ABSTRACT

Effects of Four Weeks of Daily Soy Milk or Dairy Milk Ingestion on the Exercise Induced Inflammatory and Oxidative Responses in Plasma and Skeletal Muscle in a Post-Menopausal Female Population

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The process of senescence is associated with increasing inflammation and subsequent oxidative stress in the body, both of which can exert negative health effects at local and systemic levels. Attenuation of such processes with novel dietary countermeasures has major public health implications. Soyfoods, as a source of high quality protein, minimal saturated fat, and unique composition of isoflavones may improve such indices, although such effects in healthy older women are not well delineated. To explore this supposition, a single-blind, randomized, controlled trial was conducted on 31 post-menopausal women at Baylor University, Waco TX. After a two week run-in period, subjects were randomly assigned to consume three servings of vanilla soy (n=16) or reduced-fat dairy (n=15) milk per day for four weeks. Parameters of systemic inflammation (TNF-α, IL-1β, IL-6) and oxidative stress (SOD, GPx, COX-2) as well as expression of local inflammation-responsive genes (TNF-α, IL-1β, IL-6, COX-2, NF-κB) were measured prior to supplementation, at four weeks post supplementation, and after an eccentric exercise bout performed to elicit an inflammatory

response. A significant group by time effect for plasma TNF-α was observed ($p = 0.04$), with TNF-α values for the soy group appearing to stay consistent during the exercise period, while the TNF-α values for the dairy group increased post-supplementation, decreased from T2-T4, and then returned to baseline by T6. Significant time effects were observed for plasma SOD ($p < 0.0001$) and IL-6 ($p < 0.0001$), and muscle expression of IL-6 ($p < 0.01$) and IL-1β ($p<0.01$). Despite good dietary compliance, overall results from our study do not support the notion that four weeks of daily soy milk ingestion can attenuate systemic or local elevations in markers of oxidative stress or inflammation. However, data do suggest that the downhill running protocol utilized in this study can be effective at altering systemic and local markers of inflammation, and that ingestion of soy may help to maintain plasma TNF-α levels even when exposed to a stress inducing stimulus; although, more data exploring this conjecture is certainly warranted.

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A Dissertation

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LIST OF ABBREVIATIONS

AA/DHA - ascorbic acid/dehydroascorbic acid

ATP – adenosine triphosphate

BBI - Bowman-Birk inhibitor

COX-2 – cyclooxygenase two

CVD – cardiovascular disease

CRP - c-reactive protein

d - day

DNA – deoxyribonucleic acid

DXA – dual energy x-ray absorptiometry

EC – endothelial cell

eIF-4E – eukaryotic initiation factor

ERK – extracellular receptor kinase

g – gram

GSH/GSSG - reduced glutathione/oxidized glutathione

GPx – glutathione peroxidase

h - hour

H_2O_2 - hydrogen peroxide

HDL – high density lipoprotein

IFN-γ - interferon gamma

IL-1β – interleukin one beta

IL-6 – interleukin six

JNK - c-Jun amino-terminal kinases

kg – kilogram

kJ – kilojoule

km - kilometer

LDL – low density lipoprotein

m – meter

MAPK – mitogen activated protein kinase

MCP-1 – monocyte chemoattractant protein one

mg – milligram

mL – milliliter

mmol - millimole

mRNA – messenger ribonucleic acid

NO - nitric oxide

O_2^- - superoxide anion

OH• - hydroxyl radical

$ONOO^-$ - peroxynitrite

p38 MAPK - p38 mitogen-activated protein kinase

P70-S6K - P70 ribosomal protein S6 kinase

RNS - reactive nitrogen species

ROS – reactive oxygen species

TNF-α – tumor necrosis factor alpha

SOD – superoxide dismutase

VCAM-1 - vascular cell adhesion molecule one

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To Dan

for helping me with my wings

CHAPTER ONE

Introduction

The proportion of the world's aging population is increasing at an accelerated rate. Declining fertility rates combined with steady improvements in life expectancy over the latter half of the 20th century have produced dramatic growth in the world's elderly population. As of 2000, the proportion of the population 65 years of age and older in the United States was 12.4%, and this number is projected to increase to 19.6% by 2030 (United Nations, 2002). The process of senescence is associated with increasing inflammation and subsequent oxidative stress in the body, both of which can exert negative health effects at local and systemic levels.

Locally, it is well established that oxidative stress, by way of increased free radical generation in the respiring mitochondria, causes oxidative modification and damage to protein, lipid, and DNA in skeletal muscle (Marzani, Felzani, Bellomo, Vecchiet, & Marzatico, 2005). This invariably leads to cellular dysfunction and muscle protein degradation, as well as a decline in muscle mass and function. Loss of muscle strength has been associated with increased risk of frailty, disability, and mortality (Beck et al., 2007), and is implicated in the pathogenesis of sarcopenia (Howard et al., 2007); a process that can begin as early as the fourth decade of life (Lindle et al., 1997). While the mechanisms responsible for these changes remain to be clearly defined, dysregulated oxidative stress and inflammatory processes are known to be significantly involved. Studies have shown that elevated plasma levels of certain inflammatory cytokines, most notably interleukin-6 (IL-6), are also increasingly present with

advancing age, and to a greater extent with sarcopenia (Leng, Chaves, Koenig, & Walston, 2002). Further, the appearance of IL-6 and other inflammatory markers, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), are normally associated with a number of adverse clinical outcomes including decreased strength and mobility, falls, and mortality (Ershler & Keller, 2000).

Systemically, inflammation and oxidative stress are thought to play roles in the progression of cardiovascular disease (CVD). Systemic inflammation in the body is in part reflected by increased C-reactive protein (CRP) concentrations and increased systemic levels of some cytokines. Elevated levels of these cytokines, specifically TNF-α and IL-6, have been shown to predict all-cause mortality as well as cardiovascular mortality (Volpato et al., 2001). Accumulating evidence also indicates that oxidative stress plays a major role in the initiation and progression of cardiovascular dysfunction (Droge, 2002). Generation of reactive oxygen species (ROS) has been shown to cause endothelial cell (EC) apoptosis, increased monocyte adhesion, and play a role in angiogenesis (Taniyama & Griendling, 2003), all of which are implicated in the development of CVD.

In an attempt to attenuate the age-related progression of oxidative stress and inflammation, nutritional countermeasures such as antioxidant and protein supplementation, are being studied. Of such, soyfoods, because of their high protein content and unique concentration of isoflavones (thought to possess antioxidant and estrogenic activity), have become a popular choice as a functional food. In skeletal muscle, numerous studies have compared soy and whey protein isolates on skeletal muscle anabolism (Anthony et al., 2007; Candow, Burke, Smith-Palmer, & Burke, 2006;

Phillips, Hartman, & Wilkinson, 2005) and catabolism (Box, Hill, & DiSilvestro, 2005; Elia, Stadler, Horvath, & Jakus, 2006; Hill, Box, & DiSilvestro, 2004), as both provide a high biological value protein source to the body. In young men, consumption of fluid milk (containing both casein and whey) after resistance training appears to promote greater lean mass accretion than soy (Hartman et al., 2007); however, in an older population, data suggests that increases in muscle strength and size are not influenced by the predominant source of protein consumed, rather adequate total calorie and protein intake (Haub, Wells, Tarnopolsky, & Campbell, 2002). Further, in rats, the consumption of diets containing 20% by weight isolated soy protein suppressed exercised-induced activation of proteases which can degrade myofribrillar proteins known as calpains (Nikawa et al., 2002).

Moreover, in addition to the protein itself, soyfoods contain isoflavones, polyphenolic compounds that possess estrogenic and antioxidant properties (Ross & Kasum, 2002). These added components are speculated to confer additional benefit of soy above and beyond whey. Oxidative DNA damage in men and women has been shown to be decreased after as little as three weeks of isoflavone (Djuric, Chen, Doerge, Heilbrun, & Kucuk, 2001) and soy protein (Mitchell & Collins, 1999) supplementation. In a recent study by Brown et al. (Brown, DiSilvestro, Babaknia, & Devor, 2004), the ingestion of soy and whey protein bars were found to promote exercise-induced lean body mass gain, but the soy had an added benefit of preserving antioxidant function in young men. Specifically, when compared to whey, the group consuming soy protein

bars had improved markers of plasma radical scavenging capacities and myeloperoxidase values (Brown et al., 2004), both of which signify increases in antioxidant capability in the body.

In addition to the effects on skeletal muscle, from a cardiovascular standpoint, it has been suggested that ingestion of dietary isoflavones results in improved indices and markers of cardiovascular function such as vasodilatation (Lissin, Oka, Lakshmi, & Cooke, 2004), inhibition of calcium-induced contractions (Vostal & Shafer, 1996), and decreased susceptibility of low density lipoprotein (LDL) oxidation (Steinberg, Guthrie, Villablanca, Kumar, & Murray, 2003). Taken together, because of the purported health benefits derived from both soy protein and isoflavones, there is a need for future research to address the potential synergistic effects that may exist between or among isolated soy constituents.

While inflammation, oxidative stress, and associated muscle wasting do occur as function of aging, studying these phenomena in a laboratory setting requires the employment of known methodology to up-regulate such processes in a timely and consistent manner. In clinical settings, exercise can be used to induce local and systemic inflammation and oxidative stress. Studies have shown that as little as 30 minutes of exercise can lead to a significant elevation of biomarkers of inflammation and oxidative stress in the plasma, thereby eliciting an acute phase response (Beck et al., 2007; Bloomer, Goldfarb, Wideman, McKenzie, & Consitt, 2005). Such biomarkers, namely the cytokines IL-1, TNF-α, and IL-6, mediate a variety of host responses to trauma and infection, including the generation of ROS. To date, most of these studies have been performed in men (Chen, Bakhiet, Hart, & Holtzman, 2004; Feasson et al.,

2002; Peake et al., 2005), and further research is needed to elucidate the effects that exercise and soyfood supplementation have on exercise induced inflammation and oxidative stress in older women.

Purpose of the Study

The effects of soyfoods containing protein and isoflavones on attenuating systemic and local oxidative stress and inflammation in healthy older women are not well delineated. Further, comparisons between soy milk and dairy milk supplementation with regard to these outcomes are lacking in the literature; and favorable results could have important public health implications. Therefore, the overall purpose of the proposed study is to investigate the actions of different milk-beverages on plasma and skeletal muscle markers of inflammation and oxidative stress. This study has two specific aims. Firstly, to assess the effects of the daily consumption of soy milk or dairy milk for four weeks in older females on plasma markers of oxidative stress and inflammation, and markers of local oxidative stress and inflammation in skeletal muscle. And secondly, to assess the effects of the daily consumption of soy milk or dairy milk for four weeks in older females on plasma markers of oxidative stress and inflammation and markers of local oxidative stress and inflammation in skeletal muscle after a single bout of eccentric exercise.

General Study Overview

This study will be conducted as a single blind, randomized, controlled trial. After initial cardiopulmonary assessment, participants will be matched based on physical activity level and protein intake and randomized into a group consuming soy milk (Group

A) or a group consuming dairy milk (Group B). The program will last six weeks and include a two week washout period, and three testing sessions at baseline (week two), week four and week six. The independent variables will be the supplement group (soy milk or dairy milk), the four week supplementation period, and the sampling times during the course of this study. Dependent variables will include the information collected on the 24-hour recall and four day food records, international physical activity questionnaire (IPAQ), delayed onset muscle soreness (DOMS) scale, plasma levels of inflammation and oxidative stress-responsive plasma variables [superoxide dismutase (SOD), glutathione peroxidase (GPx), cyclooxygenase-2 (COX-2), TNF-α, IL-1β, IL-6], and skeletal expression of inflammation-responsive genes [TNF-α, IL-1β, IL-6, nuclear factor kappa-B (NF-κB), COX-2].

Hypotheses

This study will test four alternative hypotheses:

> H_1: There will be a statistically significant difference in plasma levels of inflammation and oxidative stress-responsive plasma variables (SOD, GPx, COX-2, TNF-α, IL-1β, IL-6) in both groups after four weeks of nutritional supplementation.
>
> H_2: There will be a statistically significant difference in plasma levels of inflammation and oxidative stress-responsive plasma variables (SOD, GPx, COX-2, TNF-α, IL-1β, IL-6) in both groups after the eccentrically biased downhill run.
>
> H_3: There will be a statistically significant difference in the skeletal expression of inflammation-responsive genes (TNF-α, IL-1β, IL-6, NF-κB, COX-2) in both groups after four weeks of nutritional supplementation.
>
> H_4: There will be a statistically significant difference in the skeletal expression of inflammation-responsive genes (TNF-α, IL-1β, IL-6, NF-κB, COX-2) in both groups after the eccentrically biased downhill run.

Delimitations

This study will be completed using the following guidelines:

1. Thirty apparently healthy, physically active, but not trained (not engaged in an exercise program involving either resistance or endurance training at least thrice weekly for one year), post-menopausal women between the ages of 40-60 will be used as participants in the study.

2. Participants will be recruited from the Waco area by flyers posted throughout campus and through local gyms and health clubs.

3. Participants will be randomly assigned to consume either soy milk (Group A) or dairy milk (Group B).

4. Muscle biopsies from the lateral thigh will be collected before and after eccentric stress testing.

5. Fasting venous blood will be collected before and after eccentric stress testing, with the exception of the four-hour post-exercise blood draw.

6. All testing will be in the Exercise and Biochemical Nutrition Lab and Exercise and Sport Nutrition Lab at Baylor University in the Marrs-McLean Gym according to all policies and procedures within each respective laboratory.

Limitations

1. The number of participants that complete the study will be limited to those volunteers that qualify to be in the study and follow the research guidelines.

2. The sensitivity of the technologies and protocols utilized to identify quantifiable changes in the criterion variables.

3. The daily schedules of each participant and the inherent circadian rhythms that exist for all humans as a result of slightly different testing times, stresses, etc.

Assumptions

1. Participants will fast for 12 hours prior to reporting for testing.

2. Participants will be apparently healthy with no contraindications to any of the prescribed treatments involved with this protocol.

3. Participants will be physically active, but not trained (not engaged in an exercise program involving either resistance or endurance training at least thrice weekly for one year).

4. All participants will consume three cups of their respective "milk" supplement per day for four weeks.

5. All participants will limit consumption of isoflavone containing foods and dairy products the duration of the study.

6. All assay reagents and equipment used in the sample analysis are accurate and reliable in quantification of the criterion variables.

7. All methods are previously established and are accurate and reliable methods for determination of the criterion variables.

8. Randomization of participants into supplement groups will adequately control for known and unknown confounders.

Definition of Terms

1. Antioxidant - Any substance that, when present at low concentrations compared with an oxidizable substrate, can significantly delay or prevent oxidation of that substrate (Willcox, Ash, & Catignani, 2004).

2. Bowman Birk Inhibitor (BBI) – A soybean-derived protease inhibitor that has anti-inflammatory and anti-carcinogenic activities (Malkowicz et al., 2001).

3. Body Mass Index (BMI) - An index used to assess weight relative to height. The calculation for BMI is body mass (kg) divided by the square of the height (m), usually expressed in the unit kg/m^2 (Flegal & Troiano, 2000).

4. Carbonyl group – In organic chemistry, a carbonyl group is a functional group composed of a carbon atom double-bonded to an oxygen atom. An aldehyde is an organic compound containing a terminal carbonyl group (Solomons, 2003).

5. Cardiovascular Disease (CVD) - The leading cause of death in the United States. Heart disease and stroke—the principal components of cardiovascular disease—are the first and third leading causes of death in the United States, accounting for nearly 40% of all deaths (American Heart Association, 2005).

6. Caspase - A family of calcium-dependent cysteine proteases, which play essential roles in apoptosis, necrosis, and inflammation (Logue & Martin, 2008).

7. Cyclooxygenase-2 (COX-2) - An enzyme that is responsible for formation of important biological mediators called prostanoids (including prostaglandins, prostacyclin and thromboxane). It is an inducible enzyme, becoming abundant in

activated macrophages and other cells at sites of inflammation (Funk & FitzGerald, 2007).

8. Cytochrome C - A small heme protein found loosely associated with the inner membrane of the mitochondria. It is a soluble protein, unlike other cytochromes, and is an essential component of the electron transport chain, where it carries one electron (Capaldi, 1990).

9. Cytokine - Soluble extra cellular proteins or glycoproteins that are important intercellular regulators of cells engaged in innate as well as adaptive inflammatory host defenses, cell growth, differentiation, cell death, angiogenesis, and development and repair processes all to provide restoration of homeostasis (Oppenheim, 2001).

10. Delayed Onset Muscle Soreness (DOMS) – This term refers to the skeletal muscle pain (stiff, tender, and aching) that follows novel eccentric exercise. The intensity of soreness increases during the first 24 hours, peaks at 24–48 hours, and subsides within five to seven days post-exercise (Hilbert, Sforzo, & Swensen, 2003).

11. Dual-Energy X-ray Absorptiometry (DXA) - Absorptiometry in which the density or mass of a material (as bone or fat) is measured by comparing the amounts of absorption by the material of x-radiation of two different energies generated by an X-ray tube and which is used especially for determining bone mineral content (Mazess, Barden, Bisek, & Hanson, 1990).

12. E3alpha-II – Thought to be the rate-limiting step in the ubiquitin-proteasome system. E3alpha-II is highly enriched in skeletal muscle, and its expression is regulated by pro-inflammatory cytokines (Kwak et al., 2004).

13. Free Radical - Any species capable of independent existence that contains one or more unpaired electrons (Willcox et al., 2004).

14. Glutathione Peroxidase (GPx) - The general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. Intracellular and tissue levels of GPx activity affect apoptotic signaling pathway, protein kinase phosphorylation, and oxidant-mediated activation of NFκB (Lei, Cheng, & McClung, 2007).

15. Interleukin-1 Beta (IL-1β) - IL-1β is a cytokine produced by macrophages, monocytes and dendritic cells and forms an important role in inflammatory processes. Specifically, IL-1β has been shown to increase the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes, to sites of infection and to re-set the hypothalamus thermoregulatory center (Oppenheim, 2001).

16. Interleukin-6 (IL-6) – A pro-inflammatory cytokine secreted by T cells and macrophages to stimulate an immune response. IL-6 plays a key role in the acute

phase response as defined by a variety of clinical and biological features such as the production of acute phase proteins (Gabay, 2006).

17. Isoflavones – A family of phytoestrogens found chiefly in soybeans that is under investigation for its preventative health benefits as a nutritional supplement (Messina, 1999).

18. Nuclear Factor-kappa B (NF-κB) - A protein complex which is a transcription factor. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized low-density lipoprotein (LDL), and bacterial or viral antigens. NF-κB plays a key role in regulating the immune response to infection (Ji, Gomez-Cabrera, & Vina, 2007).

19. P70 Ribosomal Protein S6 Kinase (P70-S6K) - This 70 kilodalton protein kinase is a key enzyme in the Akt-mTOR signal transduction pathway. It is thought to be activated by mTOR and appears to activate ribosomal protein S6 (Yonezawa, Yoshino, Tokunaga, & Hara, 2004).

20. Proteasome – A large protein complex located in the nucleus and the cytoplasm of eukaryotic cells. The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis (Breusing & Grune, 2008).

21. Sarcopenia - A progressive loss of muscle mass that accompanies aging. On average, muscle strength peaks in the third or fourth decade of life and then begins to decline slowly but progressively, with the rate of decline accelerating in old age (Lindle et al., 1997).

22. Superoxide Dismutase (SOD) – This enzyme catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. As such, it is an important antioxidant defense in nearly all cells exposed to oxygen. Superoxide is one of the main reactive oxygen species in the cell and as such, SOD serves a key antioxidant role (Qin, Reszka, Fukai, & Weintraub, 2008).

23. Tumor Necrosis Factor Alpha (TNF-α) – A pro-inflammatory cytokine implicated in the pathogenesis of autoimmune disease, rheumatoid arthritis, septic shock, and other inflammatory disorders. It is known to exert its catabolic effects on infection and cancer (Moller, 2000).

24. Type I Muscle Fibers – Also known as slow twitch fibers, these fibers are able to generate energy for ATP re-synthesis predominately through the aerobic system of energy transfer. Generally, slow twitch fibers have relatively low myosin ATPase activity, slower calcium handling ability and shortening speed, less well developed glycolytic capacity as compared to fast-twitch fibers, and relatively large and numerous mitochondria (McArdle, Katch, & Katch, 2001).

25. Type II Muscle Fibers – Also known as fast twitch muscle fibers, this fiber type has a high capability for electrochemical transmission of action potentials, have a

high myosin ATPase activity, have rapid calcium release and uptake by an efficient sarcoplasmic reticulum, and have a high rate of cross bridge turnover (McArdle et al., 2001). Fast-twitch fibers rely on the short term glycolytic system for energy transfer. This explains why activation of these fibers predominates in anaerobic-type sprint activities that depend almost entirely on anaerobic energy metabolism (Gollnick, Parsons, Riedy, & Moore, 1983).

26. Ubiquitin (UB) – A small protein that occurs in all eukaryotic cells. Its main known function is to mark other proteins for destruction via proteolysis (Reinstein & Ciechanover, 2006).

27. Vascular Cell Adhesion Molecule 1 (VCAM-1) – This gene is a member of the immunoglobulin family and encodes a cell surface glycoprotein expressed by cytokine-activated endothelium. VCAM-1 promotes the adhesion of leukocytes and monocyte recruitment to atherosclerotic sties. Up-regulation of VCAM-1 in endothelial cells by cytokines occurs in response to TNF-α and IL-6 (Galkina & Ley, 2007).

28. VO_{2max}- Refers to the highest value of oxygen consumption measured during an exhaustive exercise test. Researchers can be sure that a subject has exercised to exhaustion if their VO_2 (as measured with an open circuit sampling system) has reached a plateau. (A plateau is defined as no further increase in VO_2 with increasing power outputs, or when the rise in VO_2 is less than 100 ml/min).

29. VO_{2peak}- If the criterion for VO_{2max} is not met, a VO_{2peak} can be determined by looking at attainment of age-predicted maximal heart rate, a rating of perceived exertion at exhaustion of greater than 19 and/or a post-exercise blood lactate of greater than eight mmol.

CHAPTER TWO

Literature Review

An Aging America

The median age of the world's population is increasing due to a decline in fertility and a 20 year increase in the average human life span during the past 50 years. Worldwide, the average life span is expected to extend another 10 years by 2050, and in the United States alone, the proportion of the population 65 years of age and older is projected to increase (from 12.4% in 2000) to 19.6% by 2030 (United Nations, 2002). In addition to the aging of the world's population, the leading causes of death in the world are also changing, shifting from infectious disease and acute illness of yesteryear, to chronic disease and degenerative illness of today. Chronic diseases, which disproportionately affect older adults, contribute to disability, diminished quality of life, and increased healthcare costs worldwide. In the United States, approximately 80% of all persons 65 years and older have at least one chronic condition, and 50% have at least two (National Center for Chronic Disease Prevention and Health Promotion, 1999). Prevention of chronic disease is a very important aspect of public health research, and timely management of these conditions has major healthcare implications.

Many theories exist as to why aging is accompanied with increasing incidence of chronic disease. In 1956, Harman proposed that reactive oxygen species (ROS) formed during normal oxygen metabolism induce macromolecular damage (Harman, 1956) and that accumulation of such products of oxidative damage as we age accounts for the progressive deleterious changes of in our body. This hypothesis was named the free

radical theory of aging, has been supported by recent studies showing that mitochondria obtained from aged muscle fibers display several functional abnormalities including increased proteolysis, ROS overproduction, and vulnerability to apoptosis (Martin et al., 2007; Marzani et al., 2005). In the body, inflammatory cytokines have been shown to promote oxidative stress via various mechanisms (Mantovani et al., 2004; Moylan & Reid, 2007), and recent scientific discovery has prompted debate as to whether inflammation and subsequent oxidative stress is a primary cause or a secondary phenomenon of chronic disease states, such as sarcopenia and cardiovascular disease (CVD) (Willcox et al., 2004).

Sarcopenia

Sarcopenia is defined as the progressive loss of muscle mass that accompanies aging. The prevalence of clinically significant sarcopenia is estimated to range from 8.8% in women aged 64-74 to 17.5% in men aged 75-84 (Morley, Baumgartner, Roubenoff, Mayer, & Nair, 2001) and in 2000, the estimated direct healthcare costs attributable to sarcopenia in the United States was $18.5 billion. According to a recent report by Janssen, a 10% reduction in the prevalence of sarcopenia could result in a savings of $1.1 billion per year in United States healthcare costs (Janssen, Shepard, Katzmarzyk, & Roubenoff, 2004).

The aging process itself accounts for 30-40% of the age-related declines in muscular strength (Rantanen, Era, & Heikkinen, 1997). On average, muscle strength peaks in the third or fourth decade of life and then begins to decline progressively with age, with the rate of decline accelerating into the seventh decade (Lindle et al., 1997). Loss of muscle strength has been associated with increased risk of frailty, disability, and

mortality (Beck et al., 2007), and has been implicated in the pathogenesis of sarcopenia (Howard et al., 2007). While the exact mechanisms responsible for these changes remain to be clearly defined, the literature suggests that dysregulated oxidative stress and inflammatory processes are significantly involved (Marzani et al., 2005).

Cardiovascular Disease

Cardiovascular disease refers to the class of diseases that involve the heart or blood vessels. The principal components of CVD, heart disease and stroke, are the first and third leading causes of death in the United States, accounting for nearly 40% of all-cause mortality (American Heart Association, 2005). These statistics translate into major healthcare costs. In 2005, the cost of heart disease and stroke in the United States exceeded $394 billion: $242 billion for health care expenditures and $152 billion for lost productivity from death and disability (American Heart Association, 2005). Further, in 2001, the cost of hospitalization for cardiovascular problems among Medicare beneficiaries topped $29 billion (Department of Health and Humans Services, 2002).

Nationwide, about one in three adults have some form of CVD, and the risk of developing this condition has been shown to increase with age (Rosamond et al., 2007). Atherosclerosis, the progressive accumulation of lipid and fibrous element in the arterial lumen, is the process by which CVD occurs. In the past, the correlation between lipids and atherosclerosis was primarily used to explain the pathophysiology of the disease; however, our understanding of this process is changing. Recently, there has been much focus on the role of inflammation and subsequent oxidative stress in the development of atherosclerosis (Libby, 2002).

Inflammation and Oxidative Stress: An Introduction

Oxidative stress refers to the situation in which there is a significant imbalance between free radicals and the antioxidant defense system, with resulting harm termed oxidative damage. The presence of unpaired electrons makes free radicals highly reactive because they require another electron to fill the orbital and become stable. Normally, the cytoplasm of mammalian cells is in a reduced state, with the redox ratios of NADPH/NADP+, reduced glutathione/oxidized glutathione (GSH/GSSG), and ascorbic acid/dehydroascorbic acid (AA/DHA) more than 100:1 (Lockwood, 2000). The cell redox network is involved in protein function and enzyme activities, and a disturbance to this balance can be destructive (Lockwood, 2000). Unbridled, production of free oxygen radicals are believed to result in damage to the proteins, lipids, and DNA in the respiring mitochondria leading to mutations in mitochondrial DNA that have been associated with a wide range of human disease (Willcox et al., 2004).

