center, Pullman, WA. The same technician performed all scans in order to reduce scan-to-scan variation.

Dietary Analysis

During the six-week period, participants recorded one three-day dietary log. Participants were instructed not to include the protein supplement. Dietary analysis was completed using The Food Processor for Windows Version 8.5 (ESHA Research, Salem, OR).

Statistical Analysis

Data were analyzed by ANOVA and ANCOVA using the General Linear Models Procedure of SAS (SAS Institute, Cary, N.C.). The initial statistical model included treatment,

week, treatment x week, age, % body fat, height, weight, BMI, and status. The final statistical model included treatment, week, treatment x week, age, % body fat, height, weight, BMI, and status. The ANCOVA analyzed for treatment, group, week, height, weight, % body fat, BMI, and exercise status included week 0 as a covariate. For the rest of the variables, ANCOVA was performed by subtracting baseline values from week 6. The statistical model included the effects of treatment, week, and interaction of treatment x week. The difference between week 6 and baseline for each data point was calculated and statistically analyzed. Statistical significance was set at $P<0.05$.

RESULTS

Subjects

Eighteen of the twenty-two participants (81%) completed the six-week study. Four participants withdrew due to circumstances unrelated to the study. The final number of participants completing the study was 10 and 8 in the whey and soy treatment groups, respectively. At the start of the experiment, there were no differences in body fat percentage, age, and athletic status between treatments groups (Table 2).

Dietary Intake Analysis

Eighteen of the twenty-two participants (81%) returned the three-day food diary. An example of the ESHA dietary analysis is presented in Table 3. During the 6-week dietary intervention, participants were instructed to not make significant changes in their normal dietary habits. In the dietary analysis, the protein from the treatment supplementation was not included as a component of dietary intake. The overall mean daily protein and caloric intake was 92±5 g and 2302 ± 132 kcal, respectively, and were not significantly different between groups (Figure 1).

Exercise Log

Participants were instructed to record either total time or total distance ran per day in their exercise log. Eighteen of the twenty-two participants (81%) returned their exercise log. There was no significant difference in total run or endurance activity. The average exercise time for all participants was 345 ± 40 minutes/week.

Table 2. Mean (± OSE) demographic characteristics of participants by treatment group

	Whey (n=10)	**Soy** (n=8)
Age	22.3 ±0 .4	20.0 ± 0.4
Baseline Body Fat (%)	23.1 ± 0.7	20.4 ± 0.7
Athletic level[1]	4.5 ± 0.4	5.5 ± 0.4

[1] Participants received a ranking based upon a 1-10 scale (10 being comparable to an elite Olympic level).

Table 3. ESHA dietary foodlist summary report.

Foodlist Summary Report
For: [redacted] Day 1
November 01, 2007

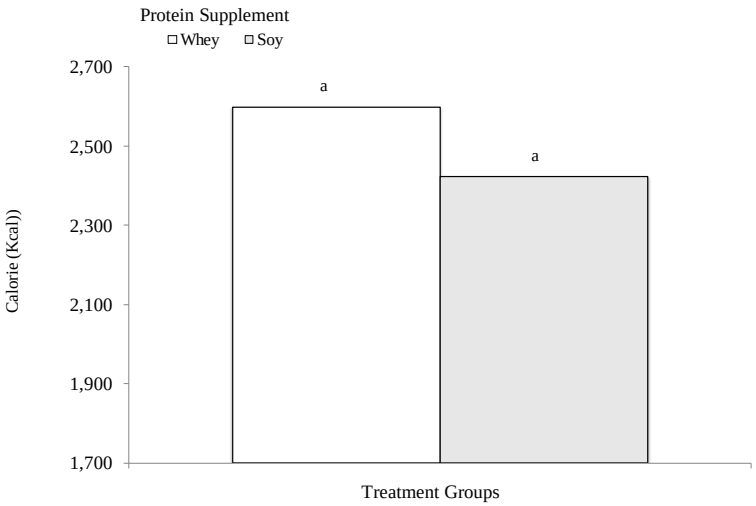
Total Weight: 1886.06 g (66.53 oz-wt.)
Serving Size: 1886.06 g (66.53 oz-wt.)
Serves: 1.00
Cost: --

Foodlist

Amount		Food Item	Cost	ESHA Code	
1	cup	HoneyBunchesOfOats HnyRstd Cereal KFT-PC	--	40292	
2.5	cup	Regular Trail Mix		--	44058
2	piece	Wheat Bread-Slc		--	42012
2	tbs	Deli Mustard-Squeeze Bottle UNI-BF	--	91811	
3	piece	Turkey Breast Lunchmeat Slice-3.5"Square	--	16160	
1	each	Fresh Iceberg Lettuce-Med FDA	--	7317	
1	piece	Fresh Red Tomatoes YrRndAvg Lrg Slc-1/2"	--	90531	
1	piece	Past Proc Swiss Cheese-0.75oz Slc		--	1458
8	each	Fresh Baby Carrots-Med		--	5439
1	each	Peanut Butter Granola Bar GML-NV		--	47593
1	each	Garden Veggie Vgtrn Burger Patty KLC-NT	--	7790	
1	each	Cheddar Cheese-Slc		--	47864
2	piece	Dill Pickles Slices		--	27013
2	tbs	Deli Mustard-Squeeze Bottle UNI-BF	--	91811	
2	tbs	Low Sodium Ketchup		--	9151
12	fl oz	Diet Dr Pepper Soda DPS		--	4797
6	piece	Toasted Corn Tortilla Chips PCO-DR	--	44223	
1	each	Whole Wheat Hamburger Buns	--	71363	
1	each	Low Fat Choc Ice Cream Sandwich UNI-SF	--	72081	

Nutrients per Serving

Calories	2943.15		Fat - Total	144.53	g
Protein	110.75	g	Saturated Fat	34.41	g
Carbohydrates	344.64	g	Vitamin A RE	1915.76	mcg
Dietary Fiber	41.31	g	Vitamin C	53.96	mg
Cholesterol	78.60	mg	Sodium	4335.01	mg
% Calories from fat	42	%	% Calories from carbs	44	%



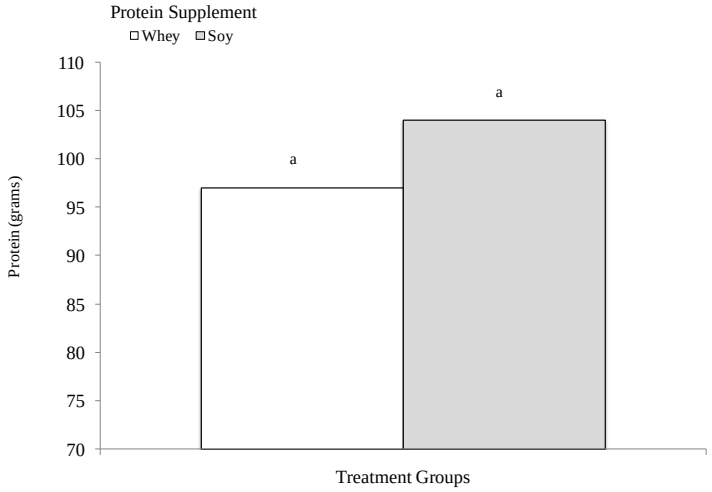


Figure 1. Calorie (Kcal) and protein (g) intake of subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA. [a] Different letters above each bar denote significant difference between treatment groups ($p<0.05$).

Total Antioxidant Capacity

There was no treatment difference in plasma TAC at the baseline period, and concentrations were within the normal range (Kampa et al., 2002). No significant treatment difference was found following 6 weeks of protein supplementation (Figure 2). However, whey and soy supplementation both appeared to decrease plasma TAC by about 8%.

Glutathione Status

There were no overall significant changes in GSH_t, GSSG, or GSH/GSSG ratio following protein supplementation. The concentration of whole blood GSH_t in the soy group remained constant between baseline and week 6. On the other hand, GSH_t concentrations in the whey group tended to decrease between week 0 and ($P<0.10$) week 6 (Figure 3). Concentrations of GSH_t were similar between the whey and soy groups at week 6 (Figure 4).

Concentrations of GSSG between week 0 and week 6 did not change significantly in either treatment groups. There was no significant treatment difference on week 6 when comparing week 0 to week 6 (Figure 5).

The calculated GSH/GSSG ratio was similar between treatment groups at week 0 and week 6 (Figure 6).

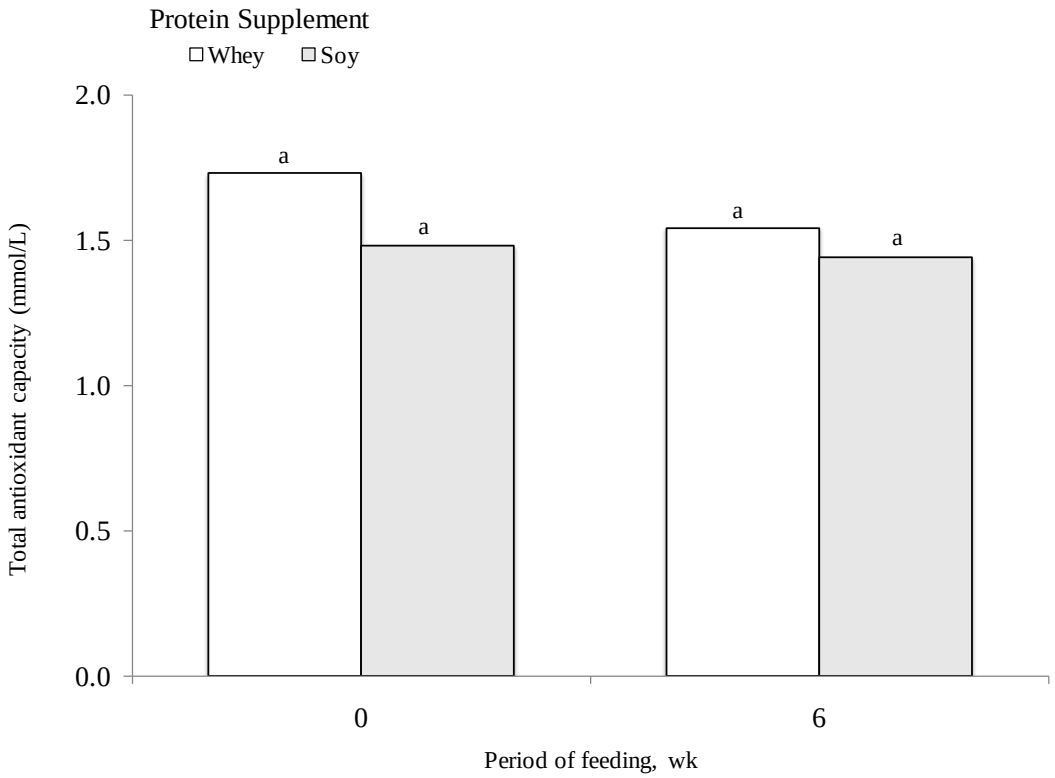


Figure 2. Total antioxidant capacity in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA. [a] Different letters above each bar denote significant difference between supplement groups ($P < 0.05$).

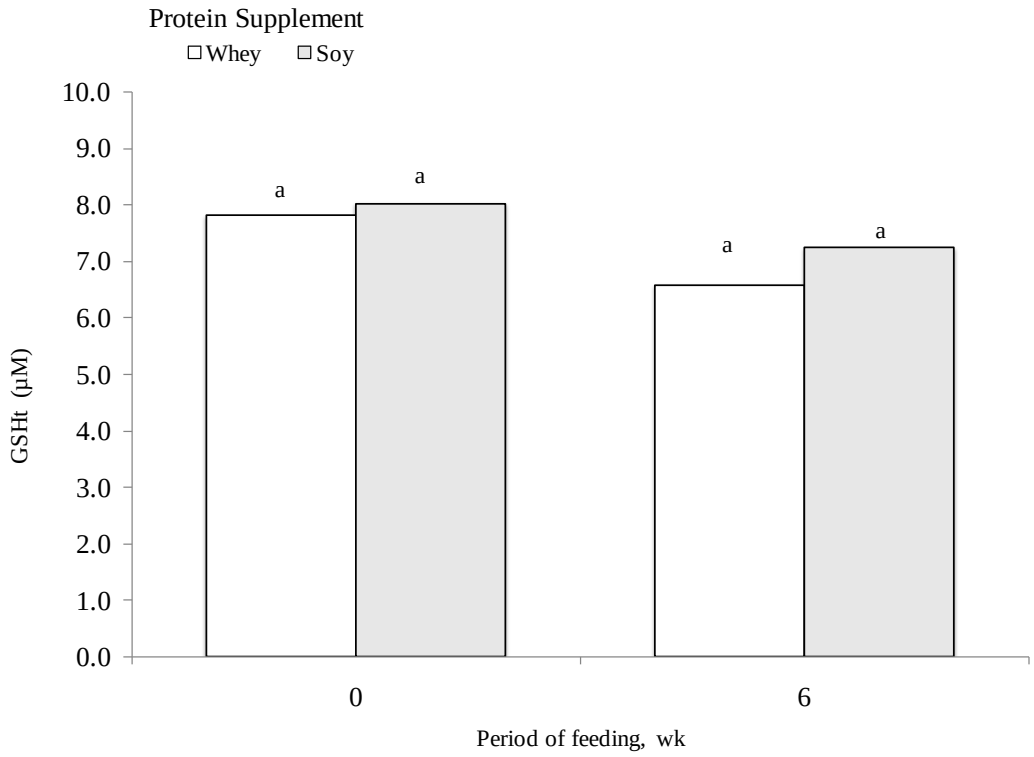


Figure 3. Whole blood concentrations of GSHt in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA. [a] Different letters above each bar denote significant difference between supplement groups ($P < 0.05$).