The most common sources of oxidants in the cell are derived from reactions that generate superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO). Once produced, these species undergo conversion to secondary highly reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as hydroxyl radical (OH•) and peroxynitrite (ONOO-). At controlled levels, ROS and RNS serve as important regulators of signal transduction and protein function. However, if left unchecked, elevated levels of ROS or RNS can damage critical cellular components such as membrane lipids, structural and regulatory proteins, and DNA (Moylan et al., 2007). Such reactions can be induced by inflammatory cytokines such as IL-1, IL-6, TNF-α,

and interferon gamma (IFN-γ) (Matthys & Billiau, 1997). Cytokines are thought to promote oxidative stress via various mechanisms, including induction of sickness which subsequently promotes malnutrition and oxidant imbalance (Mantovani et al., 2004), as well as activation of peripheral leukocytes which are known to produce excess oxidants (Moylan et al., 2007). The most common modification to proteins that oxygen free-radicals and other ROS provoke are accumulation of carbonyl groups which can arise following oxidative attack on specific amino acid residues (Hipkiss, 2006). Such structural modification can greatly compromise protein function, as well as introduce a potential for intra- and intermolecular cross-linking which may induce further deleterious effects.

Plasma and Skeletal Markers of Inflammation and Oxidative Stress

Cyclooxygenase-2 (COX-2)

An enzyme that is responsible for formation of important biological mediators called prostanoids (including prostaglandins, prostacyclin and thromboxane). It is an inducible enzyme, becoming abundant in activated macrophages and other cells at sites of inflammation (Funk et al., 2007).

Glutathione Peroxidase (GPx)

GPx is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. Intracellular and tissue levels of GPx activity affect apoptotic signaling pathway, protein kinase phosphorylation, and oxidant-mediated activation of NFκB (Lei et al., 2007).

Interleukin Six (IL-6)

IL-6 is a pro-inflammatory cytokine secreted by T-cells and macrophages to stimulate immune response. IL-6 plays a key role in the acute phase response as defined by a variety of clinical and biological features such as the production of acute phase proteins (Gabay, 2006).

Interluekin One-Beta (IL-1 β)

IL-1β is produced by macrophages, monocytes and dendritic cells and is an important part of the inflammatory response of the body against infection. This cytokine increases the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes to sites of infection. The increased body IL-1β production in peripheral tissue has also been associated with increased sensitivity to pain associated with fever (Oppenheim, 2001).

Nuclear Factor Kappa-B (NF-κB)

NF-κB is a protein complex which also acts as a transcription factor. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. NF-κB plays a key role in regulating the immune response to infection. Consistent with this role, incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection and improper immune development (Ji et al., 2007).

Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is the major extracellular scavenger of O_2^- and a main regulator of NO bioactivity in the blood vessel wall, heart, lungs, and kidney. Involvement of O_2^- has been implicated in many pathological processes, and removal of extracellular O_2^- by SOD gene transfer has emerged as a promising experimental technique to treat vascular disorders associated with increased oxidant stress (Qin et al., 2008).

Tumor Necrosis Factor-Alpha (TNF-Ŭ)

This cytokine serves a variety of functions, and is known to possess both growth stimulating and growth inhibitory properties. As an acute phase reactant, TNF-α can initiate cytokine cascades and it has been implicated in the pathogenesis of autoimmune disease, rheumatoid arthritis, septic shock, and other inflammatory disorders. The cytokine is produced by several types of cells, but especially by macrophages (Moller, 2000).

Inflammation and Oxidative Stress: Local Effects

Locally, unbridled oxidative stress in the respiring mitochondria of muscle tissue can result in a condition known as sarcopenia; defined as the degenerative loss of skeletal muscle mass and strength in senescence. Under normal conditions there is a balance between degradation and re-synthesis of skeletal muscle proteins (Mitch & Goldberg, 1996); however, with oxidative stress this balance is disrupted and favors increases protein degradation and decreases in protein synthesis. Such processes are thought to result from abnormal mitochondrial functioning, as the mitochondria are the main site of substrate oxidation, ROS production, and the initiation of programmed cell death

(Hipkiss, 2006). Further, glycolytic fibers (such as type II muscle fibers) appear to be the most affected by this process (Thompson, 1994), causing atrophy (Reynolds et al., 2002) via increased proteolytic activity (Yarasheski, 2003). It is widely accepted that the ubiquitin-proteasome pathway is the primary mechanism by which proteins are degraded during muscle atrophy. During this process proteins targeted for degradation are identified by ubiquitin and subsequently proteolyzed by the 26S proteasome (Robinson & Ardley, 2004). In physiological states, three major classes of molecules have been shown to up-regulate this system, namely cytokines, hormones, and ROS (Attaix et al., 2005; Russell, Wyke, & Tisdale, 2006).

To illustrate the ability of ROS to up-regulate this process, a recent study by Li et al. showed that exposure of skeletal muscle myotubes to hydrogen peroxide (a proxy for oxidative stress) increases the expression of important components of the proteasome proteolytic system leading to increased protein breakdown, decreased myosin expression, and increased expression of components of the ubiquitin-proteasome proteolytic pathway (Li, Chen, Li, & Reid, 2003). In addition to this role, it has been hypothesized that ROS may act as a second messenger in TNF-α induced muscle catabolism, such as the mitogen activated protein kinase (MAPK) pathway (Moylan et al., 2007). It is well established that MAPKs play a key role in cell signaling because control of numerous cellular signaling pathways is achieved via activation or deactivation of regulatory proteins through phosphorylation (Cuschieri & Maier, 2005). All eukaryotic cells possess multiple MAPK pathways, of which the best characterized are c-Jun amino-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAPK. All three pathways are structurally similar, but functionally distinct

(Johnson & Lapadat, 2002). The ERKs function in the control of cell division, the JNKs are critical regulators of transcription, and the p38 MAPKs are activated by inflammatory cytokines and play a role in the immune response (Johnson et al., 2002). ERK, JNK, and p38 have all been shown to be activated by oxidative stress and could potentially participate in pathways influencing muscle protein breakdown or myo-nuclear apoptosis (Powers, Kavazis, & McClung, 2007). This redox regulation of MAPK provides another potential link between oxidative stress and skeletal muscle atrophy.

The mitochondrial pathway of apoptosis can also be initiated via various signals, including ROS and high levels of cellular calcium. These factors induce the opening of the mitochondrial permeability transition pore and promote the release of cytochrome c from the mitochondria into the cytosol. In the cytosol, cytochrome c triggers a signaling pathway leading to the activation of caspase-9 and subsequently promotes caspase-3-mediated nuclear DNA fragmentation (Powers et al., 2007). Although the mechanism responsible for inactivity-mediated calcium overload is unknown, it is possible that the intracellular production of ROS could play a key role in disturbances in calcium homeostasis (Kandarian & Stevenson, 2002). A potential biochemical mechanism to link oxidative stress with calcium overload is that ROS-mediated formation of reactive aldehydes can inhibit plasma membrane calcium adenostine triphosphate (ATP) enzyme activity (Siems, Capuozzo, Lucano, Salerno, & Crifo, 2003). Hence, an oxidative stress induces a decrease in membrane calcium ATP enzyme activity which would impede calcium removal from the cell and promote intracellular calcium accumulation, thereby resulting in increased cellular damage (Powers et al., 2007).

Another final link between oxidative stress and muscle atrophy involves the redox regulation of the NF-κB family of transcriptional activators; all of which are expressed in skeletal muscle, and have been implicated a variety of processes, including muscle atrophy. In a pioneering study by Li and colleagues, the effects of exogenous hydrogen peroxide on NF-κB activation were studied. Authors tested the effects of TNF-α on a mouse-derived muscle cell line and on primary cultures from rat skeletal muscle. Administration of TNF-α was shown to activate binding of NF-κB to its targeted DNA sequence and stimulated total ubiquitin conjugation. Results demonstrate that ROS can induce the NF-κB signaling pathway, subsequently leading to increased myofibrillar proteolysis due to up-regulation of the ubiquitin-proteasome system (Li, Schwartz, Waddell, Holloway, & Reid, 1998).

In concert with increased degradation, ROS-regulated catabolic signaling is also thought to reduce protein synthesis (Moylan et al., 2007). Support for this hypothesis comes from several studies. In 2002, Patel and colleagues examined the effects of hydrogen peroxide on protein synthesis in hamster ovary cells. In this model, oxidative stress (i.e. hydrogen peroxide administration) was shown to decrease the activity of translational regulators including ribosomal S6 kinase and eukaryotic initiation factor eIF-4E (Patel et al., 2002). In so doing, authors demonstrated the ability of ROS to reduce translational activity and subsequent protein synthesis (Patel et al., 2002). Additionally, generation of ROS has also been shown to coincide with TNF-α induced decreases in reduced glutathione during myogenic differentiation of muscle cells. Such activation has inhibitory effects on myogenesis (Langen et al., 2002). Finally, in animal models of hind-limb unloading and denervation, increases in oxidative stress have been

shown to correlate with a significant decrease in both phosphorylated p70S6K levels and protein synthesis (Goldspink, 1976; Thomason et al., 1989).

In conjunction with oxidative stress, considerable evidence now available points to the importance of increased cytokine levels, especially IL-1β, TNF-α, and IL-6, playing a role in the pathogenesis of sarcopenia (Morley et al., 2001). A recent study conducted by Kwak and colleagues (Kwak et al., 2004) illustrates the ability of pro-inflammatory cytokines to up-regulate the primary pathway involved in muscle protein breakdown, the ubiquitin-proteasome pathway. In this study, authors examined the regulation of protein catabolism during cancer cachexia, specifically focusing on the rate limiting in the ubiquitin-proteasome system: a ligase enzyme known as E3alpha-II. In two different animal models of cancer cachexia, E3alpha-II was significantly induced at the onset and during the progression of muscle wasting, and its expression was shown to be enhanced by the presence of pro-inflammatory cytokines such as IL-6 and TNF-α (Kwak et al., 2004). Additional animal models have demonstrated that several of the cytokines are capable of causing muscle amino acid export in vivo (Fong et al., 1989; Hellerstein, Meydani, Meydani, Wu, & Dinarello, 1989). With specific regard to sarcopenia, human case-control data suggests that elevated plasma levels of certain cytokines, most notably IL-6, are increasingly present with advancing age, and to a greater extent with sarcopenia (Leng et al., 2002). Further, the appearance of IL-6 and other inflammatory markers, such as IL-1 and TNF-α, has been associated with a number of adverse clinical outcomes including decreased strength and mobility, and increased falls and mortality (Ershler et al., 2000).

The underlying mechanism explaining why increases in inflammatory cytokines may predispose someone to sarcopenia can be better understood when looking at the role of such cytokines in acute illness. In a typical acute immune response, antigen-presenting cells encountering a foreign peptide secrete IL-1β and TNF-α, which assist in the recruitment of T cells and the development of a specific immune response to the antigen. IL-1β and TNF-α have considerable effects on metabolism in acute illness, including increased or decreased secretion of insulin and counter-insulin hormones, increased gluconeogenesis, increased protein breakdown, and increased hepatic glucose production. Additionally, IL-1β and TNF-α up-regulate the production of IL-6 (Belardelli, 1995). Although these changes are largest in acute illness, in chronic inflammatory diseases, these changes can persist for months to years and are associated with loss of muscle, elevated metabolic rates, and accelerated muscle protein breakdown (Roubenoff et al., 1994). Because sarcopenia develops over many decades, only a small, sustained change in the balance of muscle protein catabolism and anabolism would be needed to exhibit a large change in body composition

Inflammation and Oxidative Stress: Systemic Effects

Systemically, up-regulation of markers of inflammation and subsequent oxidative stress can have implications on cardiovascular health. In the past, the correlation between lipids and atherosclerosis was primarily used to explain the pathophysiology of the disease; however, our understanding of this process is changing. Recently, there has been much focus on the role of inflammation and subsequent oxidative stress in the development of atherosclerosis (Libby, 2002). Systemic inflammation in the body is in part reflected by increased CRP concentrations and

increased systemic levels of some cytokines. Elevated levels of these cytokines, specifically TNF-α and IL-6, have been shown to predict all-cause mortality as well as cardiovascular mortality (Volpato et al., 2001). Moreover, it has recently been suggested that markers of inflammation, such as elevated CRP levels, are a stronger predictor of cardiovascular events than the conventional risk factor, LDL (Ridker, Rifai, Rose, Buring, & Cook, 2002).

Systemic inflammation has been shown to participate in entire spectrum of atherosclerotic development. In the primary stages of the disease, leukocyte particles adhere to vascular endothelial cells (ECs), which form the innermost surface of the artery wall. Normally, the endothelium is resistant to adhesive properties; however upon exposure to known triggers of atherosclerosis (such as a high fat diet) initiation of adhesion molecules by ECs allow for the attachment of leukocyte particles to the arterial wall (Libby, 2002). One adhesion molecule involved in this process is vascular cell adhesion molecule-1 (VCAM-1), as it binds the types of leukocytes found in early atherosclerotic plaques. In an original animal model study done by Cybulsky and colleagues, researchers demonstrated that rabbits which were fed a high fat/atherogenic diet were shown to express VCAM-1 by ECs in areas prone to lesion formation and ECs overlying early lesions (Cybulsky & Gimbrone, 1991). Further work in this area suggests that oxidized lipids are the inciting factor of VCAM-1 expression, via inflammatory pathways mediated by NFκB and other pro-inflammatory cytokines (Sever et al., 2004).

Once adhered to the arterial endothelium, monocytes disperse through the endothelial lining and enter the intima of the vessel wall. This process requires a chemoattractant gradient, such as monocyte chemoattractant protein-1 (MCP-1), a

chemoattractant cytokine that is over-expressed in human arethoma. The increasing presence of MCP-1 can then recruit monocytes, which in turn mature into macrophages within the innermost layer of an artery or vein. Macrophages engulf modified lipoproteins, cholesterol esters accumulate in the cytoplasm, and the macrophages subsequently become foam cells (Libby, 2002). Accumulation of foam cells is what ultimately leads to fatty streak development.

Mounting evidence also indicates that oxidative stress plays a major role in the initiation and progression of cardiovascular dysfunction. One of the most important ROS in the vasculature is O_2^-, as it can both exert negative effects on vascular function, and generate other reactive species (Droge, 2002). For example, reaction of O_2^- with NO generates peroxynitrite, a potentially deleterious ROS. Many functions of the endothelium are affected by ROS. The most well known is endothelium-dependent vasorelaxation, which is impaired by a loss of NO· bioactivity in the vessel wall (Mugge et al., 1991). ROS can also cause EC apoptosis, increased monocyte adhesion, and play a role in angiogenesis (Taniyama et al., 2003).

Inflammation and Oxidative Stress: Nutritional Countermeasures

In an attempt to attenuate the age-related progression of oxidative stress, inflammation, and subsequent increases in muscle proteolysis and cardiovascular risk, nutritional countermeasures such as antioxidant and protein supplementation are being studied. Of such, soy proteins have been shown to play a role in the prevention of skeletal muscle wasting, frequently seen as a result of aging (Haub et al., 2002). Further, soyfoods have been shown to confer skeletal muscle and cardioprotective benefit due to their uniquely high isoflavone content (Cassidy & Hooper, 2006).

Soy Structure, Function, and Known Health Benefits

Soybeans are a versatile plant food that provides high-quality protein, minimal saturated fat, and a rich (and essentially unique) dietary source of isoflavones. Isoflavones, or phytoestrogrens, have a very limited distribution in nature, and soybeans and soyfoods contain approximately one to three mg of isoflavones per gram. The two primary isoflavones found in soyfoods are called genistein and daidzein; and a minor isoflavone exists called glycitein (Messina, 1999). The molecular structures of genistein, daidzein, and glycitein are shown below in Figure 1.

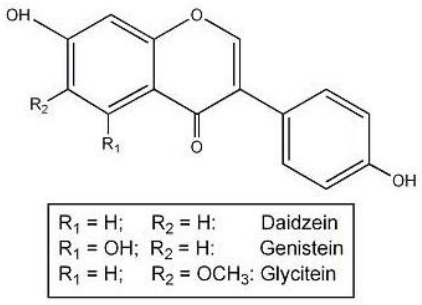

Figure 1. Molecular structure of prominent isoflavones in soy products.

Isoflavones found in soyfoods are thought to have a range of biological mechanisms, including hormonal and antioxidant properties that confer a health benefit by influencing cell signaling, gene expression, and cell division and growth (Cassidy et al., 2006). In several studies, these biological mechanisms have translated into improved indices and markers of cardiovascular function such as vasodilatation (Lissin et al., 2004), inhibition of calcium-induced contractions (Vostal et al., 1996), and decreased susceptibility of LDL oxidation (Steinberg et al., 2003).

The evidence that natural isoflavones found in soy protect against several chronic diseases is both observational and experimental (Blum, Lang, Peleg, Vigder, Israeli, Gumanovsky, Lupovitz, Elgazi, & Ben-Ami, 2003). Mechanistically, dietary isoflavones are thought to be cardio-protective because of their structural similarity to estrogen. As such, they have a high binding affinity for the primary estrogen receptor in the vascular wall, thereby enhancing endothelial function. In addition to vascular reactivity, estrogen and estrogen-like compounds are also known to have favorable effects on blood lipids and lipoprotein concentrations (Rosano, Vitale, Marazzi, & Volterrani, 2007). Several studies have suggested that intake of soy protein can reduce circulating blood lipid levels; and various mechanisms have been proposed to explain the hypolipidemic effects of soy proteins, including increased thyroxine levels, elevated hepatic LDL receptors, suppressed expression of hepatic sterol regulatory element binding protein, and modulation of hepatic thyroid hormone receptor (Xiao, Wood, & Gilani, 2006).

Although the exact mechanism by which soy lowers blood lipids remains unclear, in 1999, the FDA approved a food label claim that 25 grams of soy protein per day may help prevent coronary heart disease based on a reduction in blood lipids and lipoproteins (Sacks et al., 2006). This claim was substantiated in numerous clinical trials, including the seminal 1995 meta-analysis by Anderson et al. which showed that an average intake of 47 grams per day soy protein resulted in a 9% decrease of total cholesterol, a 13% decrease of LDL cholesterol, a 10% decrease of triglyceride, and a moderate 2.4% increase of high density lipoprotein (HDL) (Anderson, Johnstone, & Cook-Newell, 1995). Although initial estimates of the hypocholesterolemic effects of soy protein are

now known to be too high, recent meta-analyses indicate soy protein directly lowers blood low-density-lipoprotein cholesterol (LDL-C) levels by 3-5% (Sacks et al., 2006; Zhan & Ho, 2005). Over a period of many years, even this modest reduction can, in theory, reduce coronary heart disease (CHD) risk by as much as 10% (Law, Wald, Wu, Hackshaw, & Bailey, 1994). Moreover, a more recent study suggests that as little as 20 grams of soy protein per day can lower non-HDL cholesterol and have favorable cardiovascular effects (Teixeira et al., 2000).

In addition to these findings, clinical trials have shown that phytoestrogen containing soy supplementation is associated with a reduction in blood pressure. As of 2004, of a total of twelve clinical studies that examined the effects of soyfoods or soy protein on blood pressure in healthy adults, eight reported a statistically significant reduction in blood pressure (Zhou, 2004). Finally, it has recently been suggested that soy protein or its components may protect against other atherosclerotic cardiovascular disease risk factors such as total homocysteine, CRP, and excess body iron (Hanson et al., 2006).

In 2006, the American Heart Association put forth a scientific advisory which stated that soy products should be beneficial to cardiovascular and overall health because of their high content of polyunsaturated fats, fiber, vitamins, minerals, and low content of saturated fat (Sacks et al., 2006). According to this statement, present literature has also been shown to favor soy protein rather than soy isoflavones as the cardioprotective constituent, however authors clearly state that the possibility cannot be ruled out that another component in soybeans could be the active factor.

Soy and Local Inflammation and Oxidative Stress: Sarcopenia Applications

Because of its rich source of dietary protein, numerous studies have examined the ability of soy enriched diets to increase muscle synthesis and/or prevent muscle breakdown. Specifically, many of such studies have compared soy and whey protein isolates on skeletal muscle anabolism (Anthony et al., 2007; Candow et al., 2006; Phillips et al., 2005) and catabolism (Box et al., 2005; Elia et al., 2006; Hill et al., 2004), as both provide a high biological value protein source to the body. In young men, consumption of milk protein after resistance training appears to promote greater lean mass accretion than soy (Hartman et al., 2007), however, in an older population, data suggests that increases in muscle strength and size are not influenced by the predominant source of protein consumed, rather adequate total protein intake (Haub et al., 2002). In the former study, 56 healthy young men participated in a parallel three group longitudinal design in which subjects were randomly assigned to consume dairy milk, soy milk, or a maltodextrin solution (matched in calories and protein content) immediately before and again one hour after exercise. Participants were instructed to train five days per week for 12 weeks on a rotating split-body resistance exercise program. Before and after training, muscle fiber size, maximal strength, and body composition by dual-energy X-ray absorptiometry (DXA) were measured. Results showed that no between-group differences were seen in strength and type II muscle fiber area increased in all groups with training, but with greater increases in the dairy milk group than in both the soy milk and control groups. Type I muscle fiber area increased after training only in the dairy milk and soy milk groups, with the increase in the dairy milk group being greater than that in the control group. Fat and bone-free mass

increased in all groups, but with a greater increase in the dairy milk group than in both the soy milk and control groups (Hartman et al., 2007). In the latter study, Haub et al. examined whether the predominant source of protein consumed by older men influenced measures of muscle size and strength, body composition, resting energy expenditure, and skeletal muscle creatine concentrations in response to twelve weeks of resistive training. In this study, 21 men aged 65 ± 5 years were instructed to consume a lactoovovegetarian diet for two weeks, followed by a random assignment into a beef-containing diet (containing 0.6 g/kg/d protein from beef) or to continue the lactoovovegetarian diet (containing 0.6 g/kg/d protein from soy sources) throughout resistive training. Men in both groups had improvements (14-38%) in maximal dynamic strength of all the muscle groups trained with no significant difference between groups. With resistive training, cross-sectional muscle area of the vastus lateralis increased in both groups (4.2 ± 3.0% and 6.0 ± 2.6% for the lactoovovegetarian and beef containing groups, respectively) with no significant difference between groups. Body composition, resting energy expenditure, and concentrations of muscle creatine, phosphocreatine, and total creatine did not differ significantly between groups or change over time. These data suggest that increases in muscle strength and size were not influenced by the predominant source of protein consumed by older men with adequate total protein intake (Haub et al., 2002). Additionally, a study performed in rats showed that the consumption of diets containing 20% by weight isolated soy protein suppressed exercised-induced activation of proteases which can degrade myofribrillar proteins known as calpains (Nikawa et al., 2002); further supporting the notion that diets rich in soyfoods can help to preserve muscle mass and function.

In addition to the protein itself, soyfoods contain isoflavones, which are polyphenolic compounds that possess antioxidant properties (Ross et al., 2002). The added antioxidant components found in soy are speculated to confer additional health benefits to soy above and beyond those provided by whey. Oxidative DNA damage in men and women has been shown to be decreased after as little as three weeks isoflavone (Djuric et al., 2001) and soy protein (Mitchell et al., 1999) supplementation. In the former study, blood levels of oxidative damage were measured in twelve participants before and after soy isoflavone supplementation. Participants were asked to consume 50 mg of isoflavones twice daily for three weeks. Results showed that the oxidative DNA damage (as measured by 5-OHmdU) were decreased over the three week period (Djuric et al., 2001). In the latter study, 10 subjects were asked to consume one liter of soy milk per day for four weeks. Supplementation with soy resulted in a decrease in oxidative damage to DNA bases when compared to an animal protein and vegetable protein control (Mitchell et al., 1999).

Further, with regard to exercise, a recent study by Brown and colleagues (Brown et al., 2004) looked at the ability of soy containing foods to decrease exercise induced oxidative stress and promote lean mass gain. To do this, authors recruited 27 college-aged males to participate in a nine week strength training program and randomly assigned them to one of three different dietary supplement groups, whey, soy, or control. At the end of the study, subjects were evaluated for lean body mass gain and changes in antioxidant status. The latter was done using one measurement of a component of antioxidant capacity and one measurement for a component of oxidant stress. The measurement of antioxidant capacity was based on an assay called plasma antioxidant

status which assesses the ability to scavenge certain chemically generated radicals. The oxidant stress parameter measured was plasma myeloperoxidase, a measure of neutrophil activation, which is associated with increased secretion of superoxide radical (Kehrer, 1993). At the completion of the study, ingestion of soy and whey protein bars were both found to promote exercise-induced lean body mass gain, but the soy had an added benefit of preserving antioxidant function (Brown et al., 2004).

Other active components in soy, such as the Bowman-Birk inhibitor (BBI), are thought to confer additional health benefit by helping to minimize protein degradation (Hegsted, Godber, Xu, & Losso, 2002). The BBI is a polypeptide found in soy products that has been shown to inhibit the proteolytic effects of both trypsin and chymotrypsin. Unpublished studies from Louisiana State University have shown that BBI concentrations found in one cup of soy milk can prevent the activation of metalloproteinases, thereby preserving the integrity of cell membranes and overall cellular integrity (Hegsted et al., 2002). A recent study by Arbogast et al. (Arbogast et al., 2007) found that mice fed a diet supplemented with 1% BBI while undergoing 12 days of hind-limb unloading (used to induce oxidative stress), displayed less muscle atrophy and increased force production in the unloaded muscle when compared to controls (Arbogast et al., 2007). In addition, results also indicated that BBI may have scavenged superoxide anion radicals, preventing oxidative stress in the muscle (Arbogast et al., 2007). Because many different components in soyfoods have been identified, there is a need for future research to address the potential synergistic effects that may exist between isolated soy constituents.

Soy and Systemic Inflammation and Oxidative Stress: Cardiovascular Applications

Population studies suggest that up to 80% of CVD could be avoided by diet and lifestyle changes. From a dietary perspective, epidemiological and mechanistic data support the concept that phytoestrogen-rich diets have beneficial cardiovascular effects, including the reduction of cellular inflammation and oxidative stress (Cassidy et al., 2006).

CVD is partially characterized by chronic inflammation and increased expression of cell adhesion molecules. Circulating concentrations of these adhesion molecules are considered to be predictive of CVD risk because they indicate a pro-inflammatory state in the vasculature. Substantial evidence indicates that the beneficial effects of estrogen on the cardiovascular system are attributable in part to a beneficial effect on the vascular endothelium (Hwang et al., 1997). The reduction of concentrations of circulating inflammatory markers by estrogen may be one of the mechanisms by which pre-menopausal women are protected against CVD and animal studies have shown that isoflavones can improve vascular reactivity in estrogen deficient primates (Honore, Williams, Anthony, & Clarkson, 1997).

It has been suggested that ingestion of dietary isoflavones result in improved vasodilatation due in large part to the enhancement of nitric oxide (NO) synthesis (Lissin et al., 2004). NO, an important regulator of vascular homeostasis, is synthesized continuously in endothelial cells by the constitutive enzyme NO synthase from the substrate L-arginine. NO plays an important role in vascular inflammation, and thus the ability of dietary isoflavones to affect NO bioavailability may decrease vascular inflammation and slow the progression to atherosclerosis (Blum et al, 2003).

Other suggested pathways that may relate dietary isoflavones to inflammatory markers of cardiovascular disease include the inhibition of calcium-induced contractions by way of genistein supplementation (Vostal et al., 1996). The isoflavone genistein has been found to inhibit pathways involving tyrosine kinase, thereby decreasing vasoconstriction. By competitively inhibiting the ATP binding site on tyrosine kinase, genistein can interrupt signal transduction pathways, such as those associated with receptor-mediated contractions, which could lead to vascular relaxation and subsequent vasodilatation (Zerrouk et al., 1999).

The presence of chronic inflammatory conditions has shown, in aerobic organisms, to produce ROS (Leeuwenburgh & Heinecke, 2001). Attenuation of oxidation by dietary soy has received recent support in the literature. Specifically, the antioxidant properties of phytoestrogens are the result of their phenolic structure, which resembles 17β-estradiol. Genistein, the predominate isoflavone in soy, has been shown to contribute to a decrease in the oxidative susceptibility of LDL (Steinberg et al., 2003). Such observations have led to the hypothesis that it is the oxidization of LDL by free radicals which initiates the process of atherosclerosis (Willcox et al., 2004). In sum, the antioxidant properties of isoflavones found in soy, as well as the ability of soy to decrease vascular inflammation, thereby reducing the generation of free radical species, are thought to confer significant health benefits.

Inflammation, and Oxidative Stress: Eccentric Exercise

In clinical settings, exercise can be used to induce oxidative stress, inflammation, and subsequent muscle proteolysis. Severe physical exercise or new training regimens may cause muscular damage by increasing both muscle proteases and ROS, especially if

eccentric exercise is involved (Elia et al., 2006; Feasson et al., 2002; Williams et al., 1999). Studies have shown that as little as 30 minutes of exercise can lead to a significant elevation of biomarkers of oxidative stress in the plasma, thereby eliciting an acute phase response (Beck et al., 2007; Bloomer et al., 2005). Such biomarkers, (namely the cytokines: IL-1 ¡, TNF-α, and IL-6) mediate a variety of host responses to trauma and infection, including skeletal muscle proteolysis (Cannon et al., 1991).

Eccentric muscle damage has been reported to effect muscle strength by reducing it by 50% or more for 24 hours following exercise and dependent upon the amount of damage, may take five to 10 days to gradually recover (Feasson et al., 2002). Exercise induced damage mainly reflects degradation of myofibrillar proteins, such as myosin and actin (Nikawa et al., 2002). There are three major proteolytic pathways in muscle protein catabolism: lysosomal, ATP, and ubiquitin dependent, and calcium dependent (Nikawa et al., 2002; Tada & Yokogoshi, 2002). Such pathways are known to be up-regulated by biomarkers of inflammation and oxidative stress. Specifically, activation of the immune marker, IL-6 appears to be a key factor in the activation of proteolysis in response to eccentric exercise. Contracting muscle is one of the major sites of IL-6 production, and IL-6 is believed to induce proteolysis by activating the acidic protease cathepsin B+L and other proteasome activities in skeletal muscles cells (Feasson et al., 2002).

One example of eccentric exercise known to up-regulate inflammatory and oxidative responses is downhill running. A recent study by Peake and colleagues examined changes in plasma cytokines and the capacity of neutrophils to generate ROS in response to in *vitro* stimulation after downhill running were measured (Peake et al.,

2005). During this study, 10 well-trained male runners ran downhill on a treadmill at a gradient of -10% for 45 minutes at 60% VO_{2max} and blood was sampled immediately before and after exercise, and at one and 24 hours post-exercise. Results from this study showed that immediately after exercise and at one hour post-exercise, there were significant increases in neutrophil count and plasma IL-6 concentrations. By 24 hours post-exercise, neutrophil counts and plasma IL-6 levels had returned to baseline. This study demonstrates the ability of downhill running to induce plasma markers of inflammation and oxidative stress (Peake et al., 2005).

Despite the apparent ability of downhill running protocols to induce inflammation, oxidative stress, and associated muscle proteolysis, one limitation to using this modality is that test subjects must be aerobically well trained to endure the protocol. This population may not benefit the most from studies looking to prevent oxidative stress, inflammation, and associated muscle proteolysis secondary to aging. Therefore, other less strenuous mediums, such as downhill walking, can also be used to induce inflammation and oxidative stress, and are more likely to cater to testing the population of interest. In 1986 a study was conducted by Newham et al. (Newham, Jones, & Edwards, 1986) to determine if downhill walking could induce muscle damage as measured by creatine kinase (CK). In this study, five subjects were asked to walk down a treadmill declined at 13 degrees for one hour at 1.86 miles per hour. Previously, research has established that when the gradient exceeded 10% (or six degrees), downhill walking consists almost entirely of eccentric contractions (Margaria, 1968). Venous blood samples were taken before exercise and at 24-hour intervals afterwards until the CK levels had returned to pre-exercise values. Results showed that using this protocol

induced CK levels up to 15,000 IU/liter and peak values occurred between four and seven days after exercise (Newham et al., 1986).

Eccentric Exercise and Soy Supplementation

With regard to isoflavone supplementation and exercise, the literature is scant. To date, only one study has examined the ability of isoflavone supplementation to restore altered redox homeostasis during an acute bout exercise design (Chen et al., 2004). During this study, authors tested whether or not isoflavone supplementation would affect the enzymatic antioxidant defense system in young men undergoing 80% peak oxygen consumption (VO_{2pk}) exercise on cycle ergometer for 30 minutes. Fifteen pairs of subjects were randomly assigned to receive either isoflavone extract or placebo. Subjects engaged in two 30-minute sessions of biking exercise before and after four weeks of supplementation. Results from this study show that isoflavone supplementation significantly increased pre-exercise erythrocyte SOD activity and prevented the exercise-induced decrease in activities of GPx. Authors concluded that results suggest that isoflavones can restore the altered redox homeostasis of antioxidant enzymes due to exercise (Chen et al., 2004).

Despite the lack of soy based isoflavone research, there are several reports which examine whether other nutritional constituents that function as antioxidants attenuate inflammation, oxidative stress, and associated muscle damage. Although the literature is far from conclusive, studies have shown that oxidative injury can be prevented by the intake of antioxidants, such as vitamins C and E, careotenoids, or polyphenols, not only during exercise, but also on a daily basis (Kanter, Nolte, & Holloszy, 1993; Phillips, Childs, Dreon, Phinney, & Leeuwenburgh, 2003; Takanami, Iwane, Kawai, &

Shimomitsu, 2000). As cited studies indicate, to date, most of eccentrically biased acute bout studies have been performed in men (Chen et al., 2004; Feasson et al., 2002; Peake et al., 2005) and further research is needed to elucidate the effects that exercise and nutritional supplementation, specifically whole soyfood supplementation, have on inflammation and oxidative stress in women.

Summary and Future Directions

To summarize, local and systemic inflammation and oxidative stress appear to regulate pathways implicated in the progression of sarcopenia and CVD, respectively. From a public health standpoint, the prevalence of both chronic disease states is increasing with the aging of the world population, and effective and affordable strategies to attenuate their progression are needed. In a clinical setting, local and systemic states of inflammation and oxidative stress can be induced by eccentric exercise, specifically bouts of downhill running. Nutritional countermeasures, such as soyfoods, are speculated to confer skeletal and cardio-protective benefit, although further research is needed to elucidate specific effects.

CHAPTER THREE

Methods

Participants

Thirty apparently healthy, physically active, but not trained (i.e. not engaged in an exercise program involving either resistance or endurance training at least thrice weekly for one year), post-menopausal women between the ages of 40-60 were used as participants in this study. An a priori power calculation showed that 15 participants per group were necessary to detect a significant difference between groups in markers of oxidative stress [SOD effect size of 0.8 U/g; (Chen et al., 2004)] and inflammation [IL-6 effect size of 0.24 pg/mL; (Jenkins et al., 2002)] given a type I error rate of 0.05 and a power of 0.80. All participants were cleared for participation by passing a mandatory medical screening. Only participants considered as either low or moderate risk and with no contraindications to exercise as outlined in the American College of Sports Medicine (ACSM) guidelines were eligible. All eligible participants were asked to provide oral and written informed consent based on university approved documents. Exclusion criteria included inability to follow the study protocol, active use of hormone replacement therapy (HRT), and the consumption of any dietary supplements that could affect antioxidant status (excluding multivitamins) three months prior to the start of the study.

Study Site

All supervised testing and training was conducted under the direct supervision of Dr. Darryn Willoughby in the Exercise and Biochemical Nutrition Laboratory (EBNL) in

the Department of Health, Human Performance, and Recreation at Baylor University, in Waco, TX.

Independent and Dependent Variables

Table 1 shows the general research design and time course of assessments administered in this study. The independent variables were the supplement group (soy milk or dairy milk), the four week supplementation period, and the sampling times during the course of this study. Dependent variables included the information collected on the 24-hour recall and four day food records, international physical activity questionnaire (IPAQ), delayed onset muscle soreness (DOMS) scale, inflammation and oxidative stress-responsive plasma variables (SOD, GPx, COX-2, TNF-α, IL-1β, IL-6), and skeletal expression of inflammation-responsive genes (TNF-α, IL-1β, IL-6, NF-κB, COX-2).

General Study Overview

Participants followed the study design outlined in Table 1. After completing baseline assessment and a two-week dietary washout period, in a single-blind manner, participants were provided with their dietary supplement and instructions for following the supplement protocol. Briefly, during the supplementation period, each participant was required to consume three cups of either soy milk or dairy milk per day for four weeks. After the supplementation period, subjects were asked to return to the laboratory on day 29 for testing. Participants were first weighed, handed in their four day food record, completed the IPAQ, and donated blood and muscle sample. Then, participants were asked to perform a 45-minute eccentrically biased downhill run/walk test at 60% of predetermined VO_{2max}. Upon completion of the test, subjects were asked to return to the

EBNL at four, 24, and 48 hour post testing for blood (four, 24, and 48 hour) and muscle donation (four and 24 hour only). Participants were encouraged to continue consuming their dietary supplement until the completion of the 48-hour post testing period.

Table 1

Overview of Research Design

Week 0	Week 2	Week 4	Week 6		
Entry Session	Cardiopulmonary Baseline Assessment	Follow Up Session	Downhill Running Session	4 & 24-Hours Post Exercise Session	48-Hour Post Exercise Session
Familiar-ization Session. Informed Consent Form. Health History Form. Dietary Form Instruction. Medical Clearance Height, Body Weight, and BMI. 24-Hour Dietary Recall.	Baseline Muscle Biopsy. Baseline Blood Collection. Cardiopulmonary Testing (VO_{2max}) Diet Analysis. IPAQ. Reported Side Effects. Randomization Into Supplement Groups Provision of Supplement. Instructions for Supplement Protocol.	Provision of Supplement Reported Side Effects.	Weight. Hemo-dynamic Measures. Baseline Blood Collection. Baseline Muscle Biopsy. IPAQ. Diet Analysis. DOMS Scale. 5 minute warm-up. 45 min downhill run/walk @ 60% VO_{2max} and -10% grade. Post-Exercise Blood Collection.	Hemo-dynamic Measures Blood Collection. Muscle Biopsy. Ratings of Muscle Soreness and Stiffness. Reported Side Effects. DOMS Scale.	Hemo-dynamic Measures. Blood Collection. Ratings of Muscle Soreness and Stiffness. Reported Side Effects. DOMS Scale.

Entry/Familiarization Session

Participants expressing interest in participating in this study were interviewed on the phone to determine whether they appeared to qualify to participate in this study. Participants believed to meet eligibility criteria were then invited to attend an entry/familiarization session. Once reporting to the lab, participants completed a medical history questionnaire and underwent a general physical examination to determine whether they met eligibility criteria. Participants were required to obtain medical clearance from their personal physician prior to participating in baseline assessments. Once medical clearance was obtained, participants were familiarized to the study protocol via a verbal and written explanation outlining the study design. Eligible participants who agreed to participate in the study read and signed university-approved informed consent documents and were asked to complete a 24-hour recall dietary interview and IPAQ.

Baseline Assessment

Prior to beginning the supplementation protocol, each participant had her body weight, height, and body mass index (BMI) determined. Each subject was initially assessed for aerobic fitness [maximal oxygen uptake (VO_{2max})] by performing a cardiopulmonary graded exercise test on a treadmill ergometer (Quinton®, Cardiac Science™, Bothell, WA). First, subject's skin was prepared for placement of 10 ECG electrodes. Electrode sites were cleansed with sterile alcohol gauze using a circular motion. The site was allowed to air dry or was dried with a gauze pad. Electrodes were then be placed on the right subclavicular fossa (RA), left subclavicular fossa (LA), right abdomen (RL), left abdomen (LL), 4th intercostals space at the right sternal border (V1), 4th intercostals space at the left sternal border (V2), equidistant between V2 and V4 (V3),

5th intercostal space at the midclavicular line (V4), 5th intercostal space at the anterior axillary line (V5), and 5th intercostals space at the axillarly line (V6) of the chest. The subject was then attached to a Quinton 710 ECG. Resting blood pressure, heart rate, and a 12-lead ECG were obtained. The exercise specialist then reviewed the 12-lead ECG to ensure that no contraindications for exercise testing were apparent based on the ACSM guidelines. After baseline measurements at rest were completed, the treadmill test started at a velocity of eight km/h and a 0% incline. The running speed was then increased by two km/h every three minutes until exhaustion. Table 2 illustrates the protocol that was used.

Table 2

Bruce Treadmill Test

Stage	Speed (mph)	Grade (%)	Duration (min)
1	1.7	10	3
2	2.5	12	3
3	3.3	14	3
4	4.2	16	3
5	5	18	3
6	5.5	20	3
7	6	22	3

Oxygen uptake (VO_2) was measured every 30 seconds via an open-circuit sampling system (Parvo Medics, Sandy, UT). VO_{2max} was determined if two of the following criteria were met: respiratory exchange ratio (RER) $\geq$ 1.15 and/or RPE $\geq$ 19 on the RPE scale and/or maximum heart rate within $\pm$ 10 beats of age-predicted maximum

(HR_{max}). If such criteria were met, the highest level of VO_2 was defined as $VO_{2\ max.}$ If such criteria were not met, then the highest VO_2 reached was termed VO_{2peak} rather than VO_{2max}. Heart rate was continuously monitored and blood pressure determined with a mercurial sphygmomanometer every three minutes during the exercise session.

Following entry/familiarization session, participants were matched based on protein consumption and physical fitness level and randomized into either a soy milk consuming group (Group A) or a dairy milk consuming group (Group B). All participants were instructed to consume their usual diet, but limit the consumption of any soy-based or dairy-based products for two weeks, and then begin consumption of the supplement.

Dietary Records, Physical Activity, and Quality of Life

In an attempt to determine compliance with the dietary prescriptions, and also to assess the average daily macronutrient consumption of fat, carbohydrate, and protein, each participant was asked to keep four-day dietary records during weeks two and six of the study. Each participant was instructed by a Registered Dietitian how to fill out the record prior to use. The dietary records were analyzed with the Food Processor Dietary Assessment Software program (ESHA Research Inc., Salem, OR). In addition, at the entry/familiarization session, a 24-hour recall was performed by a Registered Dietitian utilizing a multiple pass method to determine "typical" calorie and protein intakes (Rumpler, Kramer, Rhodes, Moshfegh, & Paul, 2008). Participants were also asked to record weekly physical activity on an International Physical Activity Questionnaire (IPAQ) as well as assess health related quality of life (QOL) on the Short Form (36 questions; SF-36) questionnaire at weeks two and six of the study.

Supplementation Protocol

In a single-blind format, participants were matched for physical fitness level and baseline protein intake, and randomly placed into one of two dietary groups. Group A was instructed to consume a commercial vanilla soy milk product and Group B was instructed to consume a commercial reduced-fat dairy milk product. The supplement groups consumed three cups of their respective supplements per day, for a period of four weeks. Both supplements were matched as closely as possible for total caloric and protein content (Table 3).

Table 3

Supplement Nutrient Profile

	Vanilla Soymilk (8.25 fl oz)	Reduced Fat Dairy Milk (8 fl oz)
Calories (kcals)	130.0	120.0
Total Fat (g)	4.0	4.5
Saturated Fat (g)	0.5	3.0
Trans Fat (g)	0.0	0.0
Sodium (mg)	140.0	120.0
Carbohydrates (g)	19.0	12.0
Dietary Fiber (g)	1.0	0.0
Sugars (g)	16.0	12.0
Protein (g)	6.0	8.0
Vitamin A (%)	20	10
Vitamin C (%)	35	0
Calcium (%)	35	30
Iron (%)	6	0
Vitamin D (%)	30	25

Note. Data was obtained from the nutrition facts panel on listed on the products.

Prior to supplementation, all groups underwent a two-week washout period, in which subjects were instructed to consume their usual diet, but limit the consumption of any soy-based or dairy-based products. During the study, each participant was instructed not to consume any additional soy (or isoflavone) containing foods, limit dairy intake to

two servings per day, and avoid dairy milk (unless assigned to Group B). Participants also recorded daily supplement consumption for compliance on a compliance log provided at the baseline testing session.

Eccentrically Biased Downhill Run

Following the four week supplementation period, each participant reported back to the lab for the downhill run/walk test. Participants were instructed to refrain from aerobic exercise for 48 hours prior to the exercise session. A five minute warm-up (-10% grade and 2.0 mph speed) was performed on the treadmill prior to the 45 minute downhill running/walking exercise. The treadmill was then accelerated and participants were instructed to run/walk at 60% of their VO_{2max} (determined from the baseline cardiopulmonary test) for 45 minutes. During the exercise protocol, 60% of maximal oxygen uptake was maintained by measuring oxygen uptake every three minutes and adjusting the treadmill speed accordingly. Between 15 and 18 minutes and 27 and 30 minutes of exercise, heart rate and blood pressure was recorded. After the exercise session, subjects donated blood, received a small breakfast, and remained rested until the four-hour blood sample and muscle biopsy was taken.

Blood Collection Procedure

Venous blood samples were obtained from the antecubital vein into a 10 ml collection tube using a standard vacutainer apparatus. Blood samples were allowed to stand at room temperature for 10 minutes and then centrifuged. A total of six blood samples were obtained. For each sample, the plasma was removed and frozen at -80°C for later analysis. Blood samples were collected before and after the four week

supplementation period, immediately post-exercise and at four, 24, and 48 hours after the exercise session. Except for the four-hour post-exercise sample, all blood samples were obtained after a 12-hour fast and standardized to the same time of day for each sample.

Muscle Biopsy Procedure

Four percutaneous muscle biopsies (10-15 mg) were obtained from the middle portion of the vastus lateralis muscle of the dominant leg at the midpoint between the patella and the greater trochanter of the femur at a depth between one and two cm under local anesthesia (2% Xylocaine with epinephrine) using the fine needle aspiration technique. Attempts were made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy puncture, depth markings on the needle, and a successive puncture that was made approximately 0.5 cm to the former from medial to lateral. After removal, adipose tissue was trimmed from the muscle specimens and immediately frozen in liquid nitrogen and then stored at -80°C for later analysis. Muscle samples were collected before and after the four week supplementation period, and at four and 24 hours post-exercise. With the exception of the four-hour post-exercise biopsy, the remaining muscle samples were obtained after a 12-hour fast and standardized to the same time of day for each sample.

Assessment of Plasma Markers of Muscle Damage and Inflammation

The plasma levels of oxidative stress (SOD, GPx, COX-2) were assessed using enzyme-linked immunoabsorbent assays (ELISA) with a microplate reader (Wallac Victor-1420, Perkin-Elmer Life Sciences, Boston, MA). The plasma inflammatory markers (TNF-α, IL-1β, IL-6) were assessed using the Bio-Plex bead-based multiplex

assays system (Luminex xMAP technology) by Bio-Rad Laboratories, Inc. (Hercules, CA; #7000005KYMR).

For SOD, the principle of the enzyme immunoassay was based on multiple steps. After the preparation of the SOD standards, 10 μL of the standards were loaded onto the plate in duplicate. Ten μL of the plasma samples were then loaded in duplicate onto the plate followed by 200 μL of diluted radical detector. The reaction was then initiated by adding 20 μL of diluted xanthine oxidase to all wells being used. The plate was then covered and incubated on a plate shaker for 20 minutes, after which the absorbance was read at 450 nm on a plate reader (Wallac Victor-1420, Perkin-Elmer Life Sciences, Boston, MA) and used to calculate SOD activity. According the kit insert, when a series of 60 standard measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 3.7%.

For COX-2, the principle of the enzyme immunoassay was based on multiple steps. After reconstitution of COX-2 standards provided with the kit, 100 μL of standards and samples were added to all wells in duplicate. The plate was then incubated on a plate shaker for 90 minutes, after which all contents of the wells were aspirated and discarded. The plate was then washed four times. One hundred μL of the COX-2 detector antibody was added to each well and then the plate was set aside for another 90 minute incubation. The plate contents were aspirated and the plate was washed four times and then 100 μL of horseradish peroxidase (HRP) conjugated secondary antibody was added to each well. The plate was then set aside to incubate at room temperature for 60 minutes. The plate contents were aspirated and the plate was washed four times and then 100 μL of tetramethylbenzidine (TMB) substrate was added to each well followed

by a 30 minute incubation period. Lastly 100 μL of ELISA stop solution was added to each well and the absorbance was read at 450 nm using a plate reader (Wallac Victor-1420, Perkin-Elmer Life Sciences, Boston, MA). COX-2 concentration (ng/mL) was then calculated based on read absorbencies. According to a customer service representative from Cal-biochem, using the most frequent batch of this kit as an example and running samples in quadruplicate using the standards in the kit, the intra-assay standard deviation range was found to be 0.009-0.147 ng/mL and the percent coefficient of variation range was found to be 3.6-8.7%.

For GPx, the principle of the enzyme immunoassay was based on multiple steps. First, 120 μL of assay buffer was added to the background wells, and 100 μL of assay buffer was added to all sample wells. Twenty μL of sample was added in duplicate to the sample wells, and 50 μL of co-substrate mixture was added to all wells being used. The reaction was initiated by adding 20 μL of cumene hydroperoxidase to all of the wells being used and absorbencies were read using a micoplate reader (Wallac Victor-1420, Perkin-Elmer Life Sciences, Boston, MA) every minute at 340 nm to obtain five time points. The change in absorbance at 340 nm per minute was then calculated for each sample and used to determine the GPx activity. According the kit insert, when a series of 77 GPx measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 7.2%.

Plasma levels of IL-1β, IL-6, and TNF-α were simultaneously analyzed using Bio-Plex (Bio-Rad Laboratories, Hercules, CA) cytokine assays, which are multiplexable bead assays. The principle of this assay was based on multiple steps. First, the filter plate was blocked with 100 μl of assay buffer and then the assay buffer was removed by

vacuum filtration and washed two times. Fifty µL of vortexed mixed beads was added to each well, and 50 µL of standards and samples were all placed in their corresponding wells. The plate was then covered and sealed for 30 minutes on the plate shaker at room temperature and then washed three times with 100 µl per well of wash buffer, removing the wash buffer by vacuum filtration between each wash. Following the wash step, 25 µl of detection antibody cocktail was added to each well and the plate was covered and sealed for 30 minutes on the plate shaker at room temperature. A streptavidin-PE solution was then added at 50µl per well. The plate was again sealed and shaken on the plate for 10 minutes at room temperature. The fluid was then gently removed from the plate via vacuum filtration. The plate was then washed three times with 100 µl of wash buffer and vacuumed between washes. After washing, 125 µl of assay buffer was added to each well. The plate was then covered with aluminum foil and beads were re-suspended on a plate shaker for 30 seconds. If the plate was not read immediately, it could be stored for up to 24 hours at 4°C. Otherwise, the plate was shaken to ensure that bead settling did not occur and the Luminex 100 instrument was used per manufacturer's protocol for data acquisition. According the kit insert the intra-assay coefficient of variation for this assay was <20% and the inter-assay coefficient of variation was <30% (Millipore Bio-Rad Laboratories, Hercules, CA).

Assessment of Inflammatory and Proteolytic Gene Expression

The skeletal muscle expression of inflammation-responsive genes (TNF-α, IL-1β, IL-6, NF-κB, COX-2) was performed using real-time polymerase chain reaction (RT-PCR). To begin, the tissue was homogenized with Tri Reagent (Sigma, St. Louis, MO) and total RNA was isolated by isopropanol/ethanol extraction. Total RNA was then used

to generate a cDNA library using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), which was used as a template in the RT-PCR reaction. Oligonucleotide primers were designed according to known human mRNA sequences available on the National Center for Biotechnology Information (NCBI) genome database (Table 4). Samples were then run on an iCycler iQ RT-PCR system (Bio-Rad, Hercules, CA). The amplification profile was run for 40 cycles employing a denaturation step at 95°C for 30 seconds, primer annealing at 58°C for 30 seconds, and extension at 72°C for 30 seconds. Fluorescence was measured after each cycle resulting from the incorporation of SYBR green dye into each amplicon. The quantity of mRNA was determined relative to the expression of β-actin, and ΔC_T values were used to compare gene expression before and after the four week supplementation protocol and after the eccentric exercise bout. The specificity of the RT-PCR was demonstrated with an absolute negative control reaction containing no cDNA template, and single gene products confirmed using DNA melt curve analysis.

Upon completion of the RT-PCR run and using the principles of SDS-PAGE and LabChip (Caliper Life Sciences, Hopkinton, MA) technology, the myosin heavy chain (MHC) protein isoform composition within 20 μg muscle homogenates was determined under denaturing conditions by using an Experion Pro260 automated electrophoresis system (Bio-Rad, Hercules, CA). The Experion Pro260 analysis kit has a resolution and quantitation of 10-260 kDa proteins while also separating and detecting 2.5–2,000 ng/μl protein. The Experion Pro260 system combines electrophoresis, staining, destaining,

imaging, band detection, and basic data analysis into a single, automated step. Gel images were processed and displayed on a computer monitor and MHC bands identified by migration relative to the molecular weight marker.