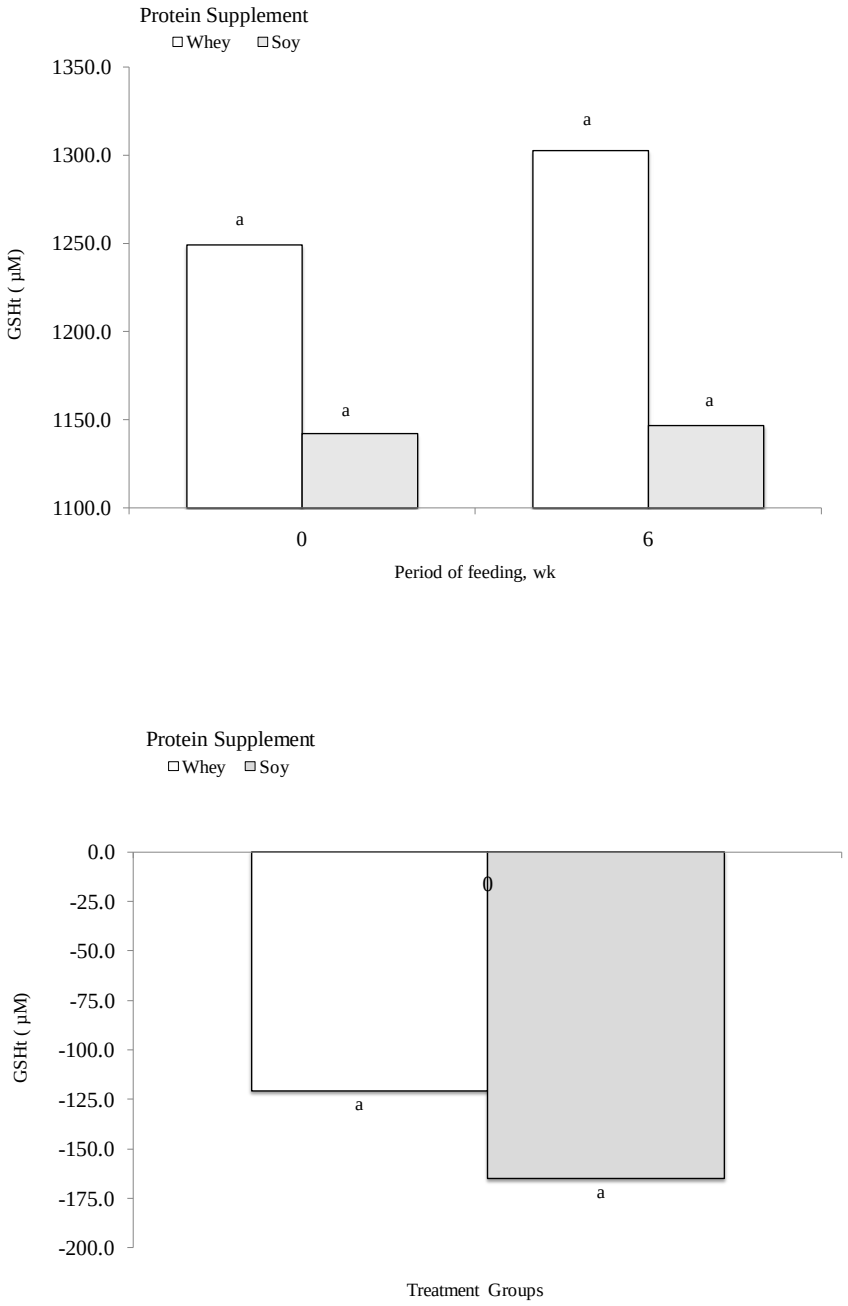


Figure 4. Whole blood concentrations of GSHt in subjects fed whey or soy protein. Data were analyzed by ANOVA (top) and ANCOVA (bottom) using the difference between wk 6 and wk 0. [a] Different letters above each bar denote significant difference ($P < 0.05$).

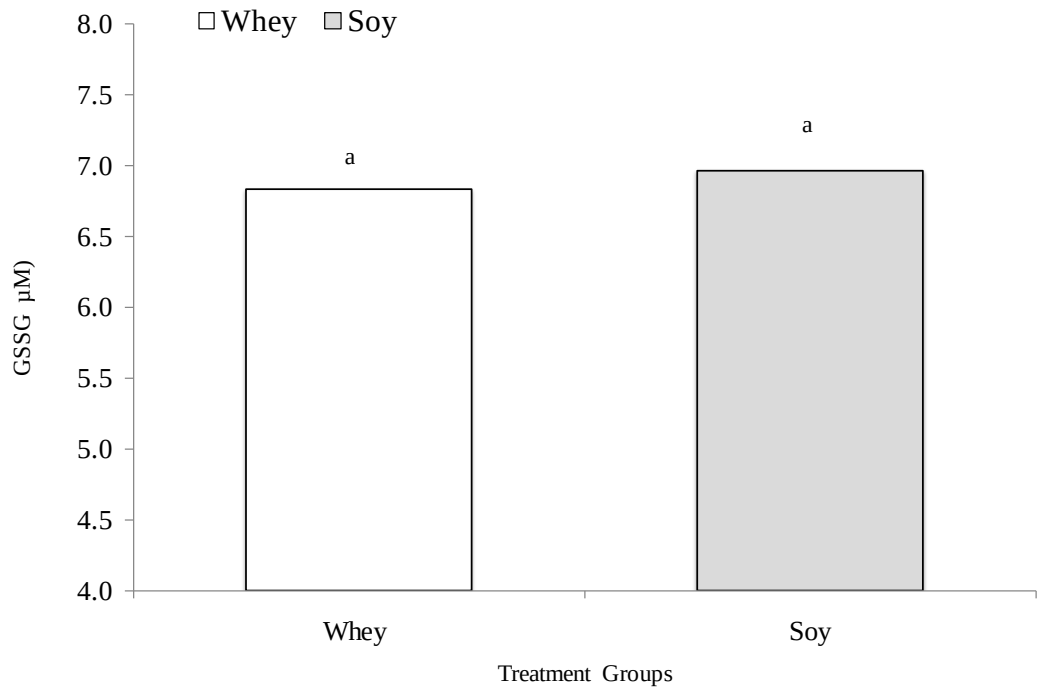


Figure 5. Whole blood concentrations of GSSG in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANCOVA using wk 0 as a covariate. [a] Different letters above each bar denote significant difference ($P < 0.05$).

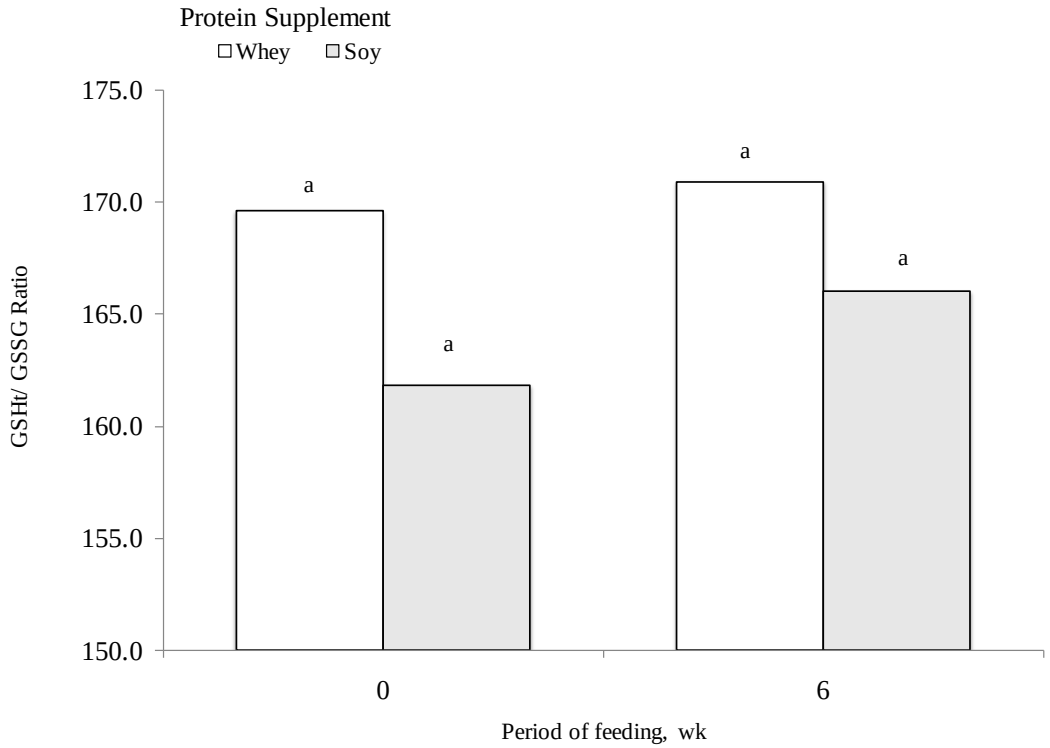


Figure 6. GSHt/GSSG ratio in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA. [a] Different letters above each bar denote significant difference between supplement groups ($P < 0.05$).

Protein Carbonyl

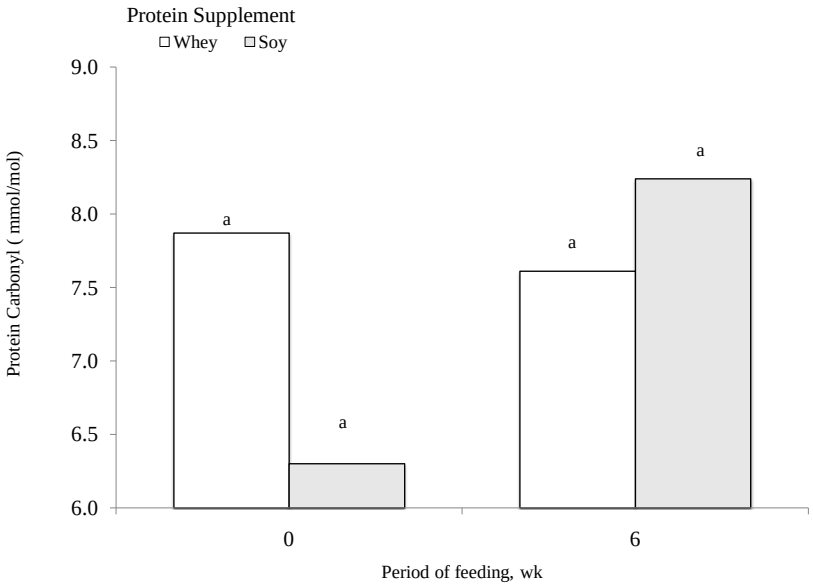
There was no significant change in protein carbonyl at week 6 in either group (Figure 7). Concentrations of plasma protein carbonyl tended ($P>0.05$) to decrease from 7.9 to 6.3 nmol/ml in participants given whey protein but tended to increase from 7.6 to 8.2 nmol/ml in those fed soy protein. However, no treatment difference was observed on week 6. Plasma protein carbonyl concentrations were within the normal range as reported by others (Ahmad et al. 2008).

8-OHdG

Plasma concentration of 8-OHdG in participants in the whey group increased ($P<0.05$) by 0.73 ng/ml from baseline whereas those in the soy group decreased by 4.40 ng/ml (Figure 8). Concentrations of 8-OHdG in both groups were above the normal range reported in human plasma (Seven et al. 2008, Manda et al. 2008). At week 6, plasma 8-OHdG was higher ($P<0.07$) in the whey group (21.04 ng/ml) compared to the soy group (17.74 ng/ml). The concentration of 8-OHdG in this study was somewhat higher than reported by others (10-14 ng/ml) (Seven et al. 2008, Manda et al. 2008).

TBARS

Plasma TBARS decreased significantly ($p<.0.01$) from baseline in participants fed whey protein (1.74 µmol/L at baseline to 0.94 µmol/L on week 6), but tended ($P>0.05$) to increase in those fed soy protein (1.29 to 1.47 µmol/L). Plasma TBARS tended ($P>0.05$) to be lower in the whey group compared to the soy group (Figure 9).