Table 4

Oligonucleotides Primers Used for PCR

Primer Name	NCBI Accession Number	Sense Sequence (5' → 3')	Anti-Sense Sequence (5' → 3')	Amplicon Size (bp)
β-Actin	NM_001101	ATC GTG CGT GAC ATT AAG	GTC ATC ACC ATT GGC AAT	102
NF Kappa-B	M58603	TAG CCA TGA TGA GCA ATA GC	CAG CCT GCC AAT GAG ATG	124
TNF-Alpha	NM_000594	CAG CAA GGA CAG CAG AGG	AGT ATG TGA GAG GAA GAG AAC C	139
IL-1β	NM_000576	TGA TGG CTT ATT ACA GTG GCA ATG	GTA GTG GTG GTC GGA GAT TCG	115
IL-6	NM_000600	GGT CCA GTT GCC TTC TCC	TGT CAA TTC GTT CTG AAG AGG	132
COX-2	M90100	TCC CTG AGC ATC TAC GGT TTG	CAT CGC ATA CTC TGT TGT GTT CC	110

Perceived Muscle Soreness Procedures

Soreness was assessed along a 10-cm scale (zero = no soreness, 10 = extreme soreness). Participants were asked to rate their level of soreness immediately prior to the eccentric exercise bout, and at four, 24, and 48 hours after exercise by drawing an intersecting line across the continuum line extending from zero to 10. The distance of each mark was measured from zero and the measurement utilized as the perceived soreness level.

Reported Side Effects from Supplements

To determine whether the participants suffered any negative side effects from the dietary supplements, participants reported via a questionnaire administered in a confidential manner whether they tolerated the supplementation protocol, as well as reported any medical problems/symptoms they may have encountered throughout the supplementation period. This questionnaire was administered at two, four, and six weeks.

Statistical Analyses

Statistical analysis for the biomarkers of interest were performed by utilizing repeated measures (group by time) ANOVAs to control for intra-individual variability for blood (2x6) and muscle (2x4) samples. Because traditional post-hoc testing in a repeated measures framework does not fully account for intra-individual variability, significant differences in mean values for main effects or interactions were explored further using profile plots and independent t-tests. All statistical procedures were performed using SAS version 9.1.3 software and a probability level of <0.05 was adopted throughout.

CHAPTER FOUR

Results

Participants

Thirty-three post-menopausal female participants began this study, one was unable to finish due to a musculoskeletal injury unrelated to the study and one was dropped from analysis due to non-compliance. Baseline demographic data for the 31 participants who completed the study are presented in Table 5. At baseline, there were no significant differences between groups with regard to age ($p = 0.37$), body mass index (BMI; $p = 0.52$), cardiopulmonary fitness level (VO_{2max}; $p = 0.37$), or baseline protein intake ($p = 0.22$). All study participants were of Caucasian descent and no significant body weight changes were observed over time ($p = 0.22$).

Table 5

Study Participant Demographics

Variable	Soy Group (n=16)	Dairy Group (n=15)
Age (yrs)	53.88±3.65	55.00±3.12
BMI (kg/m^2)	25.36±4.05	26.31±3.96
VO_{2max} (ml/kg/min)	25.60±4.86	27.22±5.07
Protein Intake (g/kg)	1.16±0.48	0.98±0.28

Note: Data are presented as means ± standard deviations

Participants were retained for analysis if they were 80% compliant with the dietary protocol. According to a Wilcoxon-rank sum test, there was no statistically

significant difference in dietary compliance among the treatment groups ($p = 0.54$). Reported side effects questionnaires administered at baseline and at weeks two, four, and six also revealed minimal and equivocal negative side effects due to the supplementation products as presented in Tables 6-9.

All participants completed the required exercise protocol, and reported significant increases in DOMS from baseline ($p < 0.01$). A significant group effect was observed ($p = 0.0133$) with the soy group reporting elevated DOMS values compared to the dairy group throughout the pre- and post-exercise period. Finally, all participants participated in all blood draws (except for one participant who missed the T3 blood draw), and all skeletal muscle biopsies during the course of the study.

Assessment for Differences in Confounding Variables

Prior to data collection, certain variables were identified as potential confounding variables. These variables included dietary intake, physical activity, and health related quality of life (QOL). To account for these variables, prior to and at the end of the supplementation period, participants were asked to complete four-day dietary food records and questionnaires pertaining to physical activity and health related QOL. Independent sample t-tests were utilized to assess the baseline differences of recorded confounding variables between the groups, and repeated measures ANOVAs were utilized to assess differences over the course of the study. Individual variable results are presented in the following sections.

Table 6

Side Effects (Soy): Frequency

	None (0 per/wk)	Minimal (1-2 per/wk)	Slight (3-4 per/wk)	Occasional (5-6 per/wk)	Frequent (7-8 per/wk)	Severe (9+ per/wk)
Dizziness						
Week 2	16					
Week 4	16					
Week 6	14	1	1			
Nausea						
Week 2	14	1				1
Week 4	15					1
Week 6	15		1			
Headache						
Week 2	12	4				
Week 4	15	1				
Week 6	13	2		1		
Fast Heart Rate						
Week 2	15	1				
Week 4	16					
Week 6	16					
Heart Skipping						
Week 2	15	1				
Week 4	15	1				
Week 6	16					
Short Breath						
Week 2	16					
Week 4	16					
Week 6	16					
Nervousness	14	1	1			
Week 2	16					
Week 4	16					
Week 6	16					
Blurred Vision						
Week 2	16					
Week 4	16					
Week 6	16					
Other						
Week 2	15			1		
Week 4	15			1		
Week 6	16					

Note: Data are presented as frequency counts.

Table 7

Side Effects (Soy): Severity

	None	Minimal	Slight	Moderate	Severe	Very Severe
Dizziness						
Week 2	16					
Week 4	16					
Week 6	14	2				
Nausea						
Week 2	14	1			1	
Week 4	15				1	
Week 6	15			1		
Headache						
Week 2	12	3		1		
Week 4	15			1		
Week 6	13	1		1		1
Fast Heart Rate						
Week 2	15	1				
Week 4	16					
Week 6	16					
Heart Skipping						
Week 2	15	1				
Week 4	15	1				
Week 6	16					
Short Breath						
Week 2	16					
Week 4	16					
Week 6	16					
Nervousness						
Week 2	14	2				
Week 4	16					
Week 6	16					
Blurred Vision						
Week 2	16					
Week 4	16					
Week 6	16					
Other						
Week 2	15			1		
Week 4	14	1	1			
Week 6	16					

Note: Data are presented as frequency counts.

Table 8

Side Effects (Dairy): Frequency

	None (0 per/wk)	Minimal (1-2 per/wk)	Slight (3-4 per/wk)	Occasional (5-6 per/wk)	Frequent (7-8 per/wk)	Severe (9+ per/wk)
Dizziness						
Week 2	14	1				
Week 4	15					
Week 6	15					
Nausea						
Week 2	15					
Week 4	14		1			
Week 6	12	1	2			
Headache						
Week 2	11	3	1			
Week 4	13	2				
Week 6	13	1		1		
Fast Heart Rate						
Week 2	12	1	1	1		
Week 4	13	2				
Week 6	14		1			
Heart Skipping						
Week 2	13	1		1		
Week 4	14	1				
Week 6	15					
Short Breath						
Week 2	13			1	1	
Week 4	13		1		1	
Week 6	14			1		
Nervousness						
Week 2	13	1	1			
Week 4	14		1			
Week 6	13	1	1			
Blurred Vision						
Week 2	14		1			
Week 4	15					
Week 6	15					
Other						
Week 2	15					
Week 4	14				1	
Week 6	13		2			

Note: Data are presented as frequency counts.

Table 9

Side Effects (Dairy): Severity

	None	Minimal	Slight	Moderate	Severe	Very Severe
Dizziness						
Week 2	14	1				
Week 4	15					
Week 6	15					
Nausea						
Week 2	15					
Week 4	15					
Week 6	13		1	1		
Headache						
Week 2	11	1	2		1	
Week 4	12	1	1	1		
Week 6	13	1	1			
Fast Heart Rate						
Week 2	11	1	2	1		
Week 4	12	2		1		
Week 6	14			1		
Heart Skipping						
Week 2	12	1	1	1		
Week 4	14			1		
Week 6	14			1		
Short Breath						
Week 2	12		1	2		
Week 4	14			1		
Week 6	14			1		
Nervousness						
Week 2	12	1	2			
Week 4	14		1			
Week 6	14		1			
Blurred Vision						
Week 2	13		2			
Week 4	15					
Week 6	15					
Other						
Week 2	14		1			
Week 4	14			1		
Week 6	13	1		1		

Note: Data are presented as frequency counts.

Dietary Intake Analyses

All participants were instructed to consume their usual diet, but limit the consumption of any soy-based or dairy-based products for two weeks, and then begin consumption of the supplement. Independent sample t-tests were utilized to analyze all relevant dietary variables prior to supplementation. Table 10 illustrates that there were no significant differences observed between groups for total daily caloric intake, macronutrient intake of protein, carbohydrate, and fat, and select dietary components known to affect antioxidant status (vitamin C, vitamin E, zinc, selenium, omega-3 fatty acids, and beta carotene) at baseline. Following supplementation, repeated measures ANOVAs determined that there existed a statistically significant group effect for omega-3 fatty acids ($p < 0.01$) with the soy group having significantly higher values than the dairy group at both time points, a time effect for selenium ($p < 0.01$) and zinc ($p < 0.01$), and a group by time interaction for beta-carotene ($p < 0.01$) and vitamin E ($p < 0.01$).

Physical Activity Analysis

All participants were instructed to maintain their normal physical activity regime prior to and during the course of the study. Prior to and after the four week supplementation period, participants were asked to record physical activity status using the validated International Physical Activity Questionnaire® (IPAQ). This questionnaire was developed as an instrument for cross-national monitoring of physical activity and inactivity and produces a continuous (weekly met-minute level) and categorical (i.e. 1 =

Table 10

Four Day Food Records

Variable	Soy Group (N=16)		Dairy Group (N=15)	
	Pre	Post	Pre	Post
Calories (kcal/d)	1582.01 ±365.75	1769.73 ±364.82	1704.29 ±401.49	1824.95 ±538.25
Carbohydrates (g/d)	184.03 ±56.03	208.89 ±55.19	206.86 ±52.50	217.29 ±69.49
Protein (g/d)	75.44 ±25.41	94.66 ±22.70	65.68 ±17.29	89.00 ±21.89
Fat (g/d)	61.07 ±20.40	67.30 ±22.97	65.80 ±20.71	78.24 ±26.00
Beta-Carotene[¥] (µg/d)	1814.44 ±1637.32	5106.45 ±3182.15	2183.00 ±3558.50	1469.54 ±2004.96
Vitamin C (mg/d)	75.86 ±45.92	77.70 ±52.59	87.80 ±70.62	70.26 ±46.75
Vitamin E[¥] (mg/d)	5.68 ±3.53	13.88 ±5.60	5.08 ±4.19	6.58 ±4.93
Selenium[§] (µg/d)	46.77 ±25.90	82.10 ±30.80	48.24 ±19.14	63.51 ±30.21
Zinc[§] (mg/d)	7.02 ±4.28	9.01 ±2.96	6.90 ±2.65	10.64 ±4.75
Omega-3[*] (g/d)	1.29 ±2.13	1.06 ±0.28	0.74 ±0.69	0.74 ±0.36

Note. Data are presented as means ± standard deviations pre and post supplementation. [*] denotes a significant group effect ($p < .01$), [§] denotes a significant time effect ($p < 0.01$), and [¥] denotes a significant group by time interaction ($p < 0.01$).

low, 2 = moderate, 3 = high) physical activity level score. Independent sample t-tests were utilized to analyze the IPAQ continuous scores at baseline, and a repeated measures ANOVA was utilized to assess changes in physical activity over time. Categorical frequency tables were also constructed, and all data is presented in Table 11. No significant differences in the continuous physical activity score were reported at baseline between groups. The group by time interaction was non-significant ($p = 0.83$); however, there was a significant time effect observed across groups ($p = 0.02$) with both groups reporting decreases in physical activity.

Table 11

International Physical Activity Questionnaire

Variable	Soy Group (N=16)		Dairy Group (N=15)	
	Pre	Post	Pre	Post
Total Met-Minutes (min/wk)[§]	5270±4719	2804±1673	5079±6823	3007±2722
Categorical Level				
High	9	6	9	7
Moderate	7	8	4	6
Low	0	2	2	2

Note. Continuous data are presented as means ± standard deviations pre and post supplementation. Categorical data are presented as frequency counts pre and post supplementation. [§]Denotes a significant time effect ($p = 0.02$).

Health-Related Quality of Life Analysis

Prior to and after the four week supplementation period, participants were asked to record health-related QOL using the validated Short Form (36) Health Survey® (SF-36). The SF-36 consists of eight scaled scores, which are the sums of the questions in

their section. Each scale is directly transformed into a zero-100 scale on the assumption that each question carries equal weight. The eight sections are: vitality, physical functioning, bodily pain, general health perceptions, physical role functioning, emotional role functioning, social role functioning, and mental health. Independent sample t-tests were utilized to analyze the SF-36 scaled scores at baseline. Furthermore, a repeated measures ANOVA was utilized to assess changes over time in each of the eight health-related quality of life sections. No differences were observed, and all health-related QOL data are presented in Table 12.

Table 12

Health Related Quality of Life Questionnaire

Variable	Soy Group (N=16)		Dairy Group (N=15)	
	Pre	Post	Pre	Post
Physical Functioning	93.13±9.29	90.00±13.78	92.00±10.66	91.33±12.88
Role-Physical	90.23±21.77	94.53±12.88	91.67±12.87	92.50±12.32
Bodily Pain	73.19±21.63	75.06±17.23	77.2±14.67	75.67±15.67
General Health	79.38±22.20	81.06±15.71	84.80±16.79	81.37±15.88
Vitality	70.70±9.66	71.88±10.70	67.50±14.41	69.58±15.28
Social Functioning	50.78±3.13	49.22±3.13	48.33±4.40	50.83±7.42
Role-Emotional	86.98±25.99	91.15±18.38	92.78±11.73	95.56±11.73
Mental Health	82.19±7.95	84.69±11.47	83.33±11.29	86.00±8.28

Note. Data are presented as means ± standard deviations pre and post supplementation.

Blood Variables

A two-way [treatment groups (2) x time point (6)] repeated measures ANOVA to control for the within-individual variation was conducted to evaluate the effects of the different supplements (soy milk or dairy milk) on various blood markers that may be involved in systemic inflammation and oxidative stress. The dependant variables that were analyzed in this multivariate analysis were: SOD, COX-2, GPx, TNF-α, IL-1β, IL-6. The within-subjects factor was time with six levels (baseline = T1, immediately before exercise = T2, immediately post-exercise = T3, four hours post-exercise = T4, 24 hours post-exercise = T5, and 48 hours post-exercise = T6). The between-subjects factor was group with two levels (soy milk or dairy milk). The group by time interaction effect, time main effect, and group main effect were analyzed using the multivariate criterion of Wilks' Lambda, and all effect sizes were calculated using eta-squared values. Because traditional post-hoc testing in a repeated measures framework does not fully account for intra-individual variability, significant differences in mean values for main effects or interactions were explored further using profile plots and independent t-tests. All data were analyzed in duplicate to reduce within-subject variability. Data points falling three standard deviations above or below the group mean were labeled as outliers and excluded from analysis (Pagano, 1998). Sample size per group and time point are listed by biomarker below.

Plasma SOD

Plasma SOD data is presented in Table 13. No significant interaction of group by time was observed in regard to plasma SOD ($p = 0.44$; effect size = 0.19), and no significant main effect of group was observed ($p = 0.99$; effect size = 0.00). However,

there was a significant main effect for time ($p < 0.0001$; effect size = 0.68). Post-hoc testing revealed a significant decrease in plasma SOD ($p < 0.0001$) from T2-T4, and then a return to baseline values for both groups as observed in Figure 2.

Table 13

Plasma SOD Activity (units/µL)

Group Assignment	T1	T2	T3	T4	T5	T6
Soy	1.26±0.62	1.25±0.46	0.92±0.36	0.90±0.59	1.22±0.66	1.30±0.41
Dairy	1.17±0.30	1.09±0.38	1.05±0.44	0.90±0.57	1.33±0.47	1.29±0.48
Overall	1.22±0.49	1.17±0.42	0.99±0.41	0.90±0.57[§]	1.27±0.57	1.29±0.44

Note. Data are presented as means ± standard deviations. All SOD activity values are expressed as units/µL. The soy group had 16 observations per time point, except for T3 in which only 15 data points were collected. The dairy group had 15 observations per time point. [§] denotes a significant time effect ($p < 0.0001$).

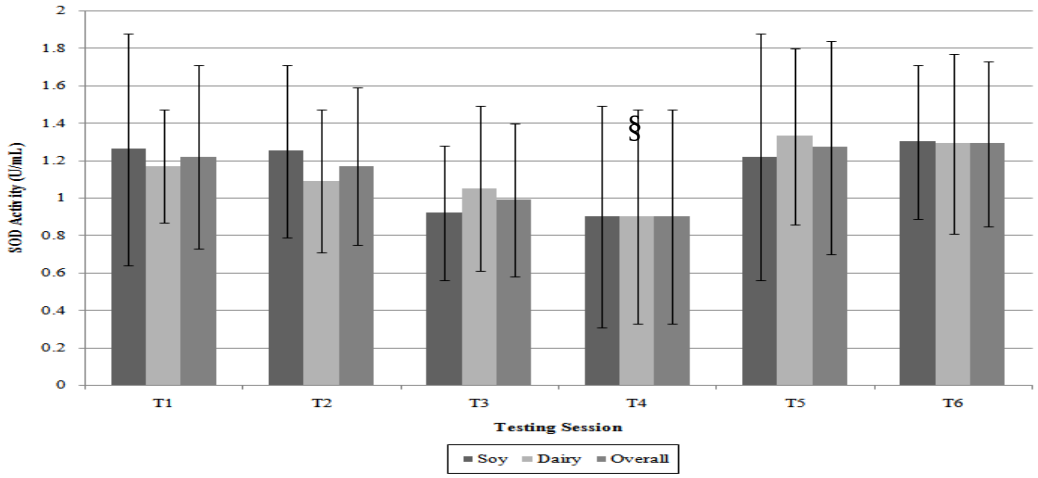

Figure 2. Column chart of SOD activity (units/µL) by time. Data are presented as means ± standard deviations and all SOD activity values are expressed as units/µL. The soy group had 16 observations per time point, except for T3 in which only 15 data points were collected. The dairy group had 15 observations per time point. § denotes a significant time effect ($p < 0.0001$).

Plasma COX-2

Data for plasma COX-2 is presented in Table 14. No significant interaction of group by time was observed in regard to plasma COX-2 ($p = 0.74$; effect size = 0.15). Additionally, no significant main effect for time ($p = 0.92$; effect size =0.07) or group ($p = 0.13$; effect size = 0.09) was observed.

Table 14

Plasma COX-2 Concentration (pg/mL)

Group Assignment	T1	T2	T3	T4	T5	T6
Soy	1.78±1.39	1.74±1.23	1.76±1.65	1.74±1.57	1.83±1.60	2.07±1.82
Dairy	1.16±1.06	1.08±0.96	1.50±1.38	1.29±0.87	1.45±1.05	1.15±1.42
Overall	1.46±1.25	1.41±1.14	1.63±1.50	1.52±1.28	1.64±1.34	1.64±1.69

Note. Data are presented as means ± standard deviations. All COX-2 values are expressed as pg/mL. The following sample sizes were observed for the soy group for T1-T6, respectively: 14, 15, 14, 15, 15, 16. The following sample sizes were observed for the dairy group for T1-T6, respectively: 15, 15, 14, 15, 15, 15.

Plasma GPx

Plasma GPx data are presented in Table 15. No significant interaction of group by time was observed in regard to plasma GPx ($p = 0.55$; effect size =0.13). Further, no significant main effect for time ($p = 0.36$; effect size = 0.17) or group ($p = 0.24$; effect size =0.04) was observed.

Table 15

Plasma GPx Activity (nmol/min/mL)

Group Assignment	T1	T2	T3	T4	T5	T6
Soy	146.31 ±35.05	140.24 ±32.31	141.57 ±25.24	143.40 ±28.80	148.44 ±35.30	151.71 ±19.22
Dairy	137.12 ±26.74	137.46 ±33.54	125.70 ±36.81	140.07 ±33.68	139.33 ±32.97	132.99 ±27.21
Overall	141.86 ±31.14	138.89 ±32.39	133.63 ±32.04	141.79 ±30.77	144.03 ±33.94	142.65 ±24.91

Note. Data are presented as means ± standard deviations. All GPx activity values are expressed as nmol/min/mL. The soy group had 16 observations per time point, except for T3 in which only 15 data points were collected. The dairy group had 15 observations per time point.

Plasma TNF-Ŭ

Data for plasma TNF-α is presented in Table 16. A significant group by time interaction was observed for plasma TNF-α ($p = 0.04$; effect size = 0.34), the dairy group values increasing at T2 and dropping through T4, with a rise to baseline values at T5 and T6. The soy group, on the other hand, maintained relatively constant and lower mean TNF-α values throughout the majority of the exercise bout, with the interaction occurring between T2 and T4 ($p = 0.02$). Data is displayed pictorially in Figure 3.

Table 16

Plasma TNF-ŭConcentration (pg/mL)

Group Assignment	T1	T2	T3	T4	T5	T6
Soy	2.79±0.93	2.71±1.01	2.77±1.16	2.86±1.02¥	3.24±1.52	2.75±0.72
Dairy	2.99±1.20	3.53±0.85	3.09±1.15	2.67±0.77¥	3.15±0.83	3.04±1.02
Overall	2.89±1.06	3.09±1.01	2.92±1.15	2.77±0.90	3.2±1.23	2.88±0.87

Note. Data are presented as means ± standard deviations. All TNF-α concentration values are expressed in pg/mL. The following sample sizes were observed for the soy group for T1-T6, respectively: 15, 16, 15, 16, 16, 16. The dairy group had 14 observations per time point. ¥ denotes a significant group by time interaction (p = 0.04).

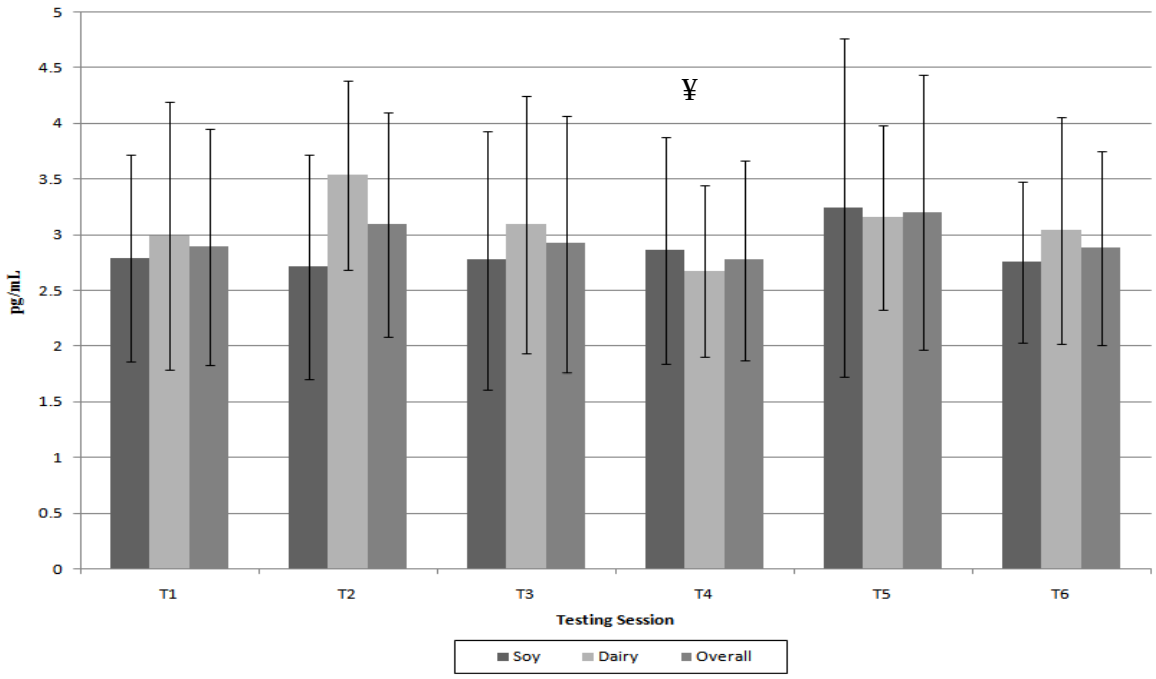

Figure 3. Column chart of TNF-α concentration (pg/mL) by time. Data are presented as means ± standard deviations and TNF- α values are expressed in pg/mL. The following sample sizes were observed for the soy group for T1-T6, respectively: 15, 16, 15, 16, 16, 16. The dairy group had 14 observations per time point. ¥ denotes a significant group by time interaction (p = 0.04).

Plasma IL-1 β

Data for plasma IL-1β is presented in Table 17. No significant interaction of group by time was observed in regard to plasma IL-1β ($p = 0.16$; effect size = 0.25). Further, no significant main effect for time ($p = 0.89$; effect size = 0.05) or group ($p = 0.79$; effect size = 0.00) was observed.

Table 17

Plasma IL-1 βConcentration (pg/mL)

Group Assignment	T1	T2	T3	T4	T5	T6
Soy	0.78±0.38	0.73±0.29	0.77±0.34	0.74±0.27	0.76±0.35	0.81±0.37
Dairy	0.74±0.22	0.77±0.22	0.75±0.22	0.78±0.22	0.70±0.23	0.73±0.30
Overall	0.76±0.30	0.75±0.25	0.76±0.28	0.76±0.24	0.73±0.29	0.77±0.33

Note. Data are presented as means ± standard deviations. All IL-1β concentration values are expressed in pg/mL. The following sample sizes were observed for the soy group for T1-T6, respectively: 15, 16, 15, 16, 16, 16. The dairy group had 15 observations per time point

Plasma IL-6

Plasma IL-6 data is presented in Table 18. No significant interaction of group by time was observed in regard to plasma IL-6 ($p = 0.56$; effect size = 0.10). There was no significant main effects of group ($p = 0.74$; effect size = 0.00); however, there was a significant main effect for time ($p < 0.0001$; effect size = 0.62). Post-hoc testing revealed a significant increase in plasma IL-6 expression from T2-T4 ($p < 0.0001$), with a subsequent return to baseline levels at T5 and T6 for both groups. Data is displayed pictorially in Figure 4.

Table 18

Plasma IL-6 Concentration (pg/mL)

Group Assignment	T1	T2	T3	T4	T5	T6
Soy	2.27±1.16	2.10±0.78	2.95±1.43	4.68±2.78	2.69±1.50	2.39±1.26
Dairy	2.22±1.29	2.48±1.63	2.94±3.73	4.43±2.96	2.39±1.23	2.30±1.02
Overall	2.24±1.20	2.27±1.24	3.45±2.82	4.56±2.82[§]	2.55±1.36	2.34±1.13

Note. Data are presented as means ± standard deviations. All IL-6 values are expressed in pg/mL. The following sample sizes were observed for the soy group for T1-T6, respectively: 15, 16, 15, 15, 16, 16. The following sample sizes were observed for the dairy group for T1-T6, respectively: 14, 14, 15, 15, 15, 15. [§] denotes a significant time effect ($p < 0.0001$).

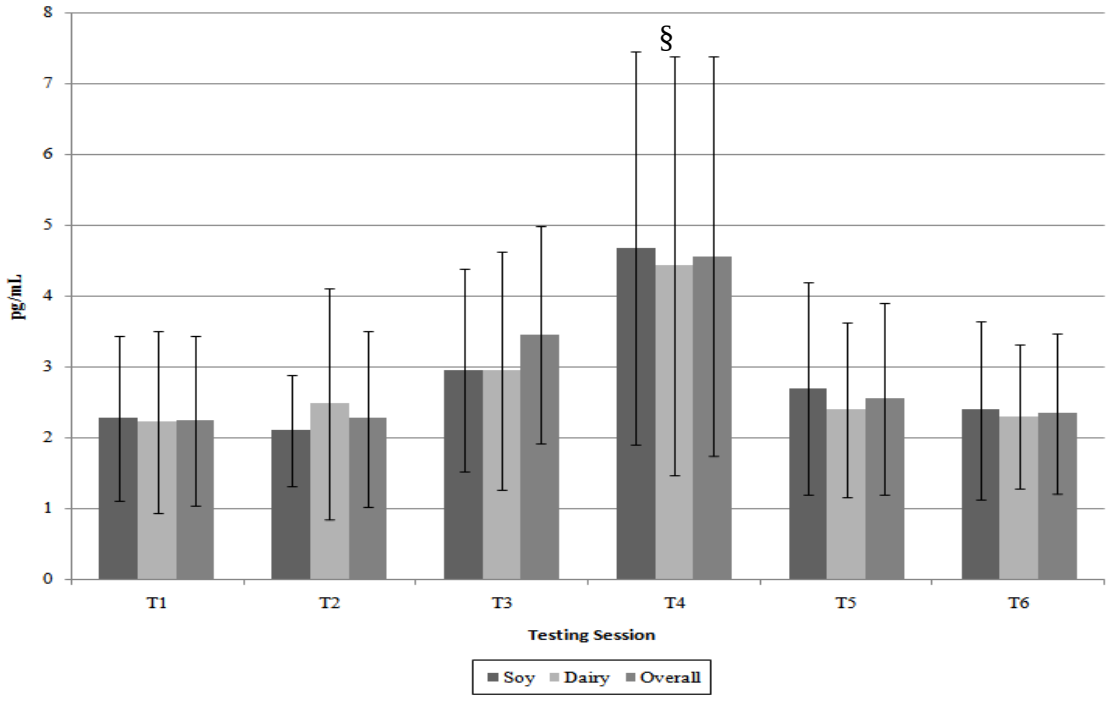

Figure 4. Column chart of plasma IL-6 concentration (pg/mL) by time. Data are presented as means ± standard deviations and all IL-6 values are expressed in pg/mL. The following sample sizes were observed for the soy group for T1-T6, respectively: 15, 16, 15, 15, 16, 16. The following sample sizes were observed for the dairy group for T1-T6, respectively: 14, 14, 15, 15, 15, 15. § denotes a significant time effect ($p < 0.0001$).

Therefore, in regard to hypothesis one, which states that there will be a statistically significant difference in plasma levels of inflammation and oxidative stress-responsive plasma variables (SOD, GPx, COX-2, TNF-α, IL-1β, IL-6) in both groups after four weeks of nutritional supplementation, the hypothesis was failed to be supported. Further, in regard to hypothesis two which states that there will be a statistically significant difference in plasma levels of inflammation and oxidative stress-responsive plasma variables (SOD, GPx, COX-2, TNF-α, IL-1β, IL-6) in both groups after the eccentrically biased downhill run, this hypothesis was also failed to be supported. However, IL-6, TNF-α, and SOD data suggest that the exercise bout was able to alter plasma markers of inflammation in both groups. Further, TNF-α data suggests that the soy group may have been better able to maintain baseline values of TNF-α compared to the dairy group, who saw a large spike in TNF-α expression post-supplementation, a subsequent drop to T4, and return to post-supplementation values by T6.

Muscle-Specific Gene Expressions

A two-way [treatment groups (2) x time point (4)] repeated measures ANOVA was conducted to evaluate the effects of the different supplements (soy milk or dairy milk) on various muscle proteins that may be involved in inflammation and oxidative stress. The dependant variables that were analyzed in this multivariate analysis were: TNF-α, IL1-β, IL-6, COX-2 and NF-κB. The within-subjects factor was time with four levels (baseline = T1, immediately before exercise = T2, four hours post-exercise = T3, and 24 hours post-exercise = T4). The between-subjects factor was group with two levels (soy milk or dairy milk). The group by time interaction effect, time main effect, and

group main effect were analyzed using the multivariate criterion of Wilks' Lambda, and all effect sizes were calculated using eta-squared values. Because traditional post-hoc testing in a repeated measures framework does not fully account for intra-individual variability, significant differences in mean values for main effects or interactions were explored further using profile plots and independent t-tests. Data points falling three standard deviations above or below the group mean were labeled as outliers and excluded from analysis (Pagano, 1998). Sample size per group and time point are listed by biomarker below.

Muscle TNF-Ŭ

Data for muscle expression of TNF-α is presented in Table 19. No significant interaction of group by time was observed in regard to muscle TNF-Ŭ(p = 0.21; effect size = 0.15). Further, there was no significant main effect of time (p = 0.21; effect size = 0.15); however, there was a moderate trend toward a significant main effect for group (p = 0.07; effect size = 0.11). Data is displayed pictorially in Figure 5.

Table 19

Muscle Gene Expression of TNF-Ŭ ⁄Actin

Group Assignment	T1	T2	T3	T4
Soy	1.00±0.05	0.97±0.06	0.94±0.06	0.99±0.08
Dairy	0.95±0.05	0.95±0.06	0.95±0.08	0.94±0.07
Overall	0.98±0.06	0.96±0.06	0.95±0.07	0.97±0.08

Note. Data are presented as means ± standard deviations. All data is expressed at the ratio between the variable of interest (TNF-α) and the internal standard (β-Actin) to control for loading artifact. The soy group had 16 observations per time point, and the dairy group had 15 observations per time point.

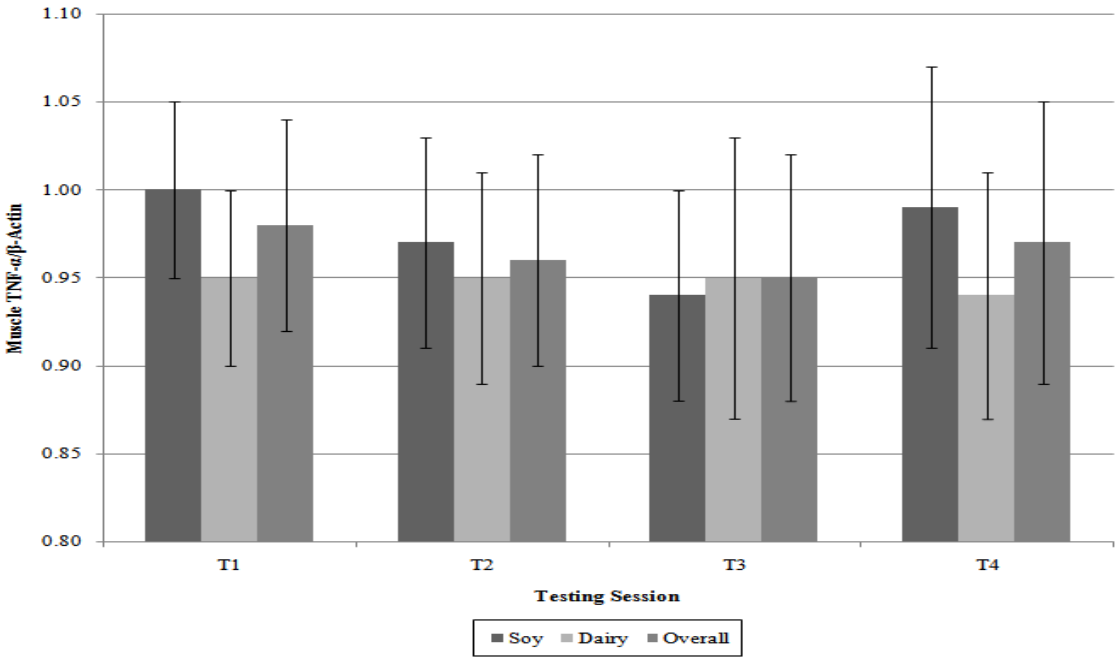

Figure 5. Column chart of muscle TNF-α/β-Actin gene expression by time. All data are presented as means ± standard deviations and all data is expressed at the ratio between the variable of interest (TNF-α) and the internal standard (β-Actin) to control for loading artifact. The soy group had 16 observations per time point, and the dairy group had 15 observations per time point.

Muscle IL-1β

Data for muscle IL-1β expression is presented in Table 20. No significant interaction of group by time was observed in regard to muscle IL-1β ($p = 0.13$; effect size = 0.19). Further, there was no significant main effect of group ($p = 0.96$; effect size = 0.00); however, there was a significant main effect for time ($p = 0.0005$; effect size = 0.48). Post-hoc testing revealed a significant difference between T2-T4 ($p = 0.001$), with a sharp decline in muscle IL-1β occurring at T3 for both groups and then returning to baseline by T4. Data is displayed pictorially in Figure 6.

Table 20

Muscle Gene Expression of IL-1 β-Actin

Group Assignment	T1	T2	T3	T4
Soy	1.06±0.07	1.02±0.06	0.95±0.06	1.05±0.08
Dairy	1.04±0.06	1.05±0.06	0.97±0.11	1.01±0.06
Overall	1.05±0.07	1.04±0.06	0.96±0.09§	1.03±0.07

Note. Data are presented as means ± standard deviations. All data is expressed at the ratio between the variable of interest (IL-1β) and the internal standard (β-Actin) to control for loading artifact. The soy group had 16 observations per time point, and the dairy group had 15 observations per time point. § denotes a significant time effect (p = 0.0005).

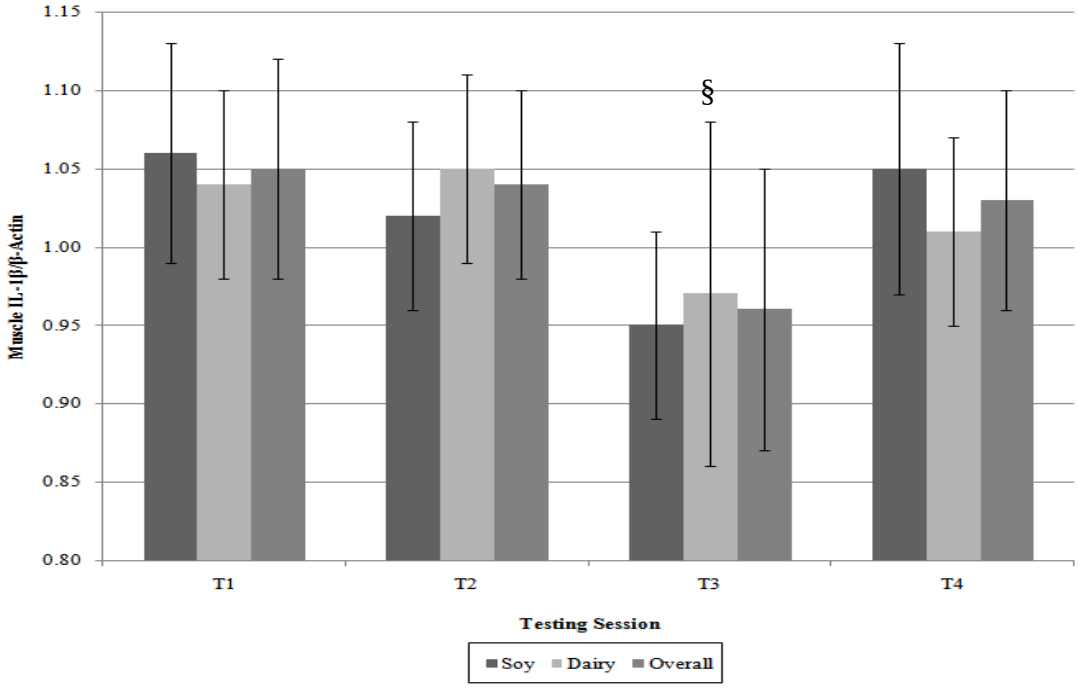

Figure 6. Column chart of muscle IL-1 β /β-Actin gene expression by time. Data are presented as means ± standard deviations. All data is expressed at the ratio between the variable of interest (IL-1β) and the internal standard (β-Actin) to control for loading artifact. The soy group had 16 observations per time point, and the dairy group had 15 observations per time point. § denotes a significant time effect (p = 0.0005).

Muscle IL-6

Data for muscle expression of IL-6 is presented in Table 21. No significant interaction of group by time was observed in regard to muscle IL-6 ($p = 0.37$; effect size = 0.11). Further, there was no significant main effect of group ($p = 0.46$; effect size = 0.02); however, there was a significant main effect for time ($p = 0.0048$; effect size = 0.38). Post-hoc testing revealed a significant difference between T2-T4 ($p = 0.003$), with a sharp decline in muscle IL-6 occurring at T3 for both groups, and values returning to baseline by T4. Data is displayed pictorially in Figure 7.

Table 21

Muscle Gene Expression of IL- 6 ⁄Actin

Group Assignment	T1	T2	T3	T4
Soy	1.05±0.05	1.03±0.05	0.98±0.05	1.03±0.09
Dairy	1.02±0.05	1.03±0.07	0.99±0.06	1.00±0.06
Overall	1.04±0.05	1.03±0.06	0.98±0.06[§]	1.01±0.08

Note. Data are presented as means ± standard deviations. All data is expressed at the ratio between the variable of interest (IL-6) and the internal standard (β-Actin) to control for loading artifact. The soy group had 16 observations per time point, and the dairy group had 15 observations per time point. [§] denotes a significant time effect ($p = 0.0048$).

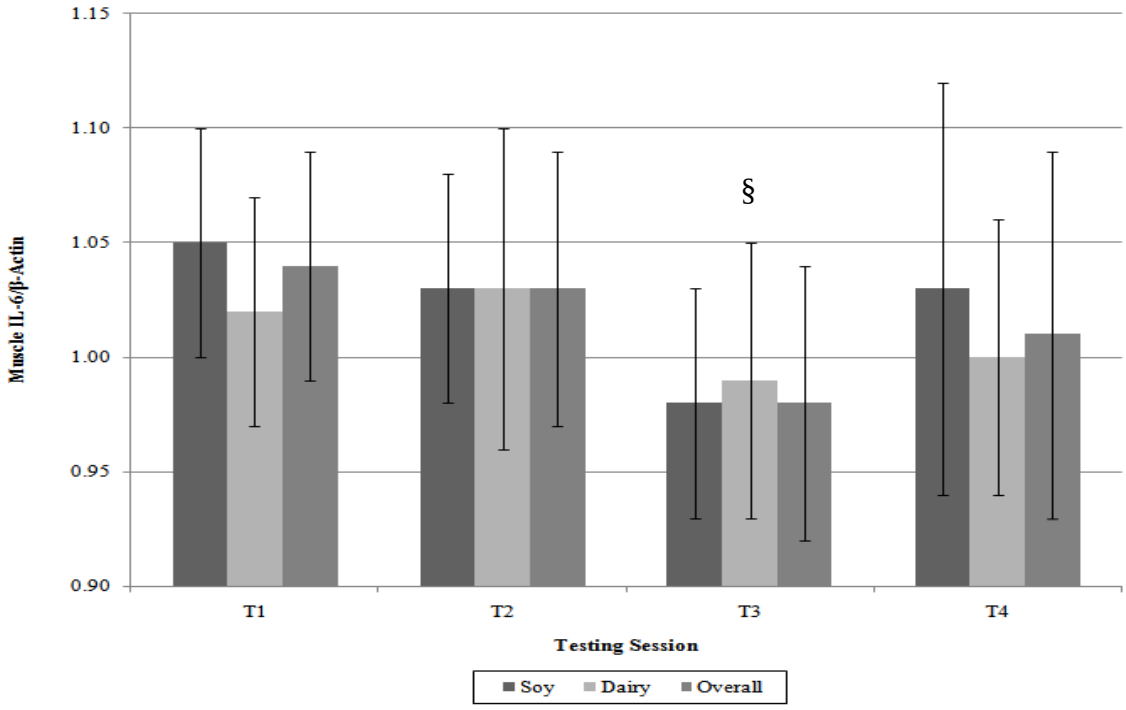

Figure 7. Column chart of muscle IL-6/β-Actin gene expression by time. Data are presented as means ± standard deviations. All data is expressed at the ratio between the variable of interest (IL-6) and the internal standard (β-Actin) to control for loading artifact. The soy group had 16 observations per time point, and the dairy group had 15 observations per time point. § denotes a significant time effect (p = 0.0048).

Muscle COX-2

Data for muscle expression of COX-2 is presented in Table 22. No significant interaction of group by time was observed in regard to muscle COX-2 (p = 0.60; effect size = 0.07). Further, there was no significant main effect of time (p = 0.22; effect size = 0.15); however, there was a trend toward a significant main effect for group (p = 0.06; effect size = 0.12). Data is displayed pictorially in Figure 8.

Table 22

Muscle Gene Expression of COX- 2 /Actin

Group Assignment	T1	T2	T3	T4
Soy	0.96±0.09	0.98±0.09	0.92±0.09	0.96±0.09
Dairy	0.93±0.09	0.93±0.09	0.89±0.11	0.89±0.10
Overall	0.94±0.09	0.96±0.09	0.91±0.10	0.92±0.10

Note. Data are presented as means ± standard deviations. All data is expressed at the ratio between the variable of interest (COX-2) and the internal standard (β-Actin) to control for loading artifact. The soy group had 16 observations per time point, and the dairy group had 15 observations per time point.

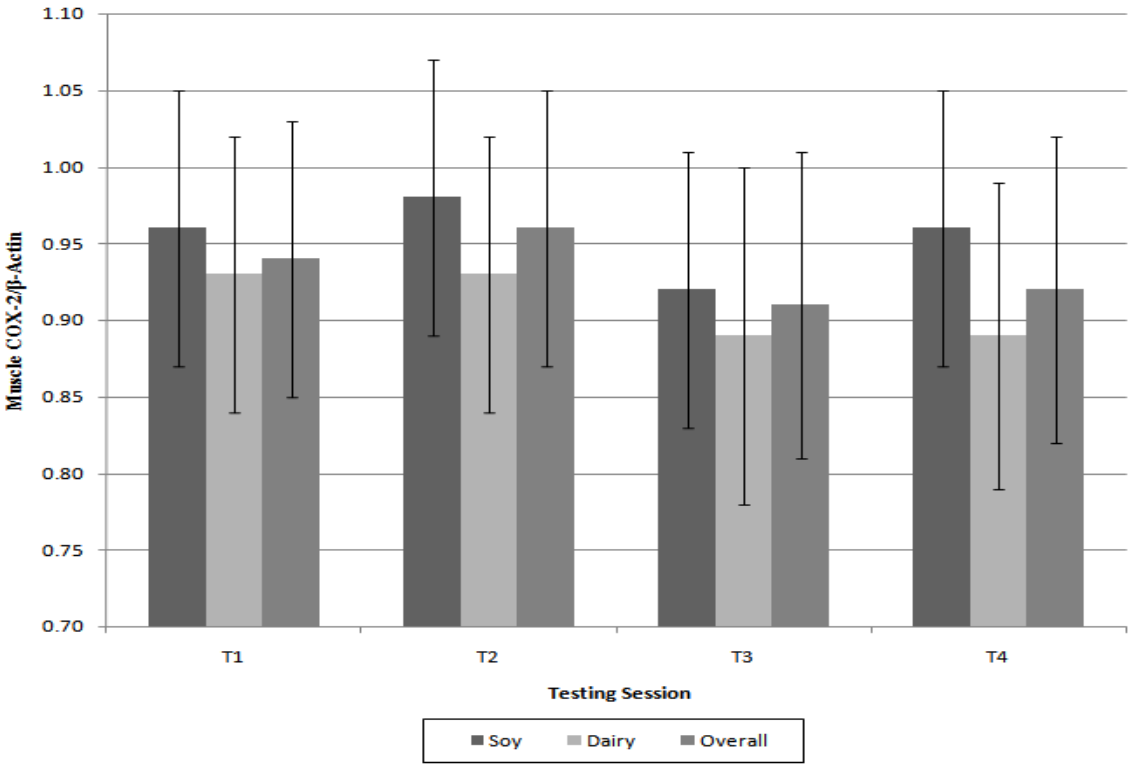

Figure 8. Column chart of muscle COX-2/β-Actin gene expression by time. Data are presented as means ± standard deviations. All data is expressed at the ratio between the variable of interest (COX-2) and the internal standard (β-Actin) to control for loading artifact. The soy group had 16 observations per time point, and the dairy group had 15 observations per time point.

Muscle NF-ə B

NF-ϑB data is presented in Table 23. No significant interaction of group by time was observed in regard to plasma NF-κB ($p = 0.41$; effect size = 0.10). In addition, no significant main effects for time ($p = 0.88$; effect size = 0.02) or group ($p = 0.77$; effect size = 0.00) was observed.

Table 23

Muscle Gene Expression of NF-ə B-Actin

Group Assignment	T1	T2	T3	T4
Soy	0.93±0.09	0.93±0.10	0.93±0.07	0.96±0.09
Dairy	0.96±0.10	0.97±0.11	0.94±0.11	0.92±0.10
Overall	0.95±0.09	0.95±0.10	0.94±0.09	0.94±0.09

Note. Data are presented as means ± standard deviations. All data is expressed at the ratio between the variable of interest (NF-κB) and the internal standard (β-Actin) to control for loading artifact. The soy group had 16 observations per time point, and the dairy group had 15 observations per time point.

Qualitative Gene Expression: Gel Electrophoresis Imaging

To assess positive amplification of mRNA (Figure 9), aliquots (20 μl) of the RT-PCR reaction mixtures were electrophoresed in 1.5% agarose gels in 1X Tris-Borate-EDTA (TBE) buffer to verify positive amplification and the gel stained with ethidium bromide (present in the TBE buffer at 1 μg/ml) and illuminated with UV transillumination (Chemi-Doc XRS, Bio-Rad, Hercules, CA, USA). Upon completion of the RT-PCR runs, data from one participant was randomly selected (soy group) and a gel image was processed and displayed on a computer monitor with MHC bands identified

by migration relative to the molecular weight marker. From the left, the molecular weight marker, β-Actin, IL-1β, IL-6, TNF-α, COX-2, and NF-κB were loaded into the 1% agarose gel. Gene expression is qualitatively expressed by the flouresence of the bands at each testing session. Generally, this image concurs with the RT-PCR time results, with fluctuations in IL-6 and IL-1β expression occurring at the different testing sessions.

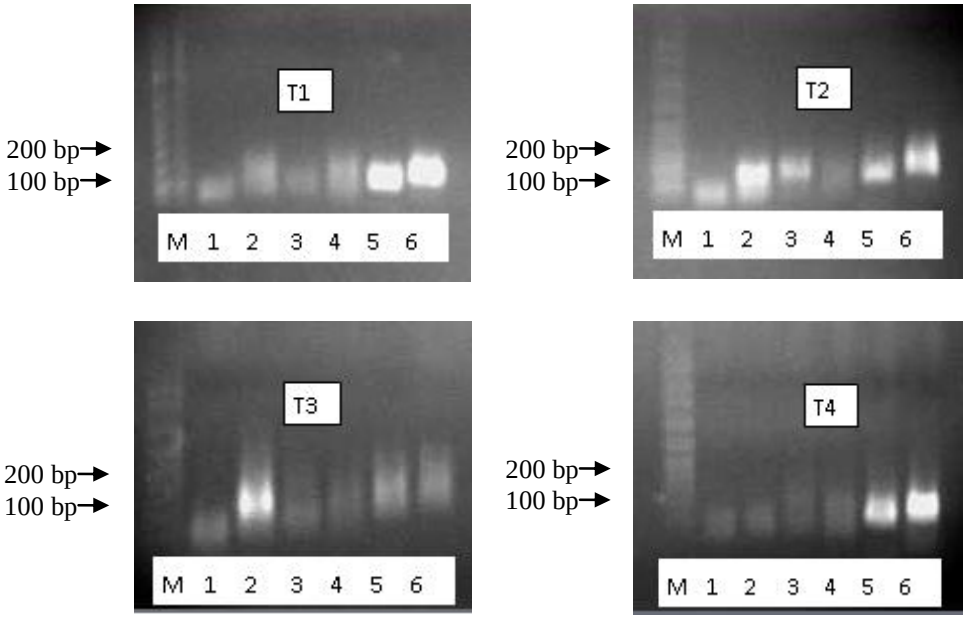

Figure 9. Illustration of PCR amplicons for the mRNA targets for one study participant at all four time points run on a 1% agarose gel. M = base pair marker, 1 = β-Actin, 2 = IL-1β, 3 = IL-6, 4 = TNF-α, 5 = COX-2, 6 = NF-κB.

Therefore, in regard to hypothesis three which states that there will be a statistically significant difference in the skeletal expression of inflammation-responsive genes (TNF-α, IL-1β, IL-6, NF-κB, COX-2) in both groups after four weeks of nutritional supplementation, the hypothesis was failed to be supported. Further, in regard to hypothesis four which states that there will be a statistically significant difference in the skeletal expression of inflammation-responsive genes (TNF-α, IL-1β,

IL-6, NF-κB, COX-2) in both groups after the eccentrically biased downhill run, the hypothesis was failed to be supported; although, the exercise bout itself did seem to have a dampening effect on IL-6 and IL-1β gene expression.

CHAPTER FIVE

Discussion

The overall purpose of the proposed study was to investigate the ability of soy milk to attenuate increases in plasma and skeletal muscle markers of inflammation and oxidative stress. This aims of this study were twofold. The first aim was to assess the effects of the daily consumption of soy milk or dairy milk for four weeks in older females on plasma markers of oxidative stress and inflammation, and markers of local oxidative stress and inflammation in skeletal muscle. The second aim was to assess the effects of the daily consumption of soy milk or dairy milk for four weeks in older females on plasma markers of oxidative stress and inflammation and markers of local oxidative stress and inflammation in skeletal muscle after a single bout of eccentric exercise designed to induce an inflammatory state. While the data collected failed to support any of the hypotheses presented, several conclusions can be drawn.

First, a significant group by time effect was observed for plasma TNF-α, with values in the dairy group increasing post-supplementation and then dropping through the post-exercise period, while TNF-α values for the soy group appeared to stay more consistent. Second, significant contrasting time effects were observed for plasma SOD and IL-6, with elevations noted in IL-6 and decreases noted in SOD post-exercise. Third, significant time effects were observed for muscle IL-6 and IL-1β gene expression, with a decrease in values for both groups from baseline to 24 hours post-exercise. Lastly, two trends toward significant group effects were observed for muscle COX-2 and TNF-α

gene expression with the soy group values being slightly higher than the dairy group values at all time points. Plausible explanations for these findings in light of related studies in the literature will be the focus of the following sections.