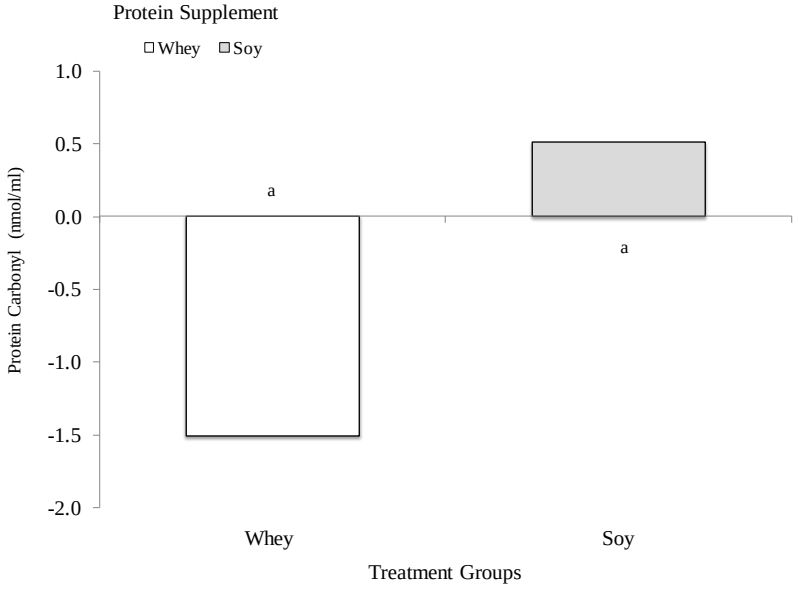


Figure 7. Plasma concentrations of protein carbonyl in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA (top) and ANCOVA (bottom) using wk 0 as a covariate. [a] Different letters above each bar denote significant difference ($P < 0.05$).

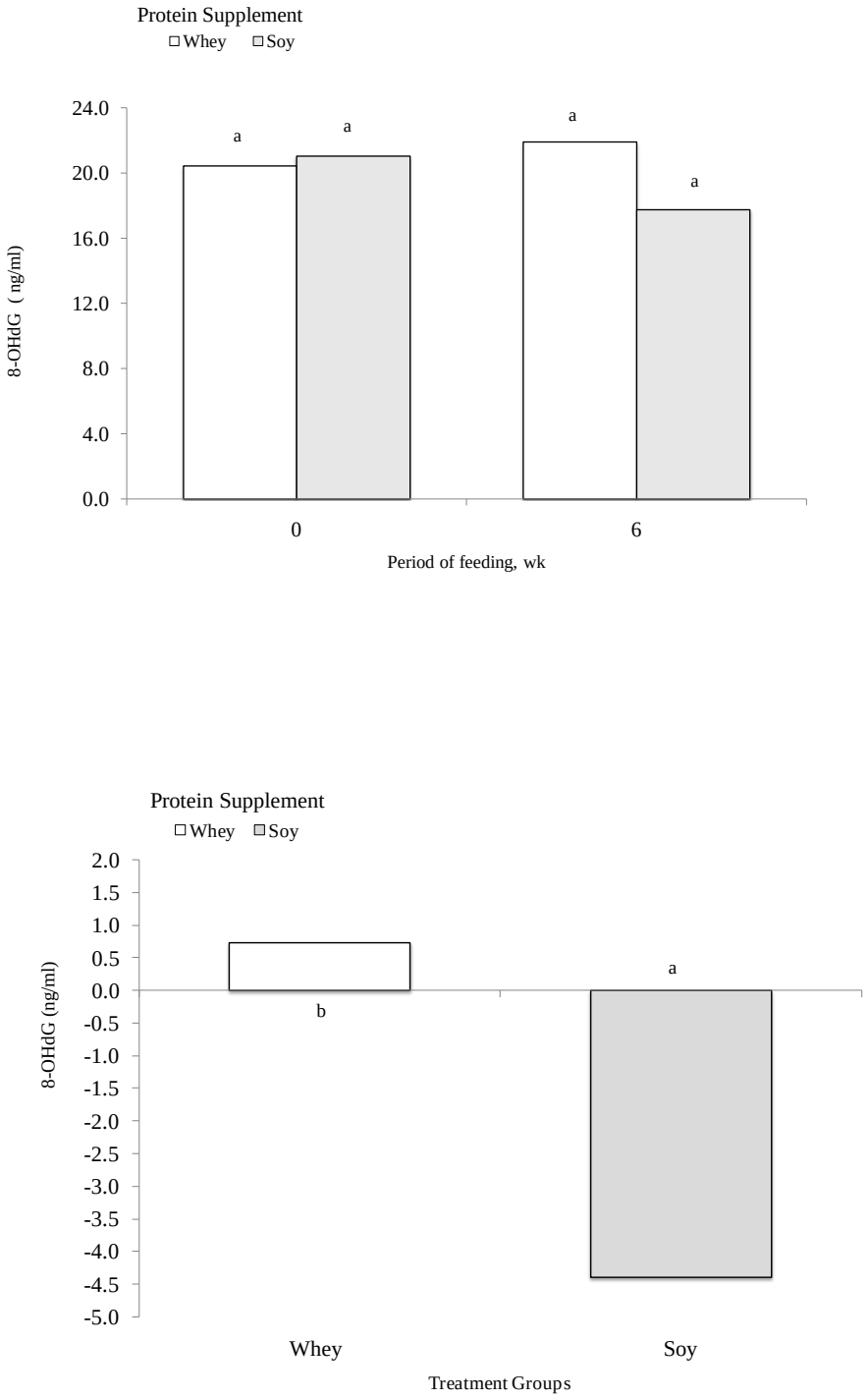


Figure 8. Plasma concentrations of 8-OHdG in subjects fed whey or soy protein in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA (top) and ANCOVA (bottom) using the difference between wk 6 and wk 0. [a b] Different letters above each bar denote significant difference ($P < 0.10$).

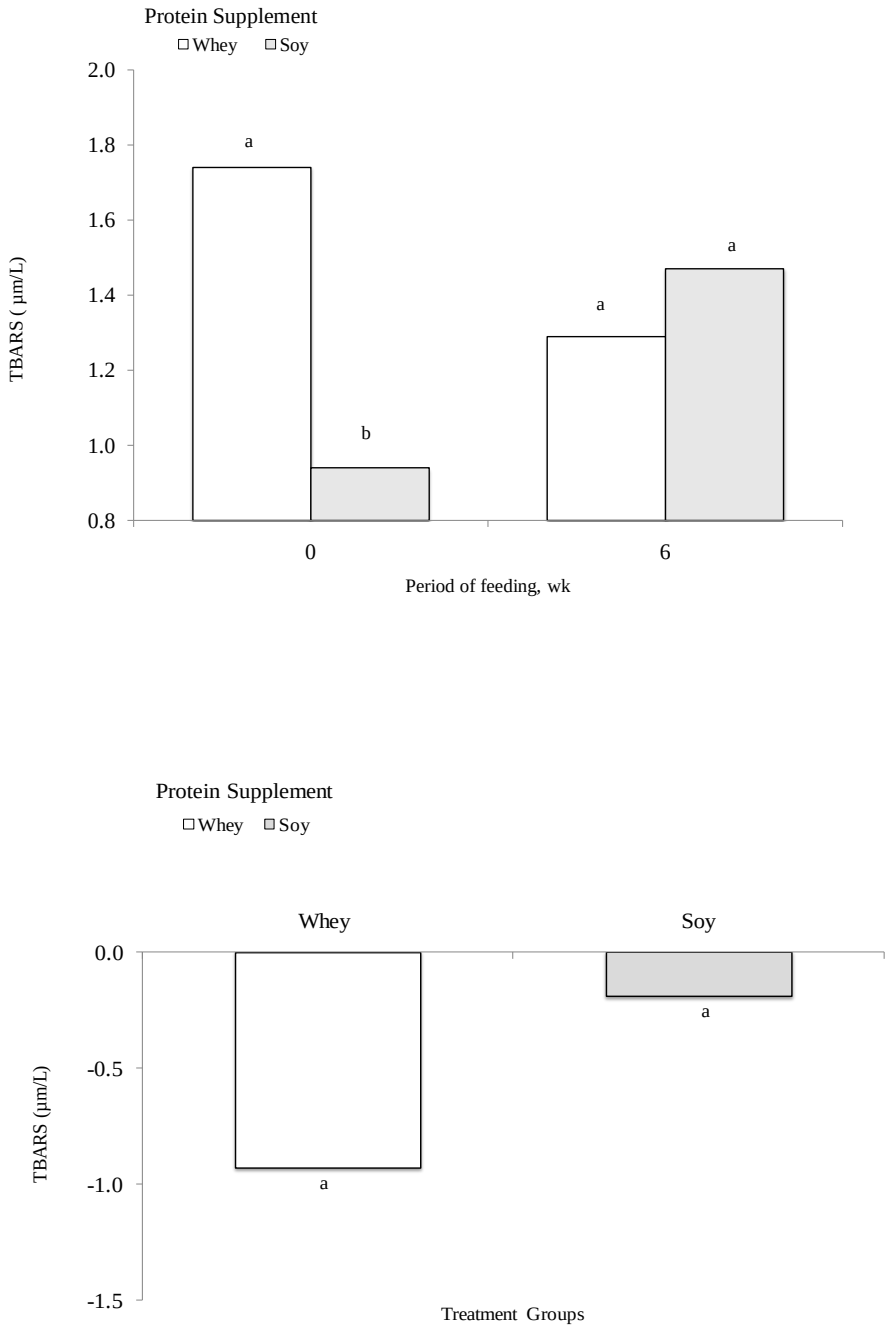


Figure 9. TBARS concentrations in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA (top) and ANCOVA (bottom) using the difference between wk 6 and wk 0. [ab] Different letters above each bar denote significant difference ($P < 0.01$).

C-Reactive Protein

Plasma CRP concentrations in participants fed the whey protein decreased ($P>0.05$) from 2587 ng/ml to 1921 ng/ml on week 6 while that in the soy group increased from 2374 to 2426 gn/ml (Figure 10). Concentrations of plasma CRP on week 6 were somewhat ($P>0.05$) decreased in the whey group compared to the soy group.

Cytokine Production

Analysis of covariance showed lower ($P<0.05$) concentrations of plasma IL-4 in the whey group on week 6 compared to the soy group. Concentrations in the whey group decreased ($P<0.05$) from 18.8 pg/ml at week 0 to 8.6 pg/ml on week 6, similarly compared, those in the soy group increased from 11.5 to 13.5 pg/ml (Figure 11).

Compared to baseline, plasma concentrations of IFN-γ increased by 8.03 and 1.40 pg/ml in whey and soy treatments (Figure 12), as analyzed by ANCOVA comparisons on week 6 were significantly different ($P<0.05$).

There was a significant ($P<0.06$) treatment difference in concentration of plasma TNF-α on week 6 (Figure 13). While plasma TNF-α increased by 2.22 pg/ml in the whey group, the concentration if the soy group decreased by 1.53 pg/ml (Figure 14).

There was no significant treatment difference in concentrations of plasma IL-1α, IL-1β, IL-2, IL-6, IL-8, and IL-10 (Table 4).

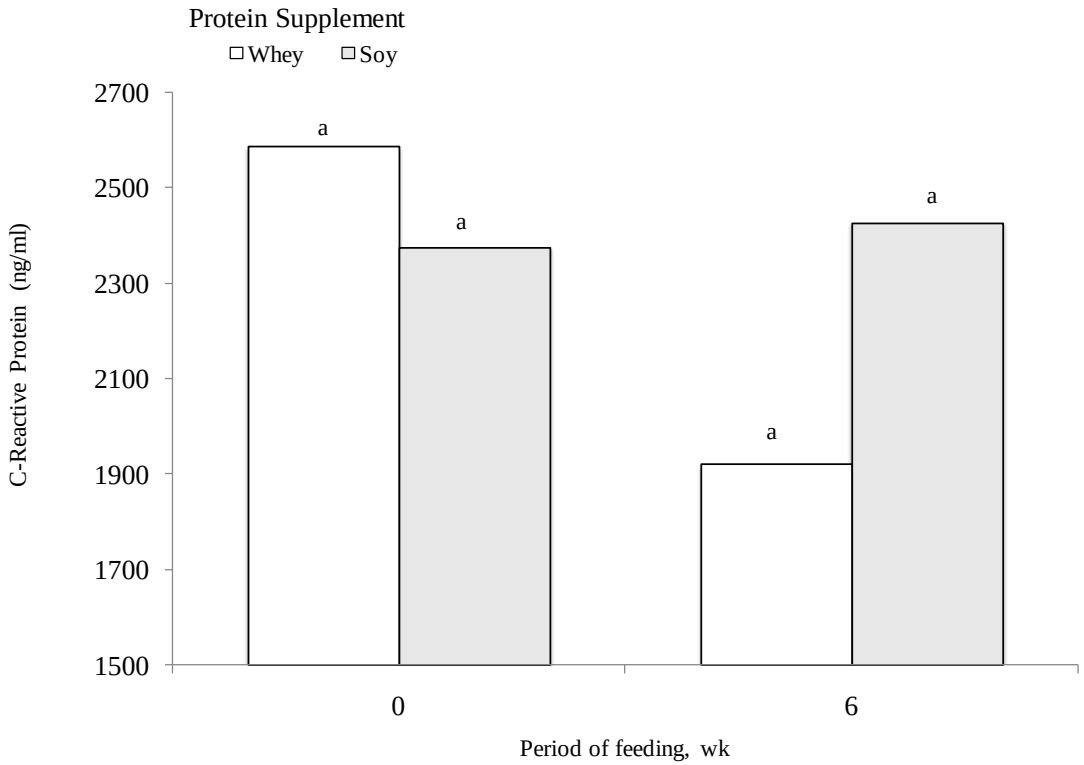


Figure 10. Plasma C-reactive protein concentrations in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA. [a] Different letters above each bar denote significant difference ($P < 0.05$).