Plasma Markers of Oxidative Stress

In 1956, Harman proposed that ROS formed during normal oxygen metabolism induce macromolecular damage (Harman, 1956) and that accumulation of such products of oxidative damage as we age accounts for the progressive deleterious changes in our body. Half a century later, his supposition still stands, as generation of ROS has been shown to cause EC apoptosis, increase monocyte adhesion, and play a role in angiogenesis (Taniyama et al., 2003), all of which are implicated in the development of CVD. Further, mitochondria obtained from aged muscle fibers display several functional abnormalities including increased proteolysis, ROS overproduction, and vulnerability to apoptosis (Martin et al., 2007; Marzani et al., 2005), contributing to sarcopenic risk. In the body, the activity of certain antioxidant enzymes [e.g., superoxide dismutase (SOD), glutathione peroxidase (GPx), cycoloxygenase-2 (COX-2)] can be assessed as indicators of the oxidative stress imposed on the tissue.

The present study failed to locate a significant effect of soy supplementation on any of the plasma antioxidant enzymes assessed. However, a time effect for SOD was noted in which levels of SOD were shown to decrease four hours post-exercise and return to baseline values two days later. This is contrary to what may be expected, as SOD generation is thought to increase during times of physical stress, like downhill running (Hollander et al., 2001). However, based on the increases in plasma IL-6 and responses to the DOMS questionnaires, it is believed that an inflammatory state was

induced, and the decrease in the marker may be due to a collective protective effect of the supplements, measurement bias, or inherent variability in measuring SOD expression.

First, there may have been a protective trend, with both the soy and dairy milk products aiding in the conversion of ROS to more inert byproducts, thereby decreasing the need (and subsequent production) of systemic SOD. From a dietary standpoint, similarities do exist between the soy and dairy milk products, including their carbohydrate, calcium, and vitamin D composition, all of which may confer benefit. In a recent animal study by Itoh (Itoh et al., 2004), dietary calcium restriction was shown to significantly up-regulate the activities of manganese-superoxide dismutase (Mn-SOD) and copper zinc-superoxide dismutase (Cu-Zn-SOD). Further, acute exercise, in addition to calcium restriction, decreased both SOD isoenzymes in the diaphragm of calcium restricted rats. Thus, it is speculative that in the present study, because of the calcium loading associated with consumption of both beverages, SOD activity may have been suppressed, which would explain the observed results. (However, because of the marked difference in the types of samples being assayed, a note of caution is warranted when extrapolating results to human plasma.) Because a placebo group was not run along side of the dairy and soy groups, it is not possible to infer the exact reason for the time effect; although, it is interesting to speculate that an unaccounted for dietary factor may be involved.

Second, due to the influence of sampling time, duration, and intensity of exercise on the expression of certain antioxidant enzymes, the critical window for observing the hypothesized rise in plasma antioxidant enzyme levels may have been "missed". Recent

studies have shown that the antioxidant defense system may indeed be reduced temporarily in response to increased ROS production, but may increase during the recovery period as a result of the initial pro-oxidant insult (Watson et al., 2005). Moreover, it is possible that the up-regulation of a different inflammatory or oxidative stress marker may be blunting the expected rise in SOD activity, although this supposition is quite speculative.

Lastly, the large variability in the expression of markers assessed in our study may have contributed to this counter-intuitive finding. Conflicting findings have been reported with regard to SOD, with investigators noting increases (Buczynski, Kedziora, Tkaczewski, & Wachowicz, 1991; Chen, Hsu, & Lee, 1994), decreases (Tozzi-Ciancarelli, Penco, & Di Massimo, 2002), and no change (Elosua et al., 2003) post-exercise. (Similar findings have also been reported for GPx (Akova et al., 2001; Buczynski et al., 1991; Laaksonen et al., 1999; Miyazaki et al., 2001; Vider et al., 2001), and at the time of this investigation, no studies were identified that measured plasma COX-2 expression post-exercise in humans.) Repeated use of this study design with a larger sample size may yield more conclusive results.

In the only comparably designed study, Chen et al. (Chen et al., 2004) found that isoflavone supplementation significantly increased pre-exercise erythrocyte SOD activity and prevented the exercise-induced decrease in activity of GPx. Authors concluded that results suggest that isoflavones can restore the altered redox homeostasis of antioxidant enzymes due to exercise (Chen et al., 2004). While it is unfortunate that the present results do not support the findings by Chen et al., differences in the

intervention product (isolated soy isoflavone versus whole soyfood), isoflavone dosage (150 mg/d versus ~100 mg/d), and exercise modality (cycling versus downhill running) may explain the differences.

Plasma Markers of Inflammation

The cytokines IL-1β, IL-6 and TNF-α are pleiotropic molecules that play major roles in the inflammatory process (Bradley, 2008; Trikha, Corringham, Klein, & Rossi, 2003) and have been linked to cardiovascular morbidity (Mendall et al., 1997; Ridker, Hennekens, Buring, & Rifai, 2000; Ridker et al., 2000) and mortality (Volpato et al., 2001). There is a notion that soyfoods, in part because they contain isoflavones, exert lipid-independent vascular benefits. In vitro and some animal data indicate one such benefit may be a decrease in circulating levels of cytokines and soluble adhesion molecules, elevated levels of which are associated with development of atherosclerosis (Blann, Faragher, & McCollum, 1997; Shai et al., 2006; Volpato et al., 2001). The inflammatory cytokines are known to induce the expression of cellular adhesion molecules, which mediate the adhesion of leukocytes to the vascular endothelium, thus initiating the cascade of the atherosclerotic process (Willerson & Ridker, 2004).

Two recent studies that support the effectiveness of soy consumption on the modulation of pro-inflammatory cytokine expression were performed by Azadbakht and Jenkins. In a crossover study by Azadbakht et al. (Azadbakht et al., 2007), 42 post-menopausal women with metabolic syndrome consumed in random order one of three diets: the Dietary Approaches to Stop Hypertension (DASH) diet, the DASH diet in which one serving of red meat was replaced with soy flour, or one in which one serving was replaced by soy nuts. The soy flour and soy nuts provided 15 and 11 g protein and

about 50 and 60 mg isoflavones, respectively. Final TNF-α, but not IL-6 levels were significantly lower in the soy nut group compared to the soy protein and control groups ($p < 0.01$), however, the actual decrease (~13%) from baseline in the soy nut group was almost identical to the decrease in the control group. Furthermore, in direct contrast to these findings are those from a four week study by Jenkins et al. (Jenkins et al., 2002), in which the effects of high and low isoflavone-containing soyfoods on pro-inflammatory cytokines were assessed in hypercholesterolemic men and women. Although there was no treatment effect on IL-6 or TNF-α, a significant interaction was noted between diet and gender. In women on the high isoflavone diet (73 vs. 10 mg/day), final serum IL-6 values were significantly higher than the control group ($p = 0.013$). Although contrary to what one might have expected, it is important to note that IL-6 can have both pro- or anti-inflammatory actions, and thus increases in this cytokine may actually have a protective effect, especially with regard to certain cancer development (De Benedetti et al., 1997; Smith, Gunnell, & Holly, 2000).

In the present study, a significant group by time interaction was observed for TNF-α and a time effect was observed for IL-6. With regard to TNF-α, post-supplementation values were found to be elevated in the dairy group compared to baseline, followed by a decrease in mean values to T4, and a return to post-supplementation values for the dairy group by T5. The soy group on the other hand, did not appear to fluctuate much from baseline in the post-supplementation or post-exercise period. Thus, it appears that with regard to this pro-inflammatory cytokine, of which

elevations are thought to promote chronic disease, the soy group may have fared better than the dairy group; although, further exploration of this conjecture is certainly warranted.

Although a significant group by time interaction was not observed for plasma IL-6, a marked increase in post-exercise IL-6 concentrations (specifically T3-T4) was observed for both groups, suggesting that the exercise bout was successful in inducing inflammation. No time or group trends were observed for plasma IL-1β; however, it has been clearly shown that IL-6 rises in accordance with both IL-1β and TNF-α, as each stimulates the synthesis and release of IL-6. Furthermore, the synthesis of IL-6 has been shown to dampen the expression of IL-1β and TNF-α (Barton, 1997). As a result, it is speculative that there may have been a time effect for IL-1β; although, the time points from which the samples were obtained may not have corresponded with peak expression of this biomarker relative to IL-6.

Overall, the plasma cytokine analysis results for the present study add to a mixed body of literature that suggests the potential of soyfoods to influence pro-inflammatory cytokine expression; although, clearly more research is needed. Future studies should address potential confounding factors such as the type of materials used for intervention, dosage, serum isoflavone level, and subject characteristics such as genotype and inflammatory status in their methodology, as such factors have been identified as contributing to the equivocal findings in the literature (Beavers, Jonnalagadda, & Messina, 2009).

Skeletal Muscle Markers of Inflammation

The cytokines IL-1β, TNF-α, and IL-6, mediate a variety of host responses to trauma and infection, including skeletal muscle proteolysis (Cannon et al., 1991). In acute illness IL-1β and TNF-α expression has been shown to considerably alter cellular metabolism. Such effects include increased secretion of insulin and counter-insulin hormones, gluconeogenesis, protein breakdown, hepatic glucose production, and expression of transcription factors, such as NF-κB, known to down-regulate myogenic regulatory factors and up-regulate the ubiquitin proteolytic system **(**Remick & Friedland, 1997). Moreover, the activation of these pro-inflammatory cytokines is also believed to induce oxidant imbalance (Mantovani et al., 2004). Although ROS generation can be beneficial, as they are used by the immune system as a way to attack and kill pathogens, when produced in excess they damage all components of the cell, including proteins, lipids, and DNA. Locally, an increase in ROS directly translates into an increase in protein degradation and a decrease in protein synthesis.

The present study did not show a significant group by time interaction for any of muscle markers assessed. However, a surprising time effect was noted for IL-6 and IL-1β, in which a decrease in the gene expression of these inflammatory cytokines was observed post-exercise (T2 to T3), with a subsequent return to baseline value observed for both groups by T4. Typically, IL-6 released from muscle increases up to 100-fold during exercise, and its production has been shown to result in increased anti-inflammatory cytokine expression (IL-1ra and IL-10), but decreased TNF-α and IL-1β production (Hoene & Weigert, 2008; Pedersen, Akerstrom, Nielsen, & Fischer, 2007). However, recent studies have shown an apparent paradoxical relationship between the

systemic and local expression of IL-6. Specifically, data have shown that higher systemic levels of IL-6 result in a down-regulation of locally expressed IL-6 in muscle (Serrano, Baeza-Raja, Perdiguero, Jardi, & Munoz-Canoves, 2008). Biologically this may occur as the stimulation of the IL-6 receptor is known to activate the Janus kinase signal transducer and its subsequent signaling pathway. This activation can then induce the suppressor to cytokine signaling protein, which will subsequently inhibit IL-6 expression (Jones, Richards, Scheller, & Rose-John, 2005; Kamimura, Ishihara, & Hirano, 2003). Therefore, it is possible that the blunting of skeletal IL-6 gene expression observed in this study may have been due to the drastic up-regulation of IL-6 systemically.

Additionally, expression of systemic IL-6 has been shown to dampen systemic IL-1β and TNF-α expression. Specifically, the administration of IL-6 in humans has been shown to cause the induction of the soluble receptors IL-1ra and p55 that inhibit IL-1β and TNF-α activity (Barton, 1997). Although speculative, this knowledge coupled with the observation that systemic IL-6 up-regulation paradoxically down-regulates local IL-6 expression, may also explain the reduction in local IL-1β observed in this study. That is, elevations in systemic IL-6 may be blunting local IL-1β. Lastly, because IL-6 released from muscle has been shown to result in decreased TNF-α and IL-1β production (Hoene et al., 2008; Pedersen et al., 2007), perhaps due to the timing of muscle sampling, elevations in IL-1β (and possibly TNF-α) were missed.

Finally, much like SOD, the blunting in IL-6 and IL-1β may be due to a protective, confounding dietary component or interaction of components found in both nutritional supplements. In a recent study by Steensburg, authors found that in post-

exercise muscle samples, those with the lowest glycogen content expressed the highest levels of IL-6 mRNA (Steensberg et al., 2001). Therefore, if we can assume the converse to be true, the purported elevated carbohydrate ingestion observed in both groups in this study may have blunted local IL-6 production. Further, diets high in calcium have been shown to suppress adipose tissue IL-6 mRNA expression (Zemel & Sun, 2008), and this finding may be applicable to muscle gene expression of the cytokine. Unfortunately, without a placebo group, we are unable to ascertain if both nutritional interventions were protective of the induction of these enzymes, and future research is needed to elucidate this supposition.

It is worth noting that despite our randomization scheme, it appears that the soy group entered into the study with marginally elevated local levels of COX-2 and TNF-α, as evidenced by the marginal group effects observed for both markers at baseline and the continued elevations of these markers throughout the exercise sampling periods. This roughly correlates to the elevation in DOMS values reported by the soy group as well. While such discrepancies may have caused a "blunting" of the dietary treatment effect in the soy group, given the marginal significance of these findings, the large number of variables assessed, and the significant intra-individual variability within these measures, the most likely explanation for this finding can be attributed to type one error.

Conclusion and Future Directions

Overall, the present study does not support the notion that four weeks of daily soy milk ingestion can attenuate systemic or local elevations in markers of oxidative stress or inflammation. However, data do suggest that the downhill running protocol utilized in this study can be effective at altering systemic expression biomarkers

associated with oxidative stress and inflammation (specifically SOD, TNF-α, and IL-6), and that ingestion of soy may help to maintain plasma TNF-α levels when exposed to a stress inducing stimulus; although, more data exploring this supposition is certainly warranted.

This study significantly contributes to the literature, as it is one of a handful of studies to assess the effect of whole soyfood consumption on markers of inflammation and oxidative stress in an older population. Further, it is also novel in its exercise design which is the first to utilize a downhill running model in post-menopausal women to elicit an inflammatory response. Future studies should address potential confounding dietary factors by utilizing an inert control group to determine if a different nutritional compound found in both soy and dairy milk could be the protective agent with regard to exercise induced inflammation and oxidative stress. Additionally, future studies may want to screen individuals based on baseline inflammatory status, as conceivably, in order to observe a statistically significant decline in inflammatory markers post-supplementation, these markers would need to be elevated at baseline.

Despite the subdued findings in this investigation, it is well known that the inclusion of soyfoods in a Western diet provides an excellent means by which to displace foods high in saturated fat. Taken together with established modest reductions in cholesterol and the potential for improvement in systemic cytokine expression, results from this study still warrant inclusion of soyfoods in a heart-healthy diet.

APPENDICES

APPENDIX A

Personal Demographic Form

Baylor University
Exercise and Sport Nutrition Laboratory

Personal Information

Name:

Address:

City: _____ State: _____ Zip Code _____

Home Phone: (___) _____ Work Phone: (___)_____

Beeper: (___) _____ Cellular (___) _____

Fax: (___) _____ email address: _____

Birth date: _____ / _____ / _____ Age: _____ Height: _____ Weight: _____

Exercise & Supplement History/Activity Questionnaire

1. Describe your typical occupational activities.
2. Describe your typical recreational activities
3. Describe any exercise training that you routinely participate.
4. How many days per week do you exercise/participate in these activities?
5. How many hours per week do you train?
6. How long (years/months) have you been consistently training?
7. When was the last time you ingested an anti-inflammatory medication or supplement?
8. What was the reason you were taking an anti-inflammatory product?

APPENDIX B

Medical History Form

Directions. The purpose of this questionnaire is to enable the staff of the Exercise and Sport Sciences Laboratory to evaluate your health and fitness status. Please answer the following questions to the best of your knowledge. All information given is CONFIDENTIAL as described in the Informed Consent Statement.

Name:_____ Age _____Date of Birth_____
Name and Address of Your Physician:_____

MEDICAL HISTORY

Do you have or have you ever had any of the following conditions? (Please write the date when you had the condition in blank).

____	Heart murmur, clicks, or other cardiac findings?	____	Asthma/breathing difficulty?
____	Frequent extra, skipped, or rapid heartbeats?	____	Bronchitis/Chest Cold?
____	Chest Pain of Angina (with or without exertion)?	____	Melanoma/Suspected skin Lesions?
____	High cholesterol?	____	Stroke or Blood Clots?
____	Diagnosed high blood pressure?	____	Emphysema/lung disease?
____	Heart attack or any cardiac surgery?	____	Epilepsy/seizures?
____	Leg cramps (during exercise)	____	Rheumatic fever?
____	Scarlet fever?	____	Ulcers?
____	Chronic swollen ankles?	____	Varicose veins?
____	Frequent dizziness/fainting?	____	Pneumonia?
____	Muscle or joint problems?	____	Anemias?
____	High blood sugar/diabetes?	____	Liver or kidney disease?
____	Thyroid Disease?	____	Psychological Disorders?
____	Autoimmune disease?	____	Low testosterone/hypogonadism?
____	Gluacoma	____	Nerve disease?

Do you have or have you been diagnosed with any other medical condition not listed?
_____

Please provide any additional comments/explanations of your current or past medical history.
_____

Please list any recent surgery (i.e., type, dates etc.).
_____

List all prescribed/non-prescription medications (i.e. hormone replacement therapy) and nutritional supplements you have taken in the last 3 months.

What was the date of your last complete medical exam?

Do you know of any medical problem that might make it dangerous or unwise for you to participate in this study? (Including strength and maximal exercise tests) ____ If yes, please explain:

Recommendation for Participation
____ No exclusion criteria presented. Participant is *cleared* to participate in the study.
____ Exclusion criteria is/are present. Participant is *not cleared* to participate in the study.
Signed: _____ Date: _____

APPENDIX C

Informed Consent

BAYLOR UNIVERSITY

Department of Health, Human Performance, & Recreation
Informed Consent Form

Title of Investigation: Effects of Four Weeks of Daily Soy Milk or Dairy Milk Ingestion on the Exercise-Induced Inflammatory and Proteolytic Responses in Plasma and Skeletal Muscle in a Post-Menopausal Female Population

Principal Investigator: Darryn S. Willoughby, Ph.D., FACSM, FISSN
Associate Professor, Department of HHPR,
Baylor University

Co-Investigators: Richard B. Kreider, PhD, FACSM, FISSN
Professor and Chair, Department of HHPR,
Baylor University

Matt Cooke, Ph.D.
Assistant Professor, Department of HHPR,
Baylor University

Kristen M. Beavers, MPH, RD
Department of HHPR, Baylor University

Monica C. Serra, MS, RD, ATC
Department of HHPR, Baylor University

Mark J. Messina, Ph.D.
Associate Professor, Loma Linda University

Ronald Wilson, M.D.
Exercise and Sport Nutrition Lab, Baylor University

Sponsor: WhiteWave Foods Company (Broomfield, CO).

Rationale:

Aging is associated with oxidative stress (small molecules, which can result in significant damage to cell structures) and local inflammation in skeletal muscle. This may lead to cellular dysfunction and muscle protein breakdown, as well as a decline in muscle mass and function.

Supplementation of whey (found in dairy foods) and soy proteins have been shown to play a role in the prevention of skeletal muscle wasting, frequently seen as a result of aging. Further, the added antioxidant components (i.e. isoflavones) found in soy are speculated to provide additional health benefits above and beyond whey. In clinical settings, exercise can be used to bring about oxidative stress, inflammation, and subsequent muscle breakdown.

The purpose of the proposed study is to: 1) to assess the effects of the daily consumption of soy milk or dairy milk for four weeks in aging females on plasma markers of oxidative stress and inflammation, and markers of local oxidative stress, inflammation, and muscle breakdown in skeletal muscle and 2) to assess the effects of the daily consumption of soy milk or dairy milk for four weeks in aging females on plasma markers of oxidative stress and inflammation and markers of local oxidative stress, inflammation, and muscle protein breakdown in skeletal muscle after a single session of downhill running.

Description of the Study:

I will be one of 30 apparently healthy, post-menopausal, physically active, but not trained (not engaged in an exercise program involving either resistance or endurance training at least thrice weekly for one year) females between the ages of 40-60 not undergoing hormone replacement therapy (either pharmacological or soy-based nutritional supplements) recruited to participate in the proposed study. I understand that I must first obtain medical clearance from my personal physician prior to participating in baseline assessments. During an initial familiarization session, I will be informed of the requirements of the study and sign an informed consent statement in compliance with the Human Subjects Guidelines of Baylor University and the American College of Sports Medicine. A trained individual will examine me to determine if I am qualified to participate in this study. If I am cleared to participate in the study, I will be familiarized to the testing procedures. I will report my physical activity over the past week as well as everything I have had to eat over the past 24 hours, and then be prepared to perform a maximal treadmill test. I will have my right and left shoulder, right and left part of my stomach, and several places around my upper chest and below my bra line rubbed with alcohol gauze. I understand that 10 electrocardiograph (ECG) electrodes will then be placed on my shoulders, chest, and stomach and that I will be attached to an ECG to evaluate my heart. I understand that I will then be positioned on the treadmill and a sterile mouthpiece will be placed in my mouth and a mouthpiece holder will be placed on my head. I understand that a nose clip will be placed on my nose and that the air I breathe will be measured for oxygen and carbon dioxide content. Once the equipment is attached, I will be given instructions to begin walking on the treadmill. I will then perform an exercise test that involves increasing the speed and grade I am walking on the treadmill until I reach my maximal effort. I understand that heart rate, ECG tracings, blood pressure and my ratings of exertion will be monitored throughout the test. Once I reach my maximum, I understand that I will undergo a slow walking and seated recovery period. This test will take about 30 minutes to complete. The goal of the treadmill protocol is to determine my VO_{2max}, a value that reflects my physical fitness level. The entire familiarization session will take approximately 45 minutes to complete. Once I complete the familiarization session, I will be matched based on physical activity level and baseline protein intake and randomly placed into one of two groups involving soy milk or dairy milk under single-blind conditions. All supplements will be provided in shelf-

stable containers. I will be asked to drink one cup of milk three times a day for a total of four weeks and will then be scheduled for follow-up testing.

During the four-week supplementation period I will not be allowed to change my normal exercise routine. In addition, I will be required to avoid soy-containing foods and will limit dairy-containing foods to two servings per day. I understand that my compliance in taking the supplements will be monitored by completing a supplementation frequency questionnaire daily. I understand that if I do not take my supplements I will be removed from the study.

I understand that during the supplementation period my dietary intake will not be supervised; however, I will record my dietary intake three times for four days during my supplementation period. I also understand that I will be required to report to the laboratory after the first two supplementation weeks to pick up my supplement and complete a report of side effects from supplementation questionnaire to determine if I have experienced any unexpected problems or adverse events from participating in this study. I understand that if clinically significant side effects are reported, I will be referred to discuss the problem with Darryn Willoughby, Ph.D. or Matt Cooke, Ph.D. Upon their discretion, I may be referred to discuss the matter with the ESNL physician, Dr. Ronald Wilson to determine whether any medical treatment is needed and/or whether I can continue in the study. I understand that if I fail to report my progress and health status to the research assistant I may be removed from the study.

Following the supplementation period, I will be instructed to refrain from exercise for 48 hours and fast for 12 hours prior to follow-up testing. For 48 hours prior to reporting to the lab for follow-up testing, I will record all food that I eat on dietary record forms. In addition, I will record my diet for four days prior to follow-up testing. Once I report to the lab, I will be weighted and I understand that I will then donate about three to four teaspoons of venous blood from a vein in my arm. Blood samples will be obtained using standard/sterile procedures using a needle inserted into a vein in my arm. I understand that personnel who will be taking my blood are experienced in phlebotomy (procedures to take blood samples) and are qualified to do so under guidelines established by the Texas Department of Health and Human Services. This will take about five minutes.

After the blood draw, I will then be prepared for the muscle biopsy. I understand that I will have the biopsy location identified on the thigh of my leg. The biopsy area will be cleaned with rubbing alcohol. In addition, the biopsy site will be further cleansed by swabbing the area with Betadine (fluid antiseptic). I understand that a small area of the cleaned skin approximately two cm in diameter will be anesthetized with a 0.5 mL subcutaneous injection of the topical anesthetic Xylocaine. Once the local anesthesia has taken effect (approximately two minutes) the biopsy procedure will only take 15-20 seconds. Once anesthetized, I understand that a 16-gauge fine needle aspiration biopsy will be inserted into my skin at an approximate depth of one cm to extract the muscle sample. Due to the localized effects of the anesthetic, I should feel no pain during this process; however, I should feel a pressure sensation. Once the muscle sample has been obtained, pressure will be immediately applied to the biopsy location. Due to the small puncture hole, only a standard adhesive bandage will be used to close and cover the biopsy site. I understand that I will be provided verbal and written instructions for post-biopsy care. I understand that I will have my biopsy locations inspected for infection and proper healing when I return for subsequent testing. I will be further

advised to refrain from vigorous physical activity with my leg during the first 24 hours post-biopsy. I understand that if I feel it necessary, I may take a non-prescription analgesic medication such as Acetaminophen to relieve pain if needed and that some soreness of the area may occur for about 24 hours after the biopsy. I will also be advised to avoid such medications such as aspirin, Advil, Bufferin, or Nuprin, as they may lead to bruising at the biopsy site.

Next, I will begin a downhill running treadmill protocol. The 45 minute running exercise will be preceded by a five minute walking warm-up on the -10% downhill treadmill. After the warm-up, I will be instructed to run at 60% of my VO_{2max} for 45 minutes. Between 15 and 18 minutes and 27 and 30 minutes of exercise, VO_2 and heart rate will be recorded. After the exercise session, I will donate blood, receive a small breakfast, and will remain rested until a four hour blood sample and muscle biopsy is received. I will donate another blood sample 24 and 48 hours after the treadmill protocol and another muscle biopsy after 24 hours.

I agree to do my best to: 1) follow the instructions outlined by the investigators; 2) show up to all scheduled testing times; 3) take the supplements as instructed; and 4) attempt to avoid consuming foods with high amounts of isoflavones. I agree not to take any nutritional supplements (such as Vitamins C and E, EPA/DHA, EGCG) during this study that may affect my antioxidant status. In addition, I agree not to take any non-medically prescribed medications and to report any medication that is prescribed for me to take during this study. I understand that if I take any other nutritional supplements or medications during the course of the study that may affect oxidative stress or inflammation levels that I may be removed from the study.

Exclusionary Criteria

I understand that in order to participate in the study, a trained individual will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if I 1.) am unable to follow the study protocol 2.) do not have my primary care physicianôs approval to participa exercise program involving either resistance or endurance training at least thrice weekly for one year) 4.) have engaged in hormone replacement therapy (either pharmacological or soy-based) for up to three months prior to the study 5.) have any known bleeding or metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism 6.) have a history of pulmonary disease, hypertension, hepatorenal disease, musculoskeletal disorders, neuromuscular/neurological diseases, autoimmune disease, chronic infection diseases (e.g., hepatitis or HIV), cancer, peptic ulcers, or anemia 7.) am taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (e.g, thyroid, insulin, etc), psychotropic, neuromuscular/neurological, or androgenic medications 8.) have taken nutritional supplements that may affect antioxidant status (e.g., Vitamins C and E, EPA/DHA, EGCG) within three months prior to the start of the study 9.) have smoked cigarettes within the past three years or have an average intake of two or more alcoholic drinks/day 10.) have any absolute or relative contraindication for exercise testing or prescription as outlined by the American College of Sports Medicine 11.) report any unusual adverse events associated with this study that in consultation with the supervising physician recommends removal from the study 12.) have known food allergies, allergies to anesthesia medications, or lactose intolerance.

I have reported all nutritional supplements, medically prescribed drugs, and non-medically prescribed drugs that I am presently taking. I have reported whether I have had any prior allergic reactions to topical anesthetics. I have completed medical history questionnaires and am not aware of any additional medical problems that would prevent me from participating in this study. I agree to report all changes in medical status, nutritional and/or pharmacological agents (drugs) that I take during the course of the investigation to Darryn Willoughby, Ph.D. (254-710-3504), Matt Cooke, Ph.D. (254-710-4025), Monica Serra, M.S. or Kristen Beavers, M.P.H. (254-710-3243). I understand that if I experienced any unexpected problems or adverse events from participating in this study I may be referred to discuss the problem with either Dr. Willoughby or Dr. Cooke. Upon their discretion, I may be referred to discuss the matter with Dr. Ronald Wilson to determine whether any medical treatment is needed and/or whether I can continue in the study.

Risks and Benefits

I understand that milk supplements, such as those to be investigated in this study, have been studied for various medical uses in humans and that research has demonstrated that moderate oral administration is not associated with any significant medical side effects. As with any food or nutritional supplement, possible side effects may include stomach upset, gastrointestinal distress, allergic reactions, changes in mood and vigor, and/or changes in training adaptations.

On six separate occasions during this study, I understand that I will have about three to four teaspoons of blood drawn from a vein in my forearm using a sterile needle and blood tubes by an experienced phlebotomist following a 12 hour fast. This procedure may cause a small amount of pain when the needle is inserted into my vein as well as some bleeding and bruising. I may also experience some dizziness, nausea, and/or fainting if I am unaccustomed to having blood drawn.

On four separate occasions during this study I understand that I will undergo a muscle biopsy in which a small sample of muscle will be obtained from the thigh of my dominant leg. I understand that all muscle biopsies will be performed by either Darryn Willoughby, Ph.D. or one of Dr. Willoughbyôs trained doct anesthetic (Xylocaine) will be injected into the skin of my thigh prior to the biopsy which will help prevent any pain and discomfort during the procedure. I understand that a 16-gauge fine aspiration biopsy needle will puncture my skin and then introduced one cm into my thigh. I also understand that this puncture will be so small that it will not require any stitches and will be simply closed with a standard adhesive bandage (band-aid). After the anesthetic wears off within two to three hours, I understand that the sensation at the biopsy site is comparable to that of a bruise and may persist for approximately 24 hours after the procedure. I understand that I am required to inform the study investigators if I have had any prior allergic reactions to anesthesia (e.g. while in the hospital or during a dental visit).

As a result of the exercise protocol, I understand that I will most likely experience short-term muscle fatigue. In addition, I am likely to experience muscle soreness in my thigh area for up to 24 to 48 hours after exercise. This soreness is normal and should be commensurate with the type of muscle soreness I may have felt after doing unaccustomed exercise or physical activity. I understand that if I feel it necessary I may take a non-prescription analgesic medication such as Tylenol to relieve pain if needed. I

also understand that muscle strains/pulls resulting from the eccentric exercise protocol are possible. However, potential injury due to exercise will be minimized by the study investigators ensuring that I adhere to correct exercise technique.

I understand that the main benefit that may be obtained from this study is to determine whether providing four weeks of oral supplementation with either soy milk or dairy milk is effective at reducing inflammation, oxidative stress, and muscle injury and soreness that typically result from downhill running exercise and the aging process. I also understand that all of my analyzed muscle and blood samples will be discarded in an appropriately-labeled biohazard waste disposal container.

Alternative Treatments

This is not a medical treatment. Therefore, if medical treatment is needed, I must continue to obtain treatment for any medical problem I might have from my personal physician.

Costs and Payments

If I am a Baylor University student, I will not receive any academic credit for participating in this study. Eligible participants will be paid $150 for completing the familiarization and experimental testing sessions. I also understand that I will be given free blood assessments during the course of the study as described above and may receive information regarding results of these tests if I desire.

New Information

Any new information obtained during the course of this research that may affect my willingness to continue participation in this study will be provided to me. In addition, I will be informed of any unusual/abnormal clinical findings in which medical referral to my personal physician may be warranted. If I desire, I may request that this information be provided to my physician.

Confidentiality

I understand that any information obtained about me in this research, including questionnaires, medical history, laboratory findings, or physical examination will be kept confidential to the extent permitted by law. However, I understand in order to ensure that FDA regulations are being followed, it may be necessary for a representative of the FDA to review my records from this study which may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken about my participation in this study. In addition, I understand that my records of this research may be subpoenaed by court order or may be inspected by federal regulatory authorities. I understand that data derived may be used in reports, presentations, and publications. However, I will not be individually identified unless my consent is granted in writing. Additionally, confidentiality will be maintained by assigning code numbers to my files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. I understand that once blood samples are analyzed that they will be discarded.

Right to Withdrawal

I understand that I am not required to participate in this study and I am free to refuse to participate or to withdraw from the study at any time. Further, my decision to withdraw from the study will not affect my care at this institution or cause a loss of benefits to which I might be otherwise entitled. If there is concern about my medical safety, I may be referred to seek medical attention.

Compensation for Illness or Injury

I understand that if I am injured as a direct result of taking part in this study, I should consult my personal physician to obtain treatment. I understand that the cost associated with the care and treatment of such injury will be the responsibility of me or my insurance carrier. In some cases, insurers may not reimburse claims submitted for a research-related injury resulting from medical procedures or treatments performed as part of a research study. I understand that Baylor Uni the grant sponsor have not budgeted funds to compensate me for injury or illness that may result from my participation in this study and thus will not be accountable for illness or injury acquired during the course of this study. However, I may be referred to my personal physician if any clinically significant medical/psychological findings are observed during the course of this study.

I agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage or loss I suffer as a result of my participation in this study regardless of the cause of my injury, damage or loss.

Statement on Conflict of Interest

I understand that funding for this study was obtained in part from WhiteWave Food Company (Bloomfield, CO) through a grant awarded to Baylor University. I understand that researchers involved in collecting data in this study have no financial or personal interest in the outcome of results.

Voluntary Consent

I certify that I have read this consent form or it has been read to me and that I understand the contents and that any questions that I have pertaining to the research have been, or will be answered by Darryn Willoughby, Ph.D. (Associate Professor, Department of Health, Human Performance & Recreation, 120 Marrs McLean Gymnasium, Baylor University, phone: 254-710-3504), Matt Cooke, Ph.D. (254-710-4025), Monica Serra, M.S. or Kristen M. Beavers, M.P.H. (Doctoral Research Assistant, Department of Health, Human Performance & Recreation, 123 Marrs McLean Gymnasium, Baylor University phone: 254-710-3243) or one of the other research associates. My signature below means that I am at least 18 years of age and that I freely agree to participate in this investigation. I understand that I will be given a copy of this consent form for my records. If I have any questions regarding my rights as a research subject in this study, I may contact Baylor's University Committee for Protection of Human Subjects in Research. The chairman is Dr. Matt Stanford, Professor, Department of Psychology and Neuroscience, P.O. Box 97334, Waco, TX 76798-7334, phone number 254-710-2961.

Date _____ Participant's Signature _____

I certify that I have explained to the above individual the nature and purpose of the potential benefits and possible risks associated with participation in this study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

Date _____ Investigator's Signature _____

APPENDIX D

IRB Proposal

Application to the Baylor IRB
For Review of Research/Activity Proposal

Part 1: Signature Page

1. Name Darryn S. Willoughby, PhD, FACSM, FISSN, CSCS, CISSN
2. Email Address (optional) Darryn_Willoughby@baylor.edu
3. Complete Mailing Address P.O. Box 97313
4. Position Associate Professor
5. Faculty Advisor (if researcher is Graduate Student) _____
6. Department/School HHPR/SOE
7. Telephone # x3504 FAX # x3527
8. Are you using subjects in research (Y or N) Y or in teaching exercises (Y or N)?
9. Title of the research project/teaching exercise:

   Effects of Four Weeks of Daily Soy Milk or Dairy Milk Ingestion on the Exercise-Induced Inflammatory and Proteolytic Responses in Plasma and Skeletal Muscle in a Post-Menopausal Female Population

10. Please return this signed form along with all the other parts of the application and other documentation to the University Committee for Protection of Human Subjects in Research; Dr. Matt Stanford, Chair, Department of Psychology and Neuroscience, Baylor University, P.O. Box 97334, Waco, Texas 76798-7334. If you have questions, or if you would like to see a copy of the OHRP Report on protection of human subjects in research, contact Dr. Stanford at extension 2961.

Signature of Principal Investigator

12/15/06

Date

Signature of Faculty Advisor (required if researcher is a Graduate Student)

Departmental Review: _____
Department Chair or the Chair's Designate

Part 2: Introduction & Rationale

Aging is associated with oxidative stress and subsequent local inflammation in skeletal muscle. Oxidative stress, by way of increased free radical generation, causes oxidative modification and damage to protein, lipid, and DNA in skeletal muscle. This invariably leads to cellular dysfunction and muscle protein degradation, as well as a decline in muscle mass and function. Loss of muscle strength has been associated with increased risk of frailty, disability, and mortality, (Beck et al., 2007) and has been implicated in the pathogenesis of sarcopenia (Howard et al., 2007); a process that can begin as early as the fourth decade of life (Lindle et al., 1997). While the mechanisms responsible for these changes remain to be clearly defined, dysregulated oxidative stress and inflammatory processes are known to be significantly involved. Elevated serum levels of certain cytokines, most notably interleukin-6 (IL-6), are increasingly present with advancing age, and to a greater extent with sarcopenia (Leng, Chaves, Koenig, & Walston, 2002). Further, the appearance of IL-6 and other inflammatory markers, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), has been associated with a number of adverse clinical outcomes including decreased strength and mobility, falls, and mortality (Ershler & Keller, 2000).

In an attempt to attenuate the age-related progression of oxidative stress, inflammation, and subsequent increases in muscle proteolysis, nutritional countermeasures such as antioxidants, protein, and amino acid supplementation are being studied. Of such, soy and whey proteins have been shown to play a role in the prevention of skeletal muscle wasting, frequently seen as a result of aging (Haub, Wells, Tarnopolsky, & Campbell, 2002). Numerous studies have compared soy and whey protein isolates on skeletal muscle anabolism (Anthony et al., 2007; Candow, Burke, Smith-Palmer, & Burke, 2006; Phillips, Hartman, & Wilkinson, 2005) and catabolism (Box, Hill, & DiSilvestro, 2005; Elia, Stadler, Horvath, & Jakus, 2006; Hill, Box, & DiSilvestro, 2004), as both provide a high biological value protein source to the body. In young men, consumption of milk protein after resistance training appears to promote greater lean mass accretion than soy (Hartman et al., 2007), however, in an aging population, data suggests that increases in muscle strength and size are not influenced by the predominant source of protein consumed, rather adequate total protein intake (Haub et al., 2002). Moreover, the added antioxidant components found in soy (i.e. isoflavones, saponins, and copper), are speculated to confer additional health benefits above and beyond whey.

In addition to the protein itself, soyfoods contain isoflavones, polyphenolic compounds that possess antioxidant properties (Ross & Kasum, 2002). Oxidative DNA damage in men and women has been shown to be decreased after isoflavone (Djuric, Chen, Doerge, Heilbrun, & Kucuk, 2001) and soy protein (Mitchell & Collins, 1999) supplementation.

In a recent study by Brown et al. (Brown, DiSilvestro, Babaknia, & Devor, 2004), the ingestion of soy and whey protein bars were found to promote exercise-induced lean body mass gain, but the soy had an added benefit of preserving antioxidant function. Further, in rats, the consumption of diets containing 20% by weight isolated soy protein suppressed exercised-induced activation of calpains, (proteases, which can degrade myofribrillar proteins) (Phillips et al., 2005). Additionally, due to the presence of trypsin inhibitors, such as Bowman-Birk inhibitor (BBI), soy may minimize protein degradation (Hegsted, Godber, Xu, & Losso, 2002). Unpublished studies from Louisiana State University have shown that BBI concentrations found in one cup of soy milk can prevent the activation of metalloproteinases, thereby preserving the integrity of cell membranes and overall cellular integrity (Hegsted et al., 2002). A recent study by Arbogast et al. (Arbogast et al., 2007) found that supplementing BBI in mice, that were undergoing oxidative stress through hindlimb unloading, resulted in the prevention of body weight and muscle atrophy loss, and increased force production in the unloaded muscle. In addition, results also indicated that BBI may have scavenged superoxide anion radicals, preventing oxidative stress in the muscle (Arbogast et al., 2007). There is a need for future research to address the potential synergistic effects that may exist between isolated soy constituents.

In clinical settings, exercise can be used to induce oxidative stress, inflammation, and subsequent muscle proteolysis. Studies have shown that as little as 30 minutes of strenuous exercise can lead to a significant elevation of biomarkers of oxidative stress in the serum, thereby eliciting an acute phase response (Beck et al., 2007; Bloomer, Goldfarb, Wideman, McKenzie, & Consitt, 2005). Such biomarkers, namely the cytokines IL-1, TNF-α, and IL-6, mediate a variety of host responses to trauma and infection, including skeletal muscle proteolysis (Cannon et al., 1991). To date, most of these studies have been performed in men (Chen, Bakhiet, Hart, & Holtzman G., 2004; Feasson et al., 2002; Peake et al., 2005) and further research is needed to elucidate the effects that exercise and nutritional supplementation have on oxidative stress, inflammation, and muscle proteolysis, in women.

The effects of soyfoods containing protein and phytoestrogens on attenuating systemic and local oxidative stress and inflammation, and minimizing muscle proteolysis in healthy aging women are not well delineated. Further, comparisons between soy milk, and dairy milk supplementation with regard to these outcomes are lacking in the literature, and favorable results could have important public health implications. Therefore, the overall purpose of the proposed study is to investigate the actions of different milk-beverages on plasma and skeletal muscle markers of inflammation and oxidative stress. This study has two specific aims. Firstly, to assess the effects of the daily consumption of soy milk or dairy milk for four weeks in aging females on plasma markers of oxidative stress and inflammation, and markers of local oxidative stress, inflammation, and proteolysis in skeletal muscle. And secondly, to assess the effects of the daily consumption of soy milk or dairy milk for four weeks in aging females on plasma markers of oxidative stress and inflammation and markers of local oxidative stress, inflammation, and proteolysis in skeletal muscle after a single bout of downhill running.

Part 3: Methodology
Methods

Participants

Thirty apparently healthy, aerobically fit, post-menopausal women between the ages of 40-60 will be used as participants in the study. An a priori power calculation shows that 15 participants per group are necessary to detect a significant difference between groups in markers of oxidative stress (SOD effect size of 0.8 U/g; (Chen et al., 2004)) and inflammation (IL-6 effect size of 0.24 pg/mL; (Jenkins et al., 2002)) given a type I error rate of 0.05 and a power of 0.80. All participants will be cleared for participation by passing a mandatory medical screening. Only participants considered as either low or moderate risk and with no contraindications to exercise as outlined in the American College of Sports Medicine (ACSM) guidelines will be eligible. All eligible participants will be asked to provide oral and written informed consent based on university approved documents. All supervised testing and training will be conducted under the direct supervision of Dr. Darryn Willoughby in the Exercise and Biochemical Nutrition Laboratory (EBNL) in the Department of Health, Human Performance, and Recreation at Baylor University, in Waco, TX. Exclusion criteria include inability to follow the study protocol, active use of hormone replacement therapy (HRT), and the consumption of any nutritional supplements (excluding multivitamins) three months prior to the study.

Study Site

All supervised testing and training will be conducted under the direct supervision of Dr. Darryn Willoughby in the Exercise and Biochemical Nutrition Laboratory (EBNL) in the Department of Health, Human Performance, and Recreation at Baylor University, in Waco, TX.

Independent and Dependent Variables

The independent variables will be the supplement group (soy milk or dairy milk), the four week supplementation period, and the sampling times during the course of this study. Dependent variables will include the information collected on the 24-hour recall and four day food records, international physical activity questionnaire (IPAQ), delayed onset muscle soreness (DOMS) scale, plasma markers of muscle damage (CK), myoglobin and oxidative stress (SOD), inflammation-responsive plasma markers [TNF-α, IL-1β, IL-6, SOD, glutathione peroxidase (GPx), cyclo-oxygenase-2 (COX-2)], skeletal expression of inflammation-responsive genes (TNF-α, IL-1β, IL-6, NF-KB, COX-2) and proteolytic genes (ubiquitin, atrogin-1, E2 ligase, E3 ligase, ring finger, calpain 1, calpain 2).

General Study Overview

After completing a baseline familiarization session and a two-week dietary washout period, participants will return to the ESNL during the morning hours for baseline testing. Participants will first be weighed, complete the IPAQ, and donate blood and muscle

sample. Then, participants will be asked to perform a graded exercise test to determine their VO_{2max}. After the testing session, in a single-blind manner, participants will be provided with their dietary supplement and instructions for following the supplement protocol. Briefly, during the supplementation period each participant will be required to consume three cups of either soy milk or dairy milk per day for four weeks. After the supplementation period, subjects will be asked to return to the laboratory on day 28 to perform a 45-minute eccentrically biased downhill run test at 60% of their predetermined VO_{2max}. Upon completion of the test, an immediate post-exercise blood draw will be obtained. Subjects will be asked to return to the ESNL at four, 24, and 48 hour post testing for blood (four, 24, and 48 hour) and muscle donation (four and 24 hour only). Participants will be encouraged to continue consuming their dietary supplement until the completion of the 48-hour post testing period.

Entry/Familiarization Session

Participants expressing interest in participating in this study will be interviewed on the phone to determine whether they appear to qualify to participate in this study. Participants believed to meet eligibility criteria then will be invited to attend an entry/familiarization session. Once reporting to the lab, participants will complete a medical history questionnaire and undergo a general physical examination to determine whether they meet eligibility criteria. Participants will be required to obtain medical clearance from their personal physician prior to participating in baseline assessments. Once medical clearance is obtained, participants will be familiarized to the study protocol via a verbal and written explanation outlining the study design. Eligible participants who agree to participate in the study will read and sign university-approved informed consent documents and be asked to complete a 24-hour recall dietary interview and IPAQ.

Baseline Cardiopulmonary Assessment

Prior to beginning the supplementation protocol, each participant will have her body weight, height, and body mass index (BMI) determined. Each subject will be initially assessed for aerobic fitness [maximal oxygen uptake ($VO_{2\ max}$)] by performing a cardiopulmonary graded exercise test on a treadmill ergometer (Quinton®, Cardiac Science™, Bothell, WA). First, subject's skin will be prepared for placement of 10 ECG electrodes. Electrode sites will be cleansed with sterile alcohol gauze using a circular motion. The site will be allowed to air dry or will be dried with a gauze pad. Electrodes will then be placed on the right subclavicular fossa (RA), left subclavicular fossa (LA), right abdomen (RL), left abdomen (LL), 4th intercostals space at the right sternal border (V1), 4th intercostals space at the left sternal border (V2), equidistant between V2 and V4 (V3), 5th intercostal space at the midclavicular line (V4), 5th intercostal space at the anterior axillary line (V5), and 5th intercostals space at the axillarly line (V6) of the chest. The subject will then be attached to a Quinton 710 ECG. Resting blood pressure, heart rate, and a 12-lead ECG will be obtained. The exercise specialist will then review the 12-lead ECG to ensure that no contraindications for exercise testing are apparent based on the ACSM guidelines. After baseline measurements at rest are completed, the treadmill test will start at a velocity of eight km/h and a zero % incline. The running

speed will be increased by two km/h every three minutes until exhaustion. Oxygen uptake (VO_2) will be measured every 30 seconds via an open-circuit sampling system (Parvo Medics, Sandy, UT). VO_{2max} will be determined if two of the following criteria are met: respiratory exchange ratio (RER) $\geq$ 1.15 and/or RPE $\geq$ 19 on the RPE scale and/or maximum heart rate within ± 10 beats of age-predicted maximum (HR_{max}). If such criteria are met, the highest level of VO_2 will be defined as $VO_{2\ max.}$ If such criteria are not met, then the highest VO_2 reached will be termed VO_{2peak} rather than VO_{2max}. Heart rate will be continuously monitored and blood pressure determined with a mercurial sphygmomanometer every three minutes during the exercise session.

Following entry/familiarization session, participants will be matched based on protein consumption and physical fitness level and randomized into either a soy milk or dairy milk group. All participants will be instructed to consume their usual diet, but limit the consumption of any soy-based or dairy-based products for two weeks, and then return to the ESNL for subsequent baseline testing. Participants will also be instructed to refrain from exercise for 48 hours and fast for eight-12 hours prior to baseline testing.

Dietary Records/Physical Activity

In an attempt to determine compliance with the dietary prescriptions, and also to assess the average daily macronutrient consumption of fat, carbohydrate, and protein, each participant will be required to keep four-day dietary records during weeks two and six of the study. The dietary records will be analyzed with the Food Processor Dietary Assessment Software program (ESHA Research Inc., Salem, OR). In addition, at the entry/familiarization session, a 24-hour recall will be performed by a Registered Dietitian to determine "typical" calorie and protein intakes. Participants will also be asked to record weekly physical activity on an International Physical Activity Questionnaire (IPAQ) at weeks two and six of the study.

Supplementation Protocol

In a single-blind format, participants will be matched for physical activity level and baseline protein intake and randomly placed into one of two dietary groups. Group A will consume a commercially available vanilla soy milk and group B will consume a commercially available reduced-fat dairy milk. Both groups will consume three cups of the respective supplements per day, for a period of four weeks. Both supplements will be matched as closely as possible for total caloric and protein content. Prior to supplementation, both groups will undergo a two-week washout period, in which subjects will be instructed to consume their usual diet, but limit the consumption of any soy-based or dairy-based products. During the study, each participant will be instructed not to consume any additional soy (or isoflavone) containing foods, limit dairy intake to two servings per day, and avoid dairy milk (unless assigned to Group B). Participants will also record daily supplement consumption for compliance.

Eccentrically Biased Downhill Run

Following the four week supplementation period, each participant will report to the lab for the downhill run test. Participants will be instructed to refrain from aerobic exercise for 48 hours prior to the exercise session. A five minute warm-up (-10% grade and two km/hr) will be performed on the treadmill prior to the 45 minute downhill running exercise. The treadmill speed will then be increased to 60% of their VO_{2max} (as determined at the baseline cardiopulmonary assessment) for 45 minutes., During the exercise protocol, 60% of maximal oxygen uptake will maintained by measuring oxygen uptake every three minutes and adjusting treadmill speed accordingly. Between 15 and 18 minutes and 27 and 30 minutes of exercise, heart rate and blood pressure will be recorded. After the exercise session, subjects will donate blood, receive a small breakfast, and will remain rested until the four-hour blood sample and muscle biopsy is taken.

Blood Collection Procedure

Venous blood samples will be obtained from the antecubital vein into a 10 ml collection tube using a standard vacutainer apparatus. Blood samples will be allowed to stand at room temperature for 10 min and then centrifuged. A total of six blood samples will be obtained. For each sample, the plasma will be removed and frozen at -20°C for later analysis. Blood samples will be collected before and after the four week supplementation period, immediately post-exercise, and four, 24, and 48 hours after the exercise session. Except for the four-hour post-exercise sample, all blood samples will be obtained after a 12-hour fast and standardized to the same time of day for each sample.

Muscle Biopsy Procedure

Four percutaneous muscle biopsies (10-15 mg) will be obtained from the middle portion of the vastus lateralis muscle of the dominant leg at the midpoint between the patella and the greater trochanter of the femur at a depth between one and two cm under local anesthesia (2% Xylocaine with epinephrine) using the fine needle aspiration technique. For subsequent biopsies, attempts will be made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy puncture, depth markings on the needle, and a successive puncture that will be made approximately 0.5 cm to the former from medial to lateral. After removal, adipose tissue will be trimmed from the muscle specimens and immediately frozen in liquid nitrogen and then stored at -80°C for later analysis. With the exception of the four-hour post-exercise biopsy, all muscle samples will be obtained after a 12-hour fast and standardized to the same time of day for each sample.

Assessment of Serum Markers of Muscle Damage and Inflammation

The plasma levels of creatine kinase (CK) will be determined with a clinical chemistry analyzer (Dimension RXL, Dade-Behring, Inc. Newark, DE). The plasma levels of inflammation and oxidative stress (SOD, GPx, and COX-2) will be assessed with

enzyme-linked immunoabsorbent assay (ELISA) with a micoplate reader (Wallac Victor-1420, Perkin-Elmer Life Sciences, Boston, MA). The assays will be performed at 340-450 nm wavelengths against a known standard curve. TNF-α, IL-1β, and IL-6 will be assessed using the Bio-Plex bead-based multiplex assays (Luminex xMAP technology) by Bio-Rad Laboratories, Inc. (Hercules, CA; #7000005KYMR).

Assessment of Inflammatory and Proteolytic Gene Expression

The skeletal muscle expression of inflammatory (TNF-α, IL-1β, IL-6, NF-KB, COX-2) and proteolytic (ubiquitin, atrogin-1, E2 ligase, E3 ligase, ring finger, calpain 1, calpain 2) markers will be performed using real-time PCR. Oligonucleotide primers will be designed using Primer Express from known human mRNA sequences available online through the NCBI database. The quantity of mRNA will be determined relative to the expression of β-actin, and ΔCT values will be used to compare gene expression. The specificity of the PCR will be demonstrated with an absolute negative control reaction containing no cDNA template, and single gene products confirmed using DNA melt curve analysis.

Perceived Muscle Soreness Procedures

Soreness will be assessed along a 10-cm scale (zero = no soreness, 10 = extreme soreness). Participants will rate their level of soreness immediately prior to the eccentric exercise bout and at four, 24, and 48 hours after exercise by drawing an intersecting line across the continuum line extending from zero-10. The distance of each mark will be measured from zero and the measurement utilized as the perceived soreness level.

Reported Side Effects from Supplements

To determine whether the participants suffered any negative side effects from the control or experimental supplements, participants will report by questionnaire administered in a confidential manner whether they tolerated the supplement, supplementation protocol, as well as report any medical problems/symptoms they may have encountered throughout the supplementation period. This questionnaire will be administered at two, four, and six weeks.