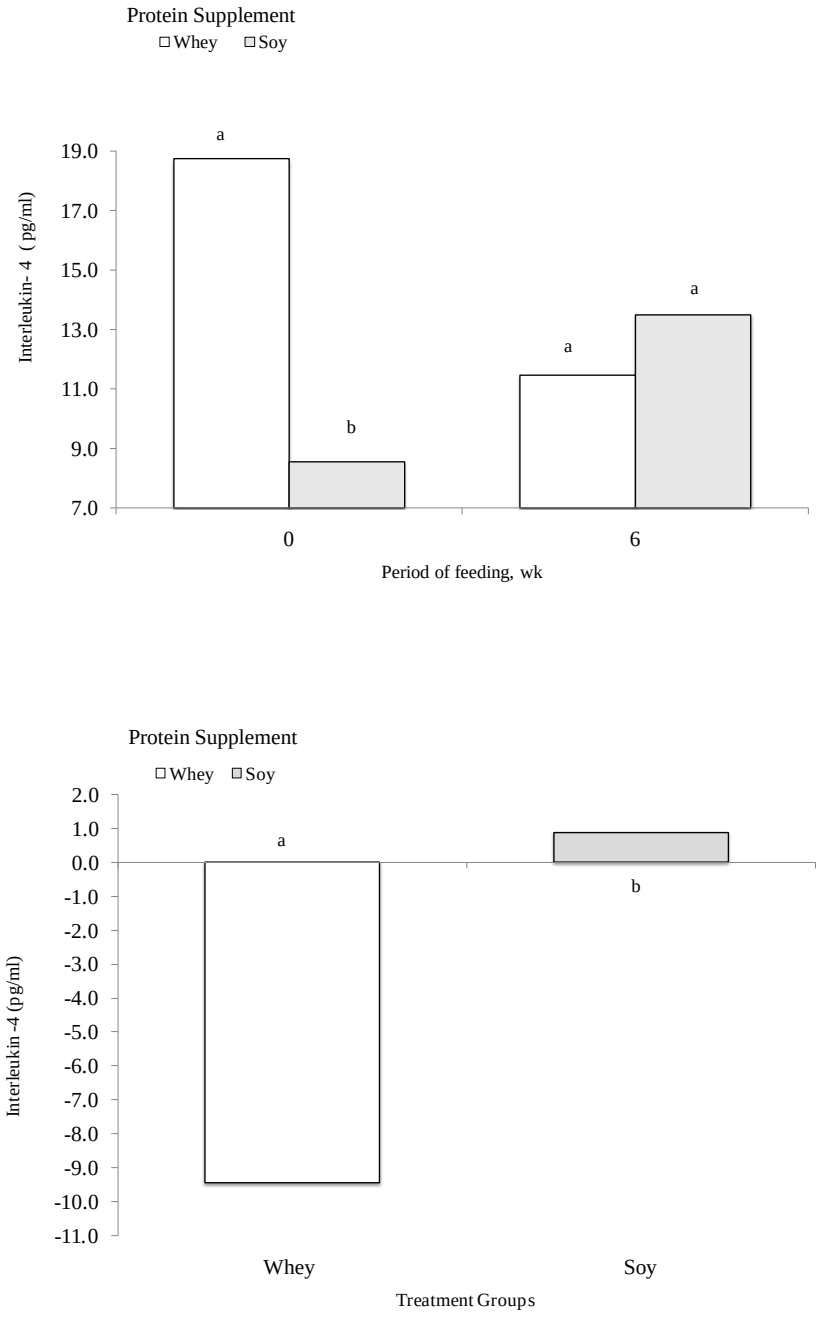


Figure 11. Plasma concentrations of IL-4 in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA (top) and ANCOVA (bottom) using the difference between wk 6 and wk 0. [a b] Different letters above each bar denote significant difference ($P < 0.10$).

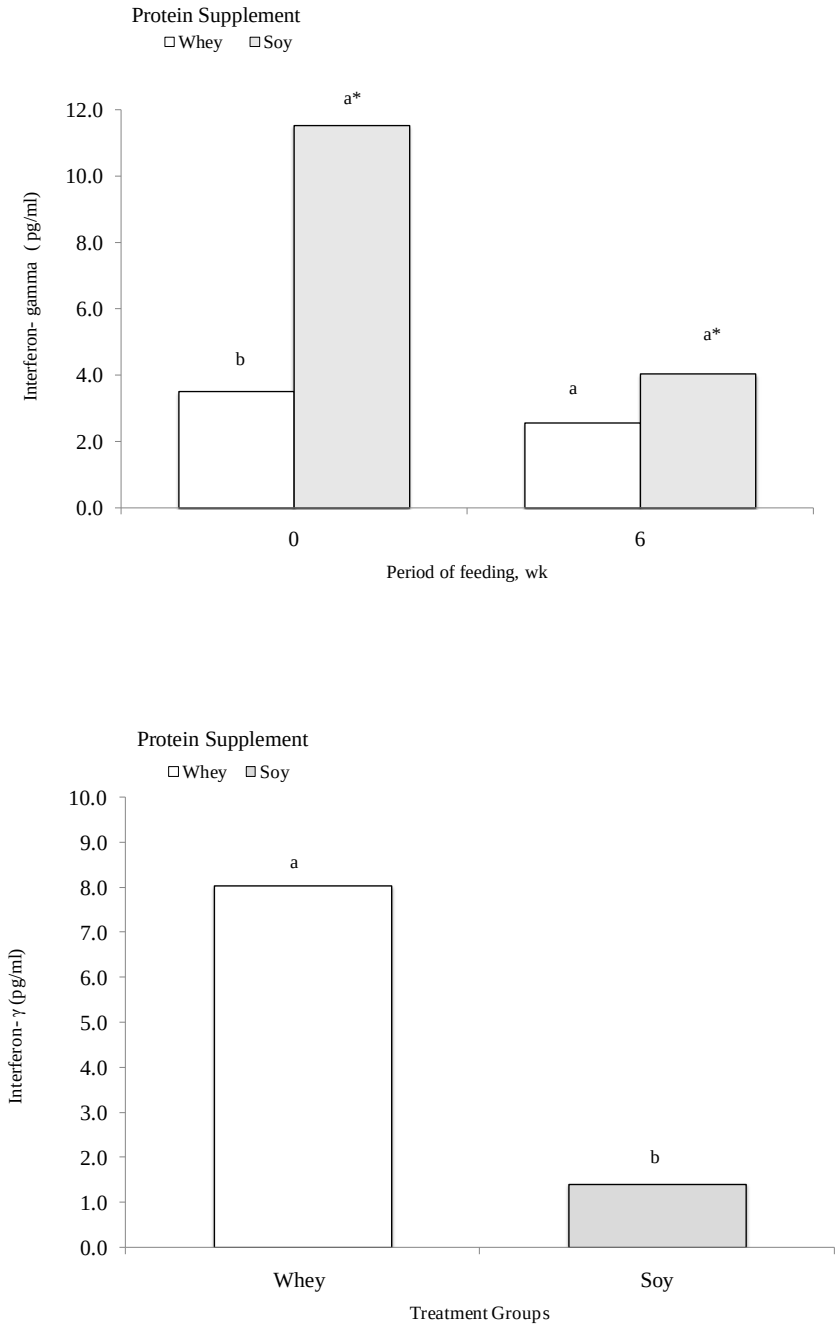


Figure 12. Plasma concentrations of IFN-γ in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA (top) and ANCOVA (bottom) using the difference between wk 6 and wk 0. [a b] Different letters above each bar denote significant difference ($P < 0.06$). [*] Different symbols above each bar denote significant difference ($P < 0.02$).

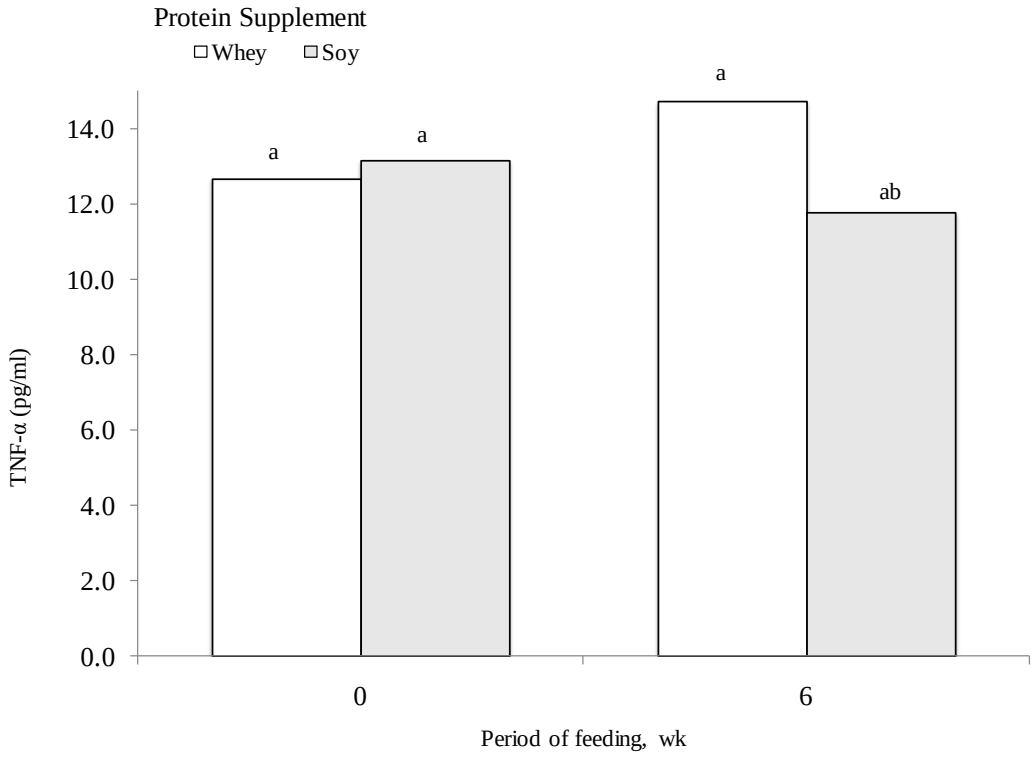


Figure 13. Plasma concentrations of TNF-α in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANOVA. [a b] Different letters above each bar denote significant difference among the supplement groups ($P < 0.02$).

Figure 14. Plasma concentrations of TNF-α in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANCOVA using the difference between wk 6 and wk 0. [a b] Different letters above each bar denote significant difference ($P < 0.07$).

Table 4. Plasma concentrations (pg/mL) of IL-1α, IL-1β, IL-2, IL-8,IL-10 of subjects fed whey or soy protein in 6 wk. Data were analyzed using difference between wk 6 – wk 0 by ANCOVA. [a] Different letters denote significant difference ($P < 0.05$).

	Whey	Soy
IL-1α	-5.37[a]	-2.44[a]
IL-1β	-6.45[a]	2.58[a]
IL-2	-0.20[a]	-0.30[a]
IL-6	10.08[a]	2.45[a]
IL-8	-1.97[a]	-1.75[a]
IL-10	-4.67[a]	0.90[a]

DXA Scan Analysis

The DXA scans provided information on bone mineral composition (BMC), bone mineral density (BMD), total lean mass, total body mass, total body fat, and % body fat (%BF) of individual body components. Each scan produced values for both right and left arms and legs, ribs, spine subsections, pelvis, trunk and head. Figure 15 illustrates a DXA scan obtained. Results from the scans also provided a table compared changes between and week 6 on total BMD and change in BMD.

Total BMC on week 6 was higher ($P<0.06$) in participants fed whey (2221 g). Those fed soy (2170 g) (Figure 16) as analyzed by ANCOVA. In fact total BMC increase by 34.9 g in whey compared as a decreased in 4.9 g in the soy group during the 6 week intervention period (Figure 17). Those changes were mostly attributed to changes in the arms, legs, and spine.

Total BMD also tended ($P>0.05$) in increase more (by 0.23 g/cm^2) in the whey group compared to the soy group (0.06 g/cm^2) after 6 weeks of supplementation (Figure 18). During the intervention period, there was a significant increase ($P<0.02$) in the right leg BMD in the whey group (Figure 19).

Total body fat between baseline and week 6 decreased ($P>0.05$) by 179 g in the whey group by increased by 380 g in the soy group (Figure 20). This difference is mainly attributed to changes in trunk fat which increased by 130 g in the whey group but increased 244 g in the soy group. However, ANCOVA revealed no significant treatment difference on week 6 (Figure 21).

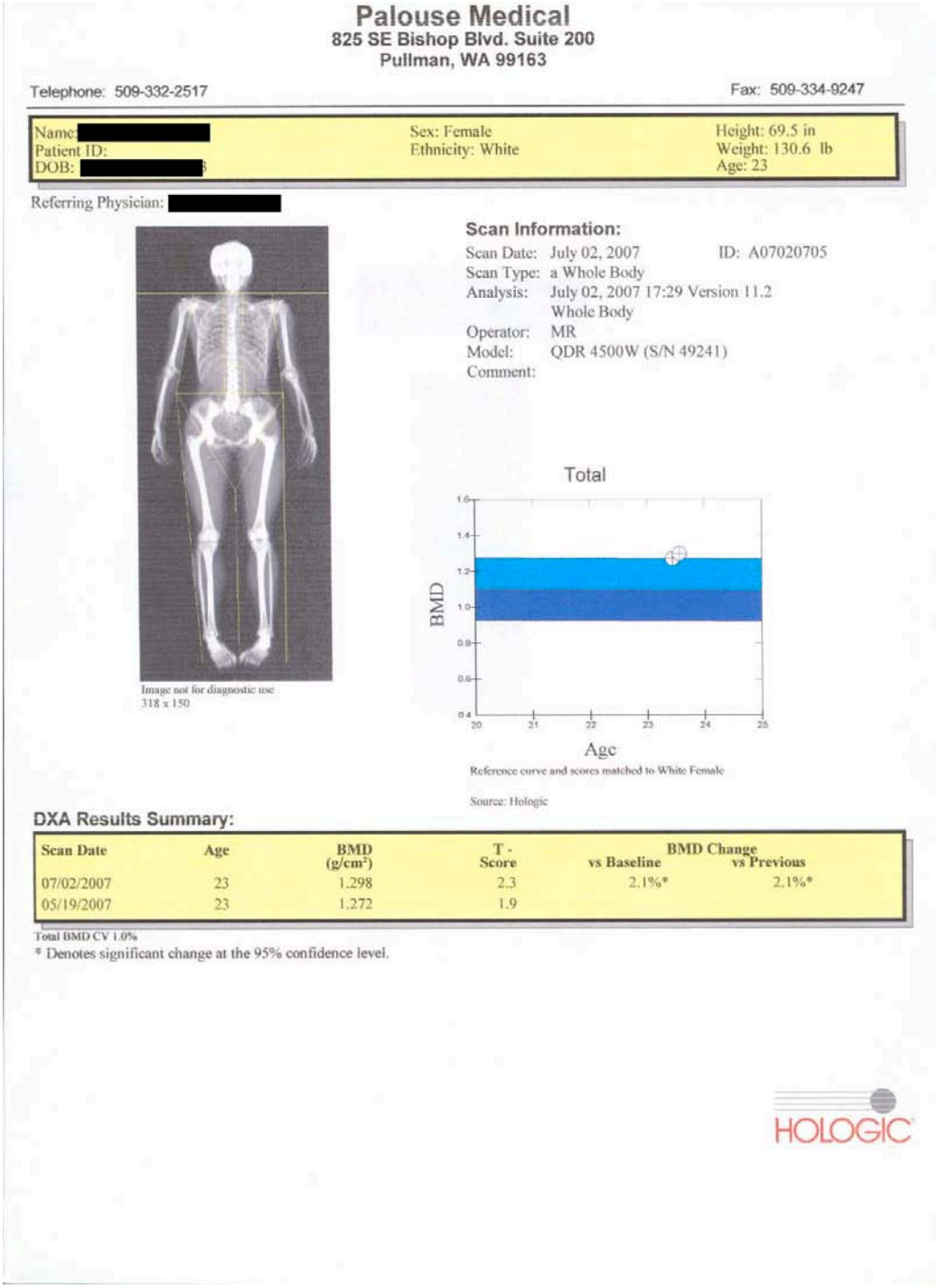
Changes in % BF mirrored those observed with total body fat. The % BF decreased ($P>0.05$) by 0.16% in the whey group but increased 0.25% in the soy group (Figure 22).

Total body mass decreased ($P<0.06$) by 217 g in the whey group compared to an increase of 933 g in the soy group. Major differences were associated with the trunk, arms, and legs.

Total lean mass reflects changes observed with total body mass. While total lean mass in participants fed whey protein decreased ($P>0.05$) by week 6, those fed soy protein showed an increase in total lean mass (Table 5).

Iron Analysis

There was no significant treatment difference in concentration of serum iron and transferrin, and in UIBC, TIBC.



Palouse Medical
825 SE Bishop Blvd. Suite 200
Pullman, WA 99163

Telephone: 509-332-2517 Fax: 509-334-9247

Name: [redacted]
Patient ID:
DOB: [redacted]
Sex: Female
Ethnicity: White
Height: 69.5 in
Weight: 130.6 lb
Age: 23

Referring Physician: [redacted]

Image not for diagnostic use
318 x 150

Scan Information:

Scan Date: July 02, 2007 ID: A07020705
Scan Type: a Whole Body
Analysis: July 02, 2007 17:29 Version 11.2
Whole Body
Operator: MR
Model: QDR 4500W (S/N 49241)
Comment:

Total

BMD

Age

Reference curve and scores matched to White Female

Source: Hologic

DXA Results Summary:

Scan Date	Age	BMD (g/cm²)	T - Score	BMD Change vs Baseline	BMD Change vs Previous
07/02/2007	23	1.298	2.3	2.1%*	2.1%*
05/19/2007	23	1.272	1.9		

Total BMD CV 1.0%
* Denotes significant change at the 95% confidence level.

HOLOGIC

Figure 15. DXA scan reports from subjects at wk 0 and wk 6.

Figure 15 DXA scan reports from subjects at wk 0 and wk 6.

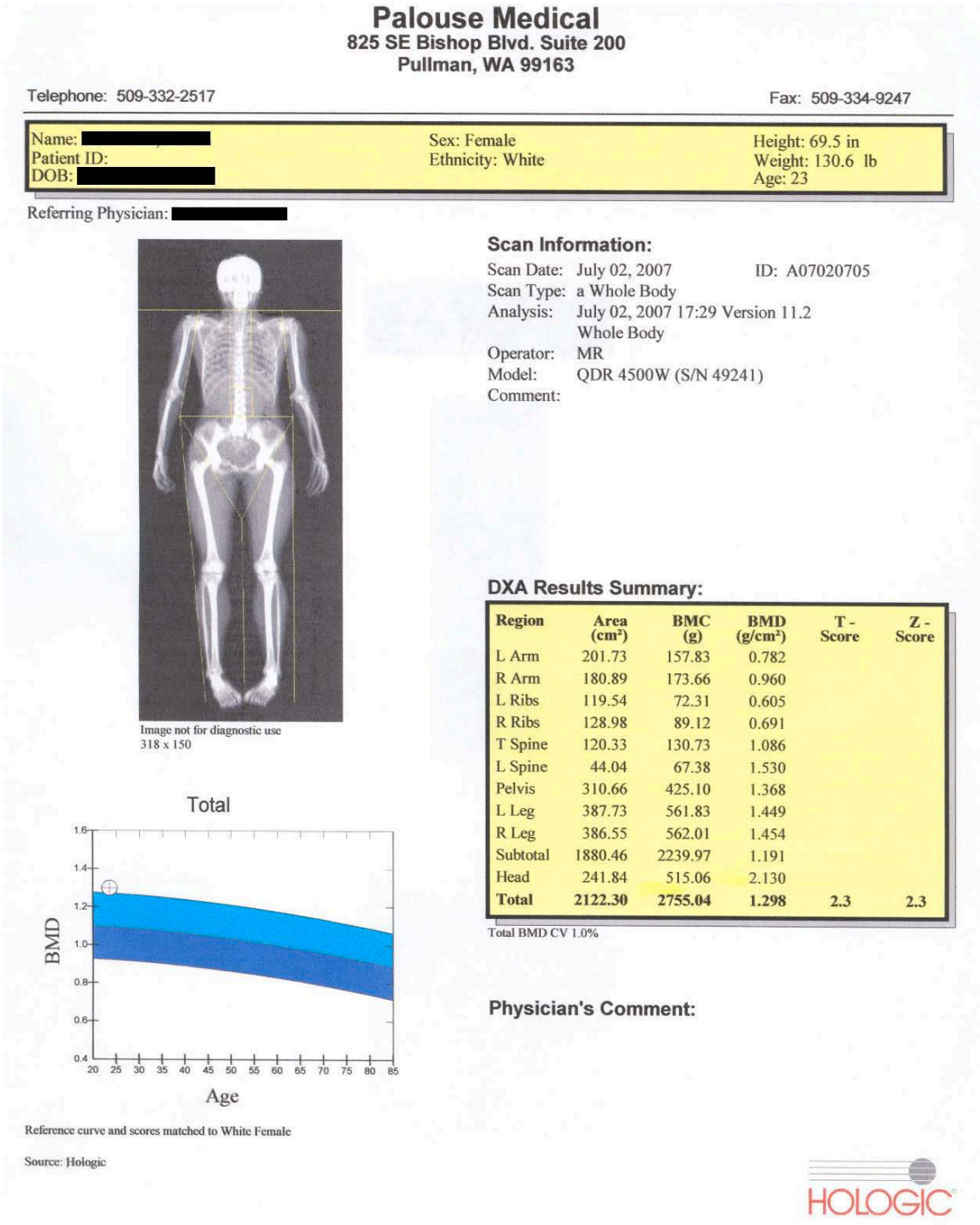


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Image not for diagnostic use
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Scan Date: July 02, 2007 ID: A07020705
Scan Type: a Whole Body
Analysis: July 02, 2007 17:29 Version 11.2
Whole Body
Operator: MR
Model: QDR 4500W (S/N 49241)
Comment:

DXA Results Summary:

Region	Area (cm²)	BMC (g)	BMD (g/cm²)	T - Score	Z - Score
L Arm	201.73	157.83	0.782		
R Arm	180.89	173.66	0.960		
L Ribs	119.54	72.31	0.605		
R Ribs	128.98	89.12	0.691		
T Spine	120.33	130.73	1.086		
L Spine	44.04	67.38	1.530		
Pelvis	310.66	425.10	1.368		
L Leg	387.73	561.83	1.449		
R Leg	386.55	562.01	1.454		
Subtotal	1880.46	2239.97	1.191		
Head	241.84	515.06	2.130		
Total	**2122.30**	**2755.04**	**1.298**	**2.3**	**2.3**

Total BMD CV 1.0%

Total

BMD

1.6
1.4
1.2
1.0
0.8
0.6
0.4

20 25 30 35 40 45 50 55 60 65 70 75 80 85

Age

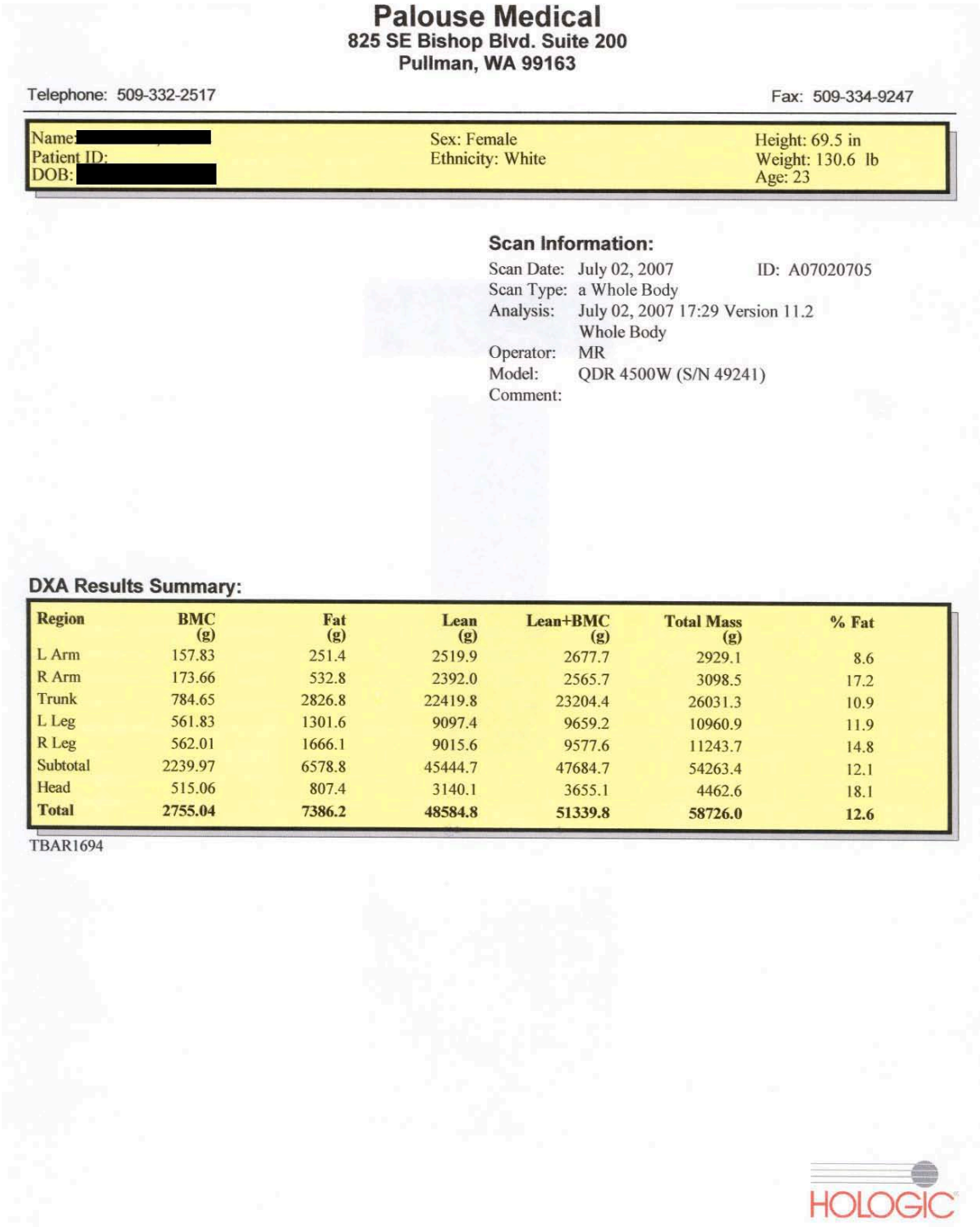
Reference curve and scores matched to White Female

Source: Hologic

Physician's Comment:

HOLOGIC

Figure 15 DXA scan reports from subjects at wk 0 and wk 6.



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Weight: 130.6 lb
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Scan Information:

Scan Date: July 02, 2007 ID: A07020705
Scan Type: a Whole Body
Analysis: July 02, 2007 17:29 Version 11.2
Whole Body
Operator: MR
Model: QDR 4500W (S/N 49241)
Comment:

DXA Results Summary:

Region	BMC (g)	Fat (g)	Lean (g)	Lean+BMC (g)	Total Mass (g)	% Fat
L Arm	157.83	251.4	2519.9	2677.7	2929.1	8.6
R Arm	173.66	532.8	2392.0	2565.7	3098.5	17.2
Trunk	784.65	2826.8	22419.8	23204.4	26031.3	10.9
L Leg	561.83	1301.6	9097.4	9659.2	10960.9	11.9
R Leg	562.01	1666.1	9015.6	9577.6	11243.7	14.8
Subtotal	2239.97	6578.8	45444.7	47684.7	54263.4	12.1
Head	515.06	807.4	3140.1	3655.1	4462.6	18.1
Total	**2755.04**	**7386.2**	**48584.8**	**51339.8**	**58726.0**	**12.6**

TBAR1694

HOLOGIC

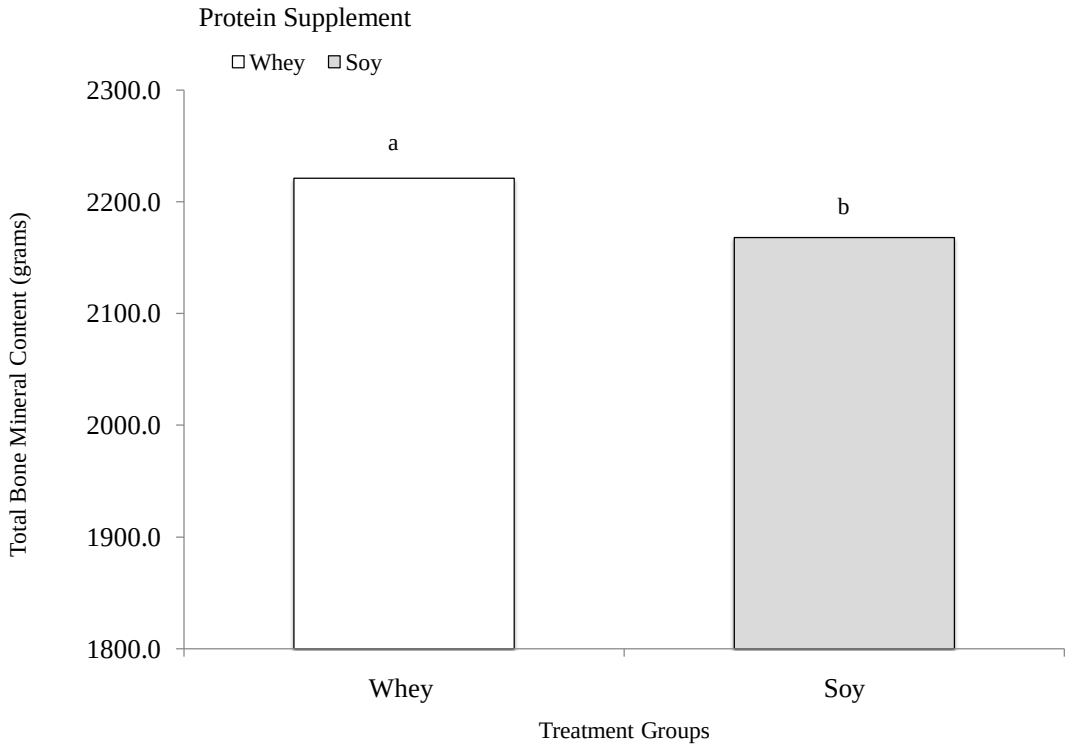

Figure 16. Total bone mineral content in subjects fed whey or soy protein for 6 weeks. Data were analyzed by ANCOVA using wk 0 as a covariate. [a b] Different letters above each bar denote significant difference ($P < 0.06$).

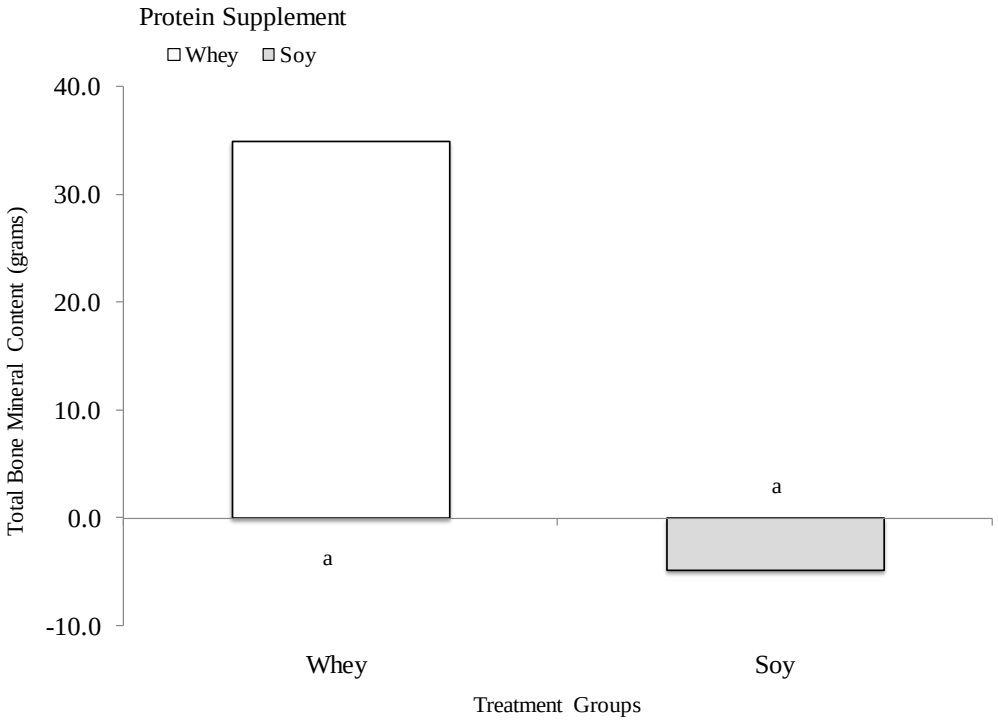

Figure 17. Total bone mineral content in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANCOVA using the difference between wk 6 and wk 0. [a] Different letters above each bar denote significant difference ($P < 0.05$).

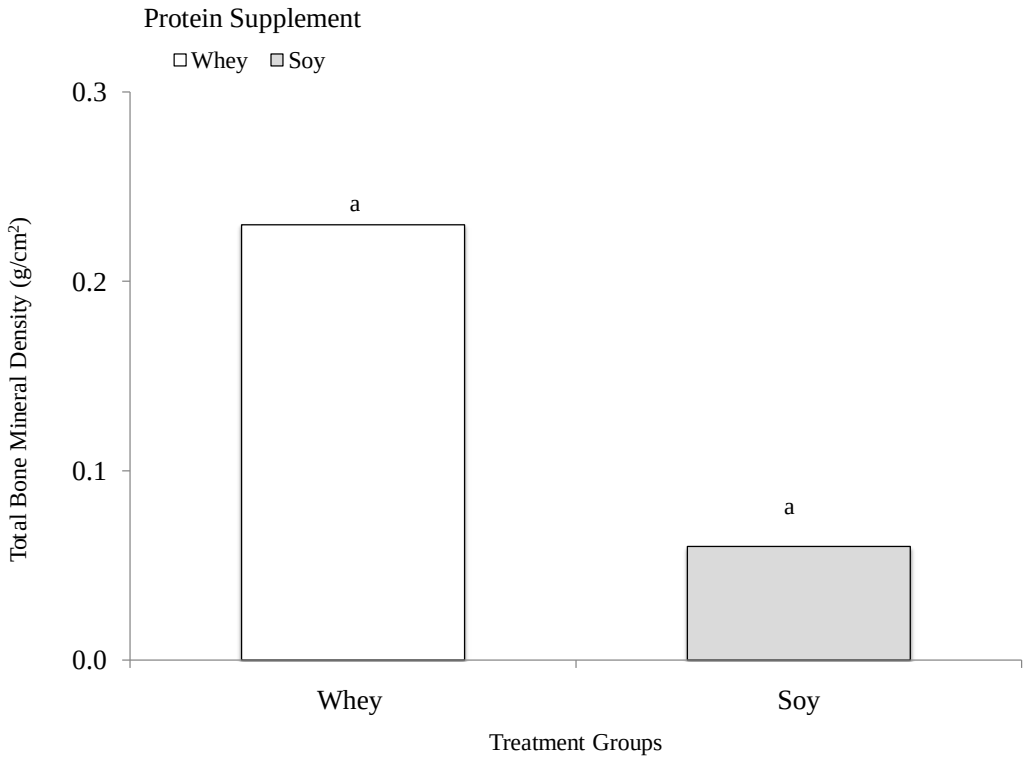

Figure 18. Total bone mineral density in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANCOVA using difference between wk 6 and wk 0. [a b] Different letters above each bar denote significant difference ($P < 0.05$).

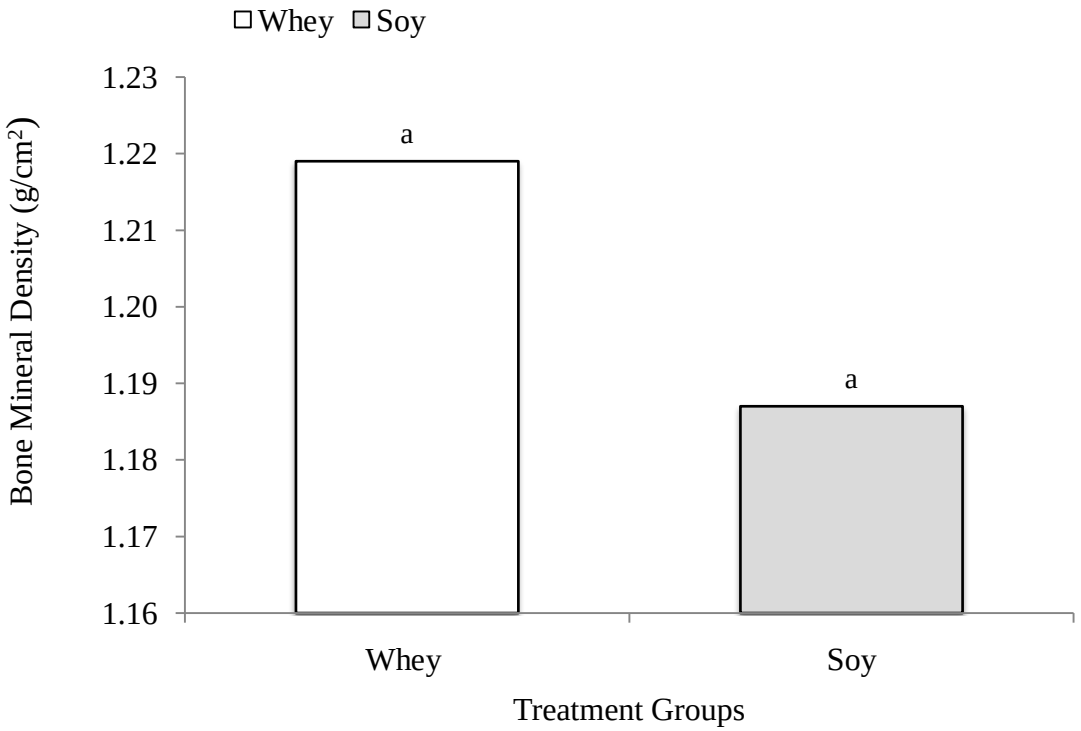

Figure 19. Right leg bone mineral density in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANCOVA using the difference between wk 6 and wk 0. [a b] Different letters above each bar denote significant difference ($P < 0.02$).

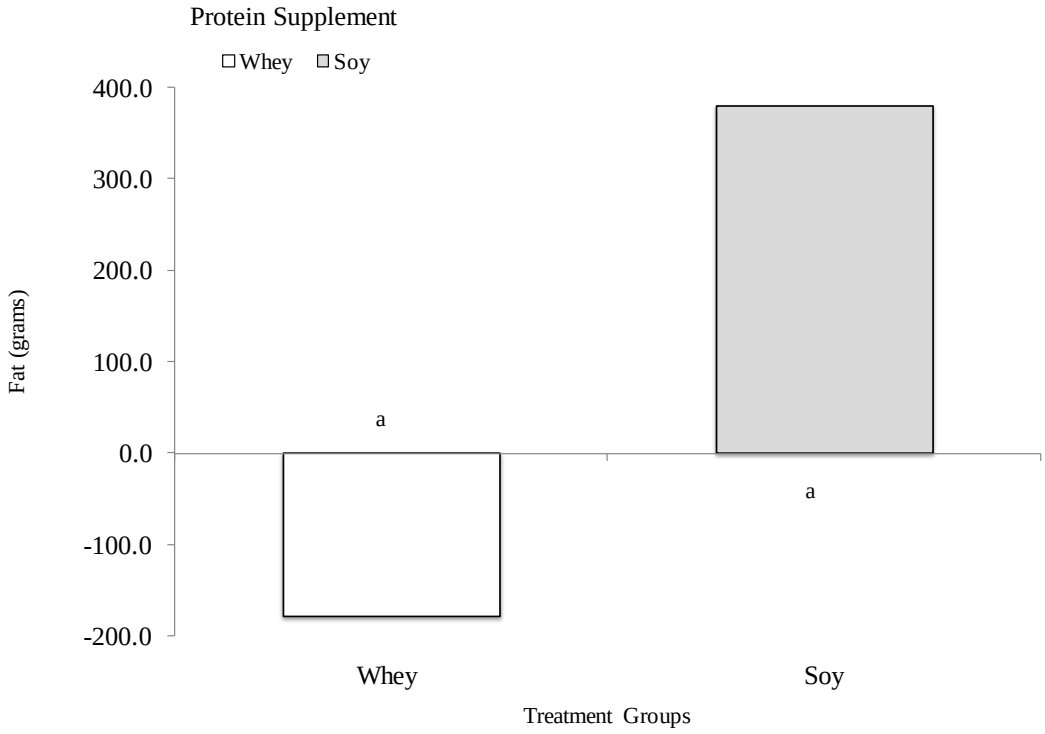

Figure 20. Total body fat in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANCOVA using the difference between wk 6 and wk 0. [a] Different letters above each bar denote significant difference ($P < 0.05$).

Figure 21. Trunk body fat in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANCOVA using the difference between wk 6 and wk 0. [a] Different letters above each bar denote significant difference ($P < 0.05$).

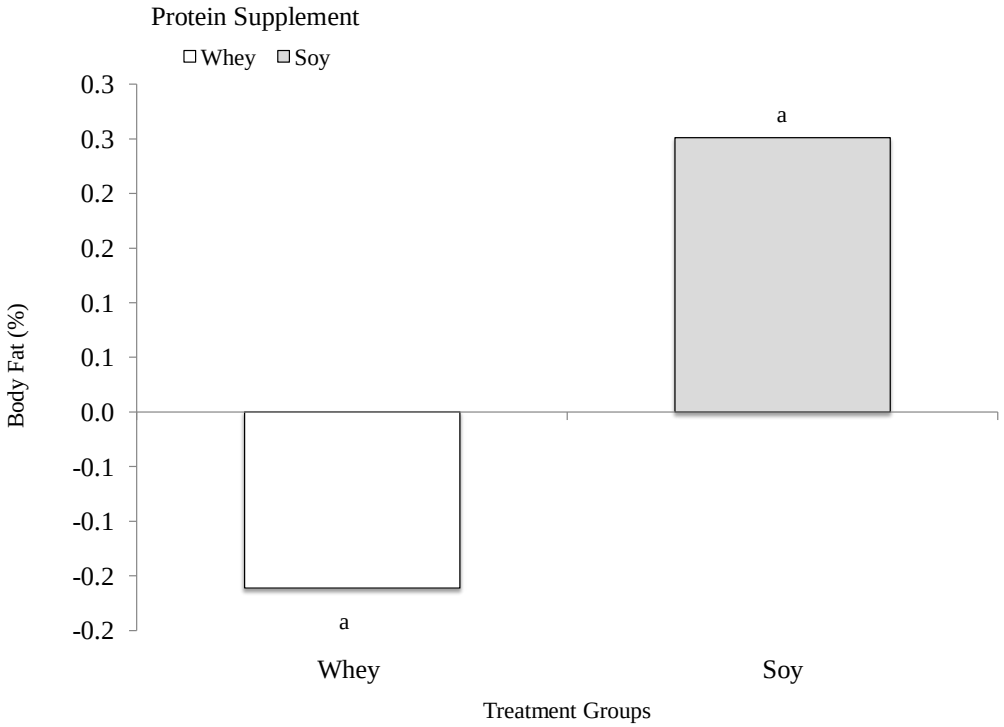

Figure 22. Percent body fat in subjects fed whey or soy protein for 6 wk. Data were analyzed by ANCOVA using the difference between wk 6 and wk 0. [a] Different letters above each bar denote significant difference ($P < 0.05$).

Table 5 Total lean mass and total body mass in subjects fed whey or soy protein for 6 wk. Data were analyzed using difference between wk 6 – wk 0 by ANCOVA. [a b] Different letters denote significant difference ($P < 0.05$).

	Total Lean Mass (grams)	Total Body Mass(grams)
Whey	-49[a]	-217[b]
Soy	587[a]	933[a]

DISCUSSION

This study is the first to evaluate the effect of soy protein isolate and whey protein isolate supplementation on the prevention of exercise-induced oxidative stress and inflammatory damage in young female endurance athletes. A limited number of studies have addressed the effects of either soy protein (Hamalainen et al. 2006, Paula et al. 2008, Ryan-Borchers et al. 2006; Si and Liu 2009, Suh et al. 2002) or whey protein (Pecquet et al. 1999, Shing et al. 2007, Saint-Sauveur et al. 2007; Yamaguchi and Uchida 2007) supplementations on immunological markers. As competition heightens, practice time is increased, and the limited recovery time tends to exacerbate the physical stress and potential for injury in an athlete. Consumption of protein supplements in young female athletes is the lowest percentile among all consumers (Erdman et al. 2007, Froiland et al. 2004; Kristiansen et al. 2005). Therefore, it was important to evaluate the potential advantages of supplemental protein in the form of whey and soy products in endurance athletes.

In this study, female athletes who consumed whey protein had lower ($P<0.05$) plasma CRP concentrations after 6 wk of supplementation. This is in contrast to those given soy protein where CRP concentrations increased ($P<0.05$) over the same period. In previous research, an increase in IL-6 concentration has been reported in endurance athletes (Drenth et al. 1995, Suzuki et al. 2000, Zaldivar et al. 2006). Elevated blood CRP has been implicated in chronic disease development and progression (Li and Fang 2004). CRP is produced by the liver in response to the inflammatory cytokines IL-6, IL-1 and TNF-α. However, there was no significant treatment difference in circulating IL-1 and IL-6 in this study. In fact, plasma concentrations of TNF-α were elevated by whey protein intervention but suppressed by soy protein intervention. Studies have shown that soy protein isolate suppressed TNF-α (Si and Liu

2009; Suh et al. 2003) and that soy isoflavones inhibited TNF-α-induced apoptosis and TNF-α induced IL-6 production (Suh et al. 2003). Supplemental α-lactalbumin and β-lactoglobulin, components of milk protein, significantly inhibited IL-6 production (Yamaguchi et al. 2007). Therefore, results from this study suggest that the anti-inflammatory action of whey protein on circulating CRP cannot be explained by the observed changes in IL-1, IL-6 or TNF-α.

The anti-oxidative action of whey protein was similarly reflected in the suppression of lipid peroxidation. After 6 wk of feeding, athletes given whey protein had lower ($P<0.01$) plasma TBARS while concentrations did not change in those given soy protein. Even though not statistically significant, plasma protein carbonyl concentrations tended to follow the same trend as plasma CRP and TBARS concentrations.

Another measure of oxidative damage is the concentration of 8-OHdG, a biomarker for DNA damage. Plasma 8-OHdG decreased during soy protein intervention but remained unchanged in those given whey protein; consequently, athletes given soy protein had lower ($P<0.07$) plasma 8-OHdG on wk 6 than those given whey protein. This is in contrast to the other anti-oxidative measures studied, CRP and TBARS. Ryan-Borchers et al. (2006) similarly reported lower plasma concentrations of 8-OHdG following soy protein supplementation to postmenopausal women. Neither treatment had any significant effect on changes in plasma total antioxidant activity, GSHt or GSSG. These results suggest that both whey and soy proteins have anti-oxidative action, albeit though different bioactive compounds and mechanisms. Antioxidants likely decrease inflammation by down-regulating the pro-inflammatory NFκB gene (Hamalainen et al. 2007), which is responsible for cytokine production in immune cells (Barnes et al. 1997, Schottelius and Baldwin 1999). Subjects given soy protein had higher ($P<0.05$) plasma concentrations of IL-4 compared those fed the whey protein on wk 6. The IL-4 targets B

cell proliferation. We did not measure changes in immunoglobulin concentrations in this study. However, Gleeson et al. (2000) reported that salivary IgA decreased after intense exercise and that lower concentrations of IgA after exercise is directly correlated with urinary tract infection. Minehira et al. (2000) reported that young rats given soy protein had lower IgA concentration compared to those fed casein; however, in aged rats fed whey protein isolate, IgA concentration was lower compared to those fed casein or soy protein. Soy isoflavones influence both non-specific and specific immune responses by influencing signal transduction of macrophages and other phagocytic cells and the activity of cytotoxic T lymphocytes (Rumsey et al. 1994, Stewart et al. 1997). Our results indicate a significant treatment difference in BMD and BMC when comparing whey and soy protein supplements. Athletes fed the whey protein had higher right leg total BMD ($P<0.05$) and BMC ($P<0.06$) when compared to those given soy protein. While research has shown a positive correlation between exercise and BMD in postmenopausal women (Kemmler et al. 2007), premenopausal female endurance athletes tend to have lower BMD than other athletes (Burrow et al. 2009; Mudd, et al. 2007). This has been attributed to the high incident of dietary deficiencies and restricted caloric intake by this population (Barrack et al. 2008, Rosen et al. 1986). As a whole, athletes have higher BMD compared to non-athletes (Heinonen et al. 1993, Slemenda et al. 1993). However, Heinrich et al. (1990) reported that female runners did not have higher BMC in the spine and femoral neck. Burrows et al. (2009) and Robinson et al. (1995) suggested an association with endurance female runners and an increase risk of fractures due to the low BMD. Our results suggest that whey protein may contribute to a higher BMD thus decreasing the incident of fractures.

Bone is in a continuous process of remodeling. Osteoclasts break down bone protein and matrix whereas osteoblasts function primarily in bone construction; this process is regulated by

numerous hormones, including estrogen, insulin and leptin (Cornish et al. 2002; Ducy et al. 2000, Hamrick et al. 2005). Milk contains the necessary components needed for bone formation and reabsorption. Uenishi et al. (2007) reported an increase in BMD mediated by the suppression of osteoclast activity following 6 wk of milk protein supplementation. The estrogenic properties of soy isoflavones are thought to have influence over the regulation of IL-6 and INF-γ on bone remodeling through the transcription factor, nuclear-factor kappa B (NF-κB). IFN-γ modulates osteoblastic activity and IL-6 regulates osteoclastic activity in the bone remodeling process (McCormick, 2007). Ye et al. (2006) reported an attenuation of bone loss in postmenopausal women following soy isoflavone supplementation over in a 24 week intervention period. Therefore, an intervention period longer than 6 wk is necessary with premenopausal women to recognize potential soy isoflavone support on bone health.

The mechanisms by which bone and fat growth are regulated are closely related as both adipocytes and osteoblasts originate from mesenchymal stem cells in the bone marrow (Turner et al. 1994). Excess adipose tissue can hinder bone growth by inhibiting osteoblastic differentiation. Under conditions of bone loss, such as induced by estrogen depletion, bone marrow adipogenesis increases (Klein et al. 1998). In this study, total BF tended to decrease ($P>0.05$) in participants fed whey protein while total BF in those fed soy protein tended to increased. The primary region with the largest difference in total BF was the trunk. When measured as %BF, participants given whey protein again averaged lower ($P>0.05$) than those fed soy protein. Therefore, whey supplementation supports the adipocyte:osteoblast balance by increasing BMD and decreasing total body fat. .

In contrast to changes in bone, subjects fed whey protein averaged lower in total body mass ($P<0.06$) compared to subjects fed the soy protein. However, there was no treatment

different in lean mass. Therefore, higher total body mass in subjects given soy protein is likely attributed to higher total body fat. Whey protein intake has been shown to increase lean body mass and increase muscle strength (Anderson et al. 2005). Whey protein provides all the essential amino acids in approximately the same proportion as muscle protein, thereby making it a high quality protein supplement (Bergstrom et al. 1974, Rasmussen et al. 2000).

Amino acid availability is an important regulator of muscle protein metabolism (Tipton et al. 2004). Muscle net protein balance must be positive (greater anabolism than catabolism) to result in a net increase in lean muscle. Lean muscle gain occurs only when sufficient amino acids are available in the intracellular pool. Whey and soy are both good sources of amino acids. Both proteins are easily digestible and have similar absorption kinetics (Anthony et al. 2007). Phillips et al. (2005) reported that whey was superior to soy in stimulating amino acid uptake during a resistance training program. Similar protein synthesis rates have been observed in exercised skeletal muscle in rats that ingested either whey or soy protein (Anthony et al. 2007). However, neither whey nor soy protein supplement influenced lean body mass in this study.

In conclusion, both whey and soy protein supplements can be beneficial to athletes. Both protein supplements provide anti-oxidative action, albeit through different bioactive compounds. While whey protein increased BMD and BMC, and decreased body fat, soy protein increased body mass; the latter is likely attributed to increase in total body fat. Further research is needed to better understand the physiological adaptations, nutrition demands, and changes during endurance athletic events.

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APPENDIX A

WASHINGTON STATE UNIVERSITY
CONSENT FORM

Title: Effects of Whey Protein in the Prevention of Exercise-induced Oxidative Stress And Inflammatory Damage in Human Athletes

RESEARCHERS

Jean Soon Park, Assistant Research Professor (335-2205)
Boon P. Chew, Professor and Scientist (335-1427)

Department of Food Science and Human Nutrition
Washington State University
Pullman, WA 99164-6376

RESEARCHERS' STATEMENT

You are being asked to participate in a research study to assess the beneficial effects of supplemental whey or soy protein in regulating the immune response and decreasing oxidative damage in athletes. The purpose of this consent form is to give you the information you will need to help you decide whether to be in the study or not. Please read the form carefully. You may ask questions about the purpose of the research, what we would ask you to do, the possible risks and benefits, your rights as a volunteer, and anything else about the research or this form that is not clear. When we have answered all your questions, you can decide if you want to be in the study or not. This process is called 'informed consent.' We will give you a copy of this form for your records.

PURPOSE AND BENEFITS

This study will provide valuable information on the health benefits of supplemental whey and soy proteins in modulating immune response and reducing oxidative stress and inflammation in athletes. Oxidative damage is associated with muscle damage and with chronic diseases such as cancer, neurodegeneration, macula degeneration, inflammation and cardiovascular disease. No benefit to individual participants is anticipated.

PROCEDURES

Upon enrollment, you will be asked to complete a brief questionnaire concerning your general health. Further, we will record your height and weight. You will receive commercially-available sources of maltodextrin, whey protein powder or soy protein powder (60 g daily in the morning) for 6 weeks. This dose is similar to that recommended by the manufacturer. A small volume of blood (20 mL; about 4

teaspoons) will be drawn on weeks 0 and 6 by a trained phlebotomist. You will be advised to restrain from taking antioxidant supplements during the study. The blood will be used to assess immune function, and oxidative and inflammatory status. You will be provided written instructions on all procedures, and there will be no financial cost to you for any aspect of this study.

RISKS, STRESS, OR DISCOMFORT

It is possible that slight bruising and discomfort may arise from blood collection. In case bruising occurs, cold compresses will be applied. Bruising poses no health problems and that the discoloration will subside. However, the risk of bruising should be minimal because only health care professionals will be allowed to perform these procedures.

COMPENSATION

Each participant will receive $100 within 2-3 weeks of their complete participation, i.e. after the week 6 blood draw. Volunteers who withdraw any time before this specified time will receive $50 for their participation.

EXCLUSIONS AND RIGHTS TO WITHDRAW

Please do not participate if you have a history of cancer, alcohol abuse, or diabetes, or if you are a smoker or are taking antibiotics or antioxidant supplements. You may choose to withdraw at any time during the study. Your participation is completely voluntary and you will not be asked to explain your reasons for withdrawing.

PARTICIPANT CONSENT

I have read and understand the information describing this study. All of my questions have been answered to my satisfaction, and this form is being signed voluntarily by me indicating my desire to participate. I am not waiving any of my legal rights by signing this form. I understand that I will receive a copy of this signed consent form.

PARTICIPANT'S STATEMENT

This study has been explained to me. I volunteer to take part in this research. I have had a chance to ask questions. If I have general questions about the research, I can ask one of the researchers listed above. If I have questions regarding my rights as a participant, I can call the WSU Institutional Review Board at (509)335-9661. This project has been reviewed and approved for human participation by the WSU IRB.

_____

Printed name of participant Signature of participant Date

_____

Printed name of witness Signature of witness Date

APPENDIX B

Interview Questionnaire

The following information is confidential.

PERSONAL/WORK INFORMATION

Last Name	First Name	M.I.	Date

Gender (M/F)	Age	Height	Weight	Occupation	Birth Date

Home Address	Apt.

City	State	Zip

Home Phone	Work Phone	E-Mail Address

Please answer the following questions:

1. How would you describe your general health?
   - o Fair
   - o Good
   - o Very good

3. Which of the following best describes your education level?
   - o High school graduate
   - o College graduate

4. Ethnicity (mark all that apply):
   - o American Indian
   - o Black
   - o Asian
   - o Hispanic
   - o Caucasian
   - o Other

5. Do you currently use tobacco in any form?
   - o No
   - o Yes

6. Have you ever used tobacco in any form?

- o No
- o Yes

If yes, how long ago did you quit? _____ years

7. Do you consume alcohol in any form?

- o No
- o Yes

If yes, please describe the type, amount, and frequency.

HEALTH INFORMATION

Current Medications and/or Vitamin Supplements

Name of Medication/Supplement	Reason for Taking		Name of Medication/Supplement	Reason for Taking

Personal Health History

Do you have, or have you ever had, any of the following? Please check Yes or No.

	Yes	No		Yes	No
Heart disease	☐	☐	Dizzy spells	☐	☐
Heart attack	☐	☐	Unusual fatigue	☐	☐
Heart surgery	☐	☐	Recent or serious illness	☐	☐
Disease of an artery	☐	☐	Skipped or rapid heart beats	☐	☐
Angioplasty	☐	☐	Chronic swelling of the feet or ankles	☐	☐
High Blood Pressure	☐	☐	Kidney disease	☐	☐
Rheumatic fever	☐	☐	Liver disease	☐	☐
Stroke	☐	☐	Shortness of breath (unexplained)	☐	☐
Claudication	☐	☐	Asthma	☐	☐
Diabetes	☐	☐	Chronic bronchitis	☐	☐
Thyroid Disease	☐	☐	Emphysema	☐	☐
Epilepsy	☐	☐	Bone or joint injury	☐	☐
Elevated cholesterol	☐	☐	Frequent choking	☐	☐
Irritable bowel syndrome	☐	☐	Reflux	☐	☐
Nausea	☐	☐	Heartburn	☐	☐
Difficulty Chewing or Swallowing	☐	☐	Anemia/blood disease	☐	☐
			Sexually transmitted disease	☐	☐

Pain, discomfort (or other anginal equivalent) in the chest, neck, jaw, arms, or other areas due to ischemia	Yes	No
	☐	☐

If you responded “Yes” to ANY item in the Personal Health History section, please explain and give dates:

Family Health History

Please describe your family history of any of the above-mentioned conditions, if applicable:

To be completed by researchers:

Body Mass Index (BMI)

Height (baseline)_____m

Weight (baseline)_____kg

Weight (post)_____kg

BMI (baseline) _____kg/m^2

BMI (post)_____kg/m^2

APPENDIX C

Three Day Food Record

A three day food record is designed to get an accurate description of your *typical* daily diet. This information will be analyzed by a computer program that estimates average daily intakes of calories, carbohydrate, protein, fat, cholesterol, fiber, vitamins, minerals, and other components of your diet. *Since this food record will be used to help you make appropriate dietary changes, it is important that you try* ***not*** *to change your usual eating pattern for these three days.* Please try to be as accurate as possible, recording all foods and beverages you eat/drink. Include the amount of food eaten, brand name, and important variations (skim, 2%, reduced fat, sugar-free, etc.). If the food is prepared at home or in a restaurant, please include a description of the preparation techniques. Recording the approximate time of your meals and snacks and your location/feelings will help us get to know the context of your eating decisions. In order to get an accurate representation of your diet, **record food intake for 2 weekdays and 1 weekend day.**

Sample Portion of 24-Hour Food Record

Time	*Amount*	*Food/Beverage Description*	*Location/Feelings*
7:00 am	1	frozen Lender's blueberry bagel	driving/rushed
	2 Tbsp	Philadelphia cream cheese, regular	
12:00 pm	2 cups	Iceberg lettuce	at desk/focused on work
	10	baby carrots	
	2 T	bacon bits	
	8	Ritz crackers	

24-Hour Food Record ***Date***

Time	*Amount*	*Food/Beverage Description*	*Location/Feelings*

24-Hour Food Record ***Date***

Time	*Amount*	*Food/Beverage Description*	*Location/Feelings*

24-Hour Food Record		***Date***	
Time	*Amount*	*Food/Beverage Description*	*Location/Feelings*

APPENDIX D

Washington State University
Food Science & Human Nutrition
DXA Scan Participant Information & Questionnaire

DATE: _____

NAME: _____

HEIGHT: _____ WEIGHT: _____

BODY FRAME: Small _____ Medium _____ Large _____

Are you pregnant? _____

Fractures History (circle if applicable):

Hip Spine Wrist Ankle Other: _____

Interfering Diagnostic Studies:

Any imaging studies performed in the last 5 days with contrast media? YES NO

Any radioactive studies in the last 3 days? YES NO

Any lower back surgery? YES NO

Metal objects in the abdominal area or in the back? YES NO

Have you taken calcium supplements within the past 48 hours? YES NO

Do you have any of the following medical conditions:

Thyroid problems ____ Asthma ____ Diabetes ____ Rheumatoid Arthritis _____ Epilepsy

____

Participant Signature: _____ Date: _____

APPENDIX E

Effects of Whey Protein in the Prevention of Exercise-induced Oxidative Stress And Inflammatory Damage in Human Athletes

Participant: _____ **Treatment**: Blue Green Yellow

Week/Day	Distance ran	Exercise time	Time drank supplement
Week 1 Day 1			
Week 1 Day 2			
Week 1 Day 3			
Week 1 Day 4			
Week 1 Day 5			
Week 1 Day 6			
Week 1 Day 7			
Week 2 Day 1			
Week 2 Day 2			
Week 2 Day 3			
Week 2 Day 4			
Week 2 Day 5			
Week 2 Day 6			
Week 2 Day 7			
Week 3 Day 1			
Week 3 Day 2			
Week 3 Day 3			
Week 3 Day 4			
Week 3 Day 5			
Week 3 Day 6			
Week 3 Day 7			
Week 4 Day 1			
Week 4 Day 2			
Week 4 Day 3			
Week 4 Day 4			
Week 4 Day 5			
Week 4 Day 6			
Week 4 Day 7			
Week 5 Day 1			
Week 5 Day 2			
Week 5 Day 3			
Week 5 Day 4			
Week 5 Day 5			
Week 5 Day 6			
Week 5 Day 7			
Week 6 Day 1			
Week 6 Day 2			
Week 6 Day 3			
Week 6 Day 4			
Week 6 Day 5			
Week 6 Day 6			
Week 6 Day 7			

APPENDIX F

Instructions for use:

1. Add cold water or juice upto the 16 ounce line on the shaker.
2. Cut corner of ziplock baggie.
3. Pour ENTIRE contents into the shaker.
4. Replace the lid.
5. Shake to mix until dissolved.
6. Drink! (you can pour into another glass if desired).
7. Record time in your log.