Statistical Analyses

Statistical analysis for the biomarkers of interest was performed by utilizing a group by time MANOVA to control for intra-individual variability for blood (2X6) and muscle (2X4) samples. Significant differences in mean values for main effects or interactions were explored further using profile plots. All statistical procedures were performed using SAS version 9.1.3 software and a probability level of <0.05 was adopted throughout.

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Research Team

Darryn S. Willoughby, PhD, FACSM, FISSN, CSCS, CISSN, CNC. Dr. Willoughby is an Associate Professor of Exercise and Muscle Physiology and Biochemistry in the Department of Health, Human Performance, & Recreation at Baylor University. He is also an Associate Professor of Baylor's Biomedical Science Institute. Dr. Willoughby is an internationally recognized exercise biochemist and molecular physiologist, and a leader in his field based on work with molecular signaling and regulation in skeletal muscle in response to exercise. He has conducted a vast amount of research focusing on the biochemical and molecular regulatory mechanisms regarding exercise performance and nutrition. Dr. Willoughby will be the principal investigator of the project. He will oversee all aspects of the study and be critically involved in quality assurance of the biochemical and clinical chemistry assays involved in the project.

Kristen M. Beavers, MPH, RD, ACSM-CPT. Mrs. Beavers is pursuing her Ph.D. in Exercise, Nutrition, and Preventative Health and serves as a research assistant in the Exercise and Sport Nutrition Laboratory. She will provide full-time assistance to the project serving as the research coordinator for the proposed research and will be responsible for assisting Dr. Willoughby with subject recruitment, day-to-day scheduling and testing, and data collection and analysis.

Monica C. Serra, MS, RD, ATC. Ms. Serra is pursuing her Ph.D. in Exercise, Nutrition, and Preventative Health and serves as a research assistant in the Exercise and Sport Nutrition Laboratory. She will provide full-time assistance to the project serving as the

research coordinator for the proposed research and will be responsible for assisting Dr. Willoughby with subject recruitment, day-to-day scheduling and testing, and data collection and analysis.

Richard B. Kreider, PhD, FACSM. Dr. Kreider is the Professor and Chair of the Department of Health, Human Performance, & Recreation at Baylor University. Dr. Kreider is an internationally recognized exercise scientist and has conducted a vast amount of research focusing on the role of exercise and nutrition and health and performance. Dr. Kreider will assist in protocol development and data analysis.

Matt Cooke, Ph.D. Dr. Cooke is an Assistant Professor of Exercise Physiology in the Department of Health, Human Performance, & Recreation at Baylor University. Dr. Cooke will assist in providing oversight, in data collection, and performing laboratory analyses.

Mark J. Messina, PhD. Dr. Messina offers a wide range of expertise on the health benefits of soy. Dr. Messina is the chairperson of the editorial advisory board and a regular columnist for The Soy Connection, he sits on scientific advisory boards for the Archer Daniels Midland Company, the United Soybean Board, and the Produce for Better Health Foundation, and teaches as an adjunct associate professor at Loma Linda University. Dr. Messina will serve as a nutrition consultant for this research study.

Ronald Wilson, MD. Dr. Wilson serves as medical supervisor for the ESNL, EBNL, and Center for Exercise, Nutrition & Preventive Health Research (CENPHR).

Procedures

Medical Monitoring

Interested participants will be invited to familiarization sessions. During this time, participants will sign consent forms and complete medical history information. Participants will then undergo a general exam by trained laboratory personnel to determine whether the subject meets entry criteria to participate in the study. This exam will include evaluating the medical and training history questionnaires and performing a general physical examination according to ACSM exercise testing guidelines. Based on this examination, participants will be assessed for their risk of cardiovascular disease and contraindications to exercise and then a recommendation will be made on whether the participant meets entry criteria and may therefore participate in the study. If deemed eligible, participants will be required to obtain medical clearance from their personal physician to participate in the study and prior to participating in baseline assessments. Trained, non-physician exercise specialists certified in CPR will supervise participants undergoing testing and assessments. A telephone is in the laboratory in case of any emergencies, and there will be no less than two researchers working with each participant during testing sessions. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts

Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance. Participants will be informed to report any unexpected problems or adverse events they may encounter during the course of the study to Darryn S. Willoughby, Ph.D. or Matt Cooke, Ph.D.. If clinically significant side effects are reported, the participants will be referred to discuss the problem with the laboratory nurse, and if deemed necessary the patient will be referred to Ronald Wilson, MD for medical follow-up. Dr. Wilson is one of the Sports Medicine physicians for Baylor University and is an adjunct Professor in the Department of HHPR. He has agreed to provide medical support and consultation for this study and to our lab. Dr. Wilson will evaluate the complaint and make a recommendation whether any medical treatment is needed and/or whether the participant can continue in the study. If Dr. Wilson feels medical follow-up is necessary, the participant will be referred to obtain medical treatment from their personal physician. This is a similar referral/medical follow-up system that Baylor athletes are provided with the exception that participants in this study will not be provided medical care. New findings and/or medical referrals of unexpected problems and/or adverse events will be documented, placed in the participants research file, and reported to the Baylor IRB committee.

Screening for Cardiopulmonary Disease Risk and Exercise Contraindications

All participants will have their risk of cardiopulmonary disease and possible contraindications to exercise assessed by qualified personnel and the laboratory nurse in accordance to standard procedures described by the American College of Sports Medicine (ACSM) (*ACSMôs Guidelines for Exercise Testing and Prescription*, 6th ed. Williams & Wilkins Publishers, 2000). Only those participants considered as low or moderate risk for cardiovascular disease with no contraindications to exercise will be considered as eligible to participate in the study. These guidelines are outlined and presented below:

ACSM Risk Stratification Criteria for Cardiovascular Disease

Low Risk. Younger individuals (men < 45 years of age; women < 55 years of age) who are asymptomatic for cardiovascular disease and possess no more than one positive cardiovascular disease risk factor.

Moderate Risk. Older individuals and/or those who are asymptomatic for cardiovascular disease and possess two or more cardiovascular disease risk factors.

High Risk. Individuals with one or more signs/symptoms suggestive or cardiovascular disease.

ACSM Criteria for Signs and Symptoms Suggestive of Cardiovascular Disease

1. Pain, discomfort in the chest, neck, jaw, arms, or other areas that may be due to myocardial ischemia.

2. Shortness of breath at rest or with mild exertion.
3. Dizziness or syncope.
4. Orthopnea or paroxysmal nocturnal dyspnea.
5. Ankle edema.
6. Palpitations or tachycardia.
7. Intermittent claudication.
8. Known heart murmur.
9. Unusual fatigue or shortness of breath with usual activities.

ACSM Absolute and Relative Contraindications to Exercise

Absolute Contraindications

1. Unstable angina.
2. Uncontrolled dysrhythmias.
3. Recent EKG changes and cardiac events.
4. Acute myocarditis or pericarditis.
5. Acute pulmonary embolism or acute myocardial infarction.
6. Severe aortic stenosis.
7. Dissecting aneurysm.
8. Acute infections.

Relative Contraindications

1. Left main coronary stenosis.
2. Severe hypertension (> 200/110).
3. Tachycardia or bradycardia.
4. Uncontrolled metabolic disease.
5. High-degree AV block.
6. Chronic infectious disease.
7. Cardiomyopahty and outflow obstructions.
8. Stenotic valve disease.
9. Ventricular aneurysm.

Blood Samples

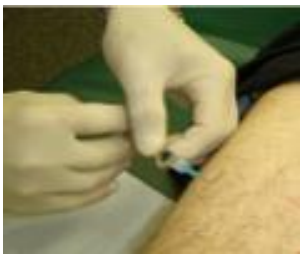
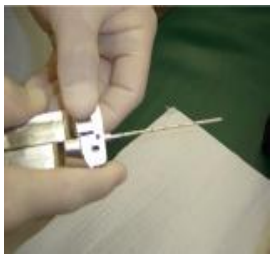
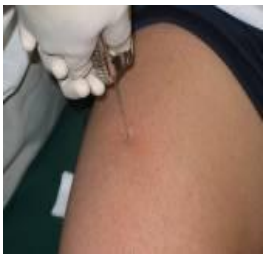
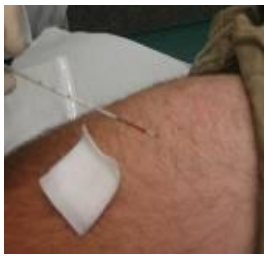
Participants will donate approximately three to four teaspoons of fasting venous blood (10-12 milliliters) during each blood draw. Blood samples will be obtained using standard phlebotomy procedures using standard sterile venipuncture of an antecubital vein by Darryn Willoughby, Ph.D., Kristen Beavers, RD, MPH, or laboratory technician's trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. Up to this point in his professional career, Dr. Willoughby has successfully performed several thousand blood draws without any complications. The phlebotomists and lab technicians will wear personal protective clothing (gloves, lab coats, etc.) when handling blood samples. Subjects will be seated in a phlebotomy chair. Their arm will be cleaned with a sterile alcohol wipe and sterile

gauze. A standard rubber tourniquet will then be placed on the brachium. An antecubital vein will be palpated and then a 23 gauge sterile needle attached to a plastic vacutainer holder will be inserted into the vein using standard procedures. Two plasma separation vacutainer tubes (green tops) will be inserted into the vacutainer holder for blood collection in succession using multiple sample phlebotomy techniques. Once samples are obtained, the vacutainer holder and needle will be removed. The needle will be discarded as hazardous waste in an appropriately-labeled plastic sharps container. The site of the blood draw will then be cleaned with a sterile alcohol wipe and gauze and a sterile Band-Aid will be placed on the site. The alcohol wipe and gauze then will be discarded in an appropriately-labeled biohazard waste receptacle. The blood collection tubes will be labeled and placed in a test tube rack. Laboratory technicians (who have received blood borne pathogen training and will be wearing personal protective clothing) will centrifuge the plasma samples, transfer plasma into labeled plasma storage containers, and store the samples at -20°C for later analysis.

Muscle Biopsies

Percutaneous muscle biopsies (approximately 10-15 mg) will be obtained from the vastus lateralis of each participant's thigh using the fine needle aspiration technique. Darryn Willoughby, Ph.D. will perform and/or directly supervise all muscle biopsy procedures. Samples will be extracted under local anesthesia of 2% Xylocaine with epinephrine from the middle portion of the muscle at the midway point between the patella and the greater trochanter of the femur. For each biopsy, muscle tissue will be extracted from the same location by using the previous location and depth markings on the needle. First, the participant will lie supine or assume a comfortable reclining position on a sterilized table. Once the biopsy site is identified, the area will be cleaned with rubbing alcohol and betadine. A small area of the skin approximately two cm in diameter will be anesthetized with a 0.5 mL subcutaneous injection of Xylocaine. Once anesthetized, a specialized 16-gauge biopsy needle will be punctured approximately one cm through the skin and connective tissue and into the muscle; the biopsy procedure will take approximately 15-20 seconds. Due to the localized effects of the anesthetic, the participant should feel no pain during this process but may feel pressure to the thigh area. Once the muscle sample has been obtained, pressure will be immediately applied to the biopsy site and an adhesive bandage immediately applied. Bleeding is minimal due to the small puncture-type opening; therefore, only a standard band-aid type bandage is needed to cover the puncture. The biopsy needles will be discarded as hazardous waste in an appropriately-labeled plastic sharps container. The site of the biopsy will be cleaned with a sterile alcohol wipe and gauze. The alcohol wipe and gauze then will be discarded in an appropriately labeled biohazard waste receptacle. The tissue sample will be stored at -80° C for future analyses. Written instructions for post-biopsy care will be given to the subjects. The participant will be instructed to leave the band-aid in place for 12 hours (unless unexpected bleeding or pain occurs) and asked to contact the lab immediately if they feel there is a problem. The time course nature of the study will allow for daily follow-up to occur in order to ensure all biopsy locations are healing correctly and free of infection. Aside from the testing sessions, the participant will be further advised to refrain from vigorous physical activity during the first 24 hours post-biopsy. These

suggestions will minimize pain and possible bleeding of the area. If needed, the subject may take non-prescription analgesic medication such as Acetaminophen to relieve pain if needed. However, medications such as aspirin, Nuprin, Bufferin, or Advil will be discouraged as these medications may lead to ecchymosis at the biopsy site. Soreness of the area may occur for about 24 hours post-biopsy.

Assessment of Hemodynamic Safety Markers (Heart Rate & Blood Pressure)

With each visit to the laboratory, participants will undergo assessment of heart rate and blood pressure. Heart rate will be determined by palpitation of the radial artery using standard procedures. Blood pressure will be assessed in the supine position after resting for five-min using a mercurial sphygmomanometer using standard procedures.

Reported Side Effects from Supplement Questionnaires

At two, four, and six weeks participants will report by questionnaire administered in a confidential manner whether they tolerated the supplement, and the supplementation protocol. Additionally, they will be asked to report any medical problems/symptoms they may have encountered throughout the supplementation period of the study.

Dietary Records/Physical Activity

Participants will bring completed four day dietary records with them upon each visit to the laboratory for the analysis of dietary intake using the Food Processor IV Nutrition Software. Participants will also be asked to complete an IPAQ at weeks two and six of the study.

Aerobic Exercise Capacity Test

Participants will first be prepared for placement of 10 ECG electrodes and a 12-lead ECG and baseline HR and BP will be collected. Participants will then be instructed to stand on a stationary treadmill. A sterile mouthpiece attached to a head harness will be secured on the subject. The participants will then have a noseclip placed on their nose. Once the participant is ready to begin the test protocol, the participant start at a velocity of eight km/h and a 0% incline. The running speed will be increased by two km/h every three minutes until exhaustion. Resting expired gases will be collected every 30 seconds via an open-circuit sampling with the Parvo Medics 2400 TrueMax Metabolic Measurement

System (Sandy, Utah). The highest level of VO_2 will be defined as $VO_{2\ max}$ or VO_{2peak} depending on whether or not defining criteria are met.

Equipment

Digital Scale.

Total body weight will be determined using a digital scale accurate to ±0.02 kg. The scale is calibrated by placing certified 25-kg weights and balancing the scale. Other than general instructions, special skills are not required to measure body weight.

Aerobic Exercise Capacity Testing

Maximal cardiopulmonary measurements will be obtained using Parvo Medics 2400 TrueMax metabolic measurement system (Sandy, Utah). Participants will run on a Quinton treadmill ergometer (Quinton®, Cardiac Science™, Bothell, WA).

Muscle Biopsy Needle

The muscle biopsy technique will be performed with a TRU-CORE® 1 Automatic Reusable Biopsy Instrument (Angiotech, Medical Device Technologies, INC., Gainsville, Florida, USA).

Plasma Inflammation/Oxidative Stress Biomarker Analysis

Blood samples will be used to assess plasma inflammation and oxidative stress levels. These assays will be performed photometrically using either a DADE Dimension RXL clinical chemistry analyzer (Dade-Behring, Inc., Newark, DE) or enzyme-linked immunoabsorbent assays (ELISA) with a Wallac Victor-1420 micoplate reader. The ELISA assays will be performed at either 340 or 450 nm wavelength against a standard curve. Prior to use each system will be calibrated with standard quality assurance protocols and known control values.

Muscle Protein Gene Expression

Muscle protein samples will be used to isolate total RNA, reverse transcribed, and then used to perform real-time polymerase chain reaction using specific oligonucleotide primers to amplify target genes using an iQ real-time PCR system (Bio-Rad, Hercules, CA).

Snack

Due to the length of the session all participants will ingest a snack (granola bar) after the eccentrically biased downhill run exercise session and lab specimen collection that is equal in caloric amount as well as macronutrient percentages.

Participants

Recruitment

Thirty apparently healthy, physically active, but not trained (not engaged in an exercise program involving either resistance or endurance training at least thrice weekly for one year), post-menopausal women between the ages of 40-60, not undergoing hormone replacement therapy (either pharmacological or soy-based nutritional supplements), will be recruited from the Waco area. A recruitment flyer that will be posted on campus, at area fitness centers, and on the Internet (http://www3.baylor.edu/HHPR/research/subjects/) and sent via campus mail is attached.

Selection Criteria

Participants will not be allowed to participate in the study if they:

1. have a body mass index lower than 19 kg/m^2 or higher than 35 kg/m^2
2. are unable to follow the study protocol.
3. do not have their primary care physician's approval to participate.
4. they cannot handle the downhill running protocol.
5. have engaged in hormone replacement therapy (either pharmacological or soy-based) for up to three months prior to the study.
6. have any known bleeding or metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism.
7. have a history of pulmonary disease, hypertension, hepatorenal disease, musculoskeletal disorders, neuromuscular/neurological diseases, autoimmune disease, chronic infection diseases (e.g., hepatitis or HIV), cancer, peptic ulcers, or anemia.
8. are taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (e.g, thyroid, insulin, etc), psychotropic, neuromuscular/neurological, or androgenic medications.
9. have taken nutritional supplements that may affect antioxidant status (e.g., Vitamins C and E, EPA/DHA, EGCG) within three months prior to the start of the study.
10. have smoked cigarettes within the past three years or have an average intake of two or more alcoholic drinks/day
11. have any absolute or relative contraindication for exercise testing or prescription as outlined by the American College of Sports Medicine.
12. report any unusual adverse events associated with this study that in consultation with the supervising physician recommends removal from the study.
13. have known food allergies, allergies to anesthesia medications, or lactose intolerance.

Compensation or Incentives

Participants completing all familiarization and testing sessions as well as turning in all required materials (i.e., dietary logs and unused supplements) in the study will be paid $150. Subjects may receive information regarding results of these tests if they desire. If subjects are Baylor students, they will not receive any academic credit for participating in this study.

Potential Risks

The nutritional supplements to be investigated in this study have been studied for various uses, both medicinal and non-medicinal in humans. Initial research has demonstrated that moderate oral administration of these compounds is not associated with any significant medical side effects.

Participants who meet eligibility criteria will be subjected to an acute downhill running protocol. Participants in this study will be experienced runners, and will be instructed not to do any aerobic exercise 48 hours prior to the exercise session. As a result of the exercise protocol, participants will most likely experience short-term muscle fatigue. In addition, they will likely experience muscle soreness in their thigh area for up to 24 to 48 hours after exercise. This soreness is normal and should be commensurate with the type of muscle soreness participants may have felt after doing unaccustomed physical activity. Muscle strains/pulls resulting from the exercise protocol are possible. However, potential injury due to exercise will be minimized by ensuring that all participants adhere to correct running technique. In addition, only Darryn Willoughby, Ph.D. and/or trained graduate student study personnel will conduct the testing and exercise procedures. Participants will be made aware of the intensity and duration of the expected soreness due to the exercise sessions. Participants will donate three to four teaspoons (six-12 milliliters) of venous blood eight times during the study using standard phlebotomy procedures. This procedure may cause a small amount of pain when the needle is inserted into the vein as well as some bleeding and bruising. The subject may also experience some dizziness, nausea, and/or faint if they are unaccustomed to having blood drawn.

Complications resulting from the muscle biopsy are rare, especially in this case where the biopsy is similar to receiving a routine intramuscular injection. As with the blood draw, however, there is a risk of infection if the subject does not adequately cleanse the area for approximately 24 hours post biopsy. While leaving the band-aid bandage in place, participants will be instructed to cleanse the biopsy area with soap and water every four to six hours, pat the area dry and reapply a fresh adhesive bandage. The participant will be further advised to refrain from vigorous physical activity with the affected leg for 24 hours after the biopsy. There is a potential risk of an allergic reaction to the Xylocaine. All subjects will be asked if they have known allergies to local anesthetics (e.g. Lidocaine, Xylocaine, etc.) that they may have been previously given during dental or hospital visits. Participants with known allergies to anesthesia medications will not be allowed to participate in the study. Trained doctoral research assistants and Darryn Willoughby, Ph.D. will perform all muscle biopsies. Up to this point in his professional

career, Dr. Willoughby has successfully performed over 500 fine needle aspiration muscle biopsies on both male and female subjects without any complications.

Researchers involved in collecting data represent trained, non-physician, certified exercise specialists (American Society of Exercise Physiologies Certified Exercise Physiologist, Certified Strength & Conditioning Specialists, and/or American College of Sports Medicine Health Fitness Instructor$_{SM}$, Exercise Technologist$_{SM}$, or Exercise Specialists$_{SM}$). All personnel involved in collecting data will be certified in CPR, which is also a condition to holding these professional certifications. A telephone and automated electronic defibrillator (AED) is located in the laboratory in case of any emergencies and there will be no less than two researchers working with each subject during testing. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance.

Potential Benefits

The main benefit that participants may obtain from this study is that if this nutritional supplement is effective in attenuating systemic and local inflammation, there is a possibility that they may gain insight into how to minimize oxidative stress, inflammation, and muscle proteolysis associated with the aging process, as well as eccentric exercise. Participants also may gain insight about their health and fitness status from the assessments to be performed. However, even if no individual benefit is obtained, participating in this study will help to determine whether ingesting this nutritional supplement affects oxidative stress, inflammation, and muscle proteolysis.

Assessment of Risk

Even though clinical data are available outlining the effects of soy milk or dairy milk and clinical safety, because they are still relatively new to the market the potential medical benefits of these supplements are not yet well delineated. However, these supplements are available in a number of over the counter products, and are generally considered safe with no negative side effects. Initial results suggest that these supplements may provide benefit at reducing oxidative stress, inflammation, and muscle proteolysis; however, additional well-controlled research is necessary before conclusions can be drawn. This study will help determine whether ingesting soy milk or dairy milk are effective at minimizing muscle damage and inflammation after an acute bout of exercise designed to induce these processes. Consequently, the risk of supplementation of these compounds at the levels to be evaluated in this study is low. The greatest risk associated with participating in this study will likely be from the muscle soreness participants will experience from participating in the eccentric exercise protocol. However, since the intensity of the exercise protocol will be no more than when individuals engaged heavily in a new or different form of physical activity, the potential benefits of subjects participating in this study outweigh the potential risks.

Compensation for Illness or Injury

Each participant will agree to indemnify and hold harmless Baylor University, its officers, directors, faculty, employees, and students for any and all claims for any injury, damage, or loss suffered as a result of participation in this study regardless of the cause of injury, damage, or loss.

Confidentiality

Information obtained from this research (including questionnaires, medical history, laboratory findings, or physical examination) will be kept confidential to the extent permitted by law. However, according to FDA regulations, records will be open to FDA representatives to review if necessary. This may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken throughout this study. Records of the research may also be subpoenaed by court order or may be inspected by federal regulatory authorities. Data derived from this study may be used in reports, presentations and publications. Participants in this study will not be individually identified unless they give their written consent. All participants will have a number to identify their results. Only the study personnel will know the subject numbers. Only study personnel will have access to the data. All data will be stored in a locked cabinet in the Exercise and Biochemistry Laboratory and only Darryn Willoughby, Ph.D. will have access to the key. All evidence of primary data will be stored for exactly three years after the completion of the study. At this time data will be destroyed in a manner that instills complete privacy to all participants of the study. Analyzed muscle and blood samples will be discarded in an appropriately-labeled biohazard waste disposal container. However, unused muscle and blood samples will be kept in a locked freezer for no longer than one year. If any subsequent analysis occurs with the samples, they will be re-coded to further instill confidentiality.

Data Presentation & Publication

Data will be presented at an appropriate scientific conference (e.g., American Dietetic Association, etc.) and published in a peer reviewed scientific journal (e.g., American Journal of Clinical Nutrition, etc.).

Statement of Conflict of Interest

Funding for this study was obtained in part from WhiteWave Foods Company (Broomfield, CO) through a grant awarded to Baylor University. Researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

APPENDIX E

Muscle Biopsy Wound Care

Wound Care for the Fine Needle Aspiration
Muscle Biopsy Procedure

Complications resulting from the muscle biopsy procedure are rare. Furthermore, after the procedure, you can reduce your risk of chance of infection by adhering to the following course of action for wound care:

For approximately 24 hours post biopsy

- leave the band-aid bandage on for 24 hours (unless unexpected bleeding or pain occurs, which should be immediately reported to the lab)
- adequately cleanse the area surrounding the band-aid bandage with soap and water every 4-6 hours, and pat the area dry
- refrain from vigorous physical activity

After 24-hours (for approximately 48 hours post biopsy)

- leave the band-aid bandage in place if necessary
- adequately cleanse the area surrounding the bandage with soap and water if necessary
- reapply a fresh adhesive band-aid bandage if necessary

At approximately 48 hours post biopsy

- leave these band-aid bandages on if unexpected bleeding or pain occurs
- return to normal hygiene practices unless complications arise

Possible Pain Side Effects

Soreness of the area may occur for about 24 hours post-biopsy. Following the procedures outlined above should significantly minimize pain and possible bleeding of the area. However, some subjects experience no significant pain post biopsy.

5 If needed, the subject may take non-prescription analgesic medication such as Acetaminophen to relieve pain if needed.

☐ Medications such as aspirin, Advil, Bufferin, or Nuprin, are discouraged as they may lead to excess bruising at the biopsy site.

If any questions or complications arise please contact:

Darryn Willoughby, Ph.D.
Room # 120 Marrs McLean Gym
(254) 710-3504
Darryn_Willoughby@baylor.edu

or

Melyn Galbreath, R.N.
Room # 120 Marrs McLean Gym
(254) 710-7277 or 7199
Melyn_Galbreath@baylor.edu

APPENDIX F

24 Hour/Four Day Food Record

Baylor University
Exercise & Sport Nutrition Laboratory

NAME _____ Date _____

INSTRUCTIONS

1. Record everything you eat for "4 days/24 hours" prior to the testing session. If you eat pretzels, record how many. If you eat a bag of chips, record the number of ounces. For drinks, record the number of cups or ounces. Record everything you drink except water.
2. Record the Food, Amount, Brand Name, and Preparation Methods. For example: baked vs. fried chicken; 1 cup of rice; 2 teaspoons of margarine; 1 cup of 2% milk; McDonald's, Healthy Choice, or Frosted Flakes.
3. Record immediately after eating. Waiting until that night may make it difficult to remember all foods and quantities.

Food (include brand)	**Method of Preparation**	**Quantity (cups, oz., no.)**
BREAKFAST:		
LUNCH:		
DINNER:		
SNACKS:		

APPENDIX G

Reported Side Effects Questionnaire

Reported Side Effects From Supplement Questionnaire

Subject: _____ **Date:** _____

Week	2	4	6
Did you avoid aerobic exercise during the supplementation period?	x		
Are you adhering to the supplementation protocol?	x		
Rate the ***frequency*** of the following symptoms according to the scale where: 0 = none 1 = minimal (1-2 per/wk) 2 = slight (3-4 per/wk) 3 = occasional (5-6 per/wk) 4 = frequent (7-8 per/wk) 5 = severe (9 or more per/wk)			
Dizziness?			
Nausea and/or upset stomach?			
Headache?			
Fast or racing heart rate?			
Heart skipping or palpitations?			
Shortness of breath?			
Nervousness?			
Blurred Vision?			
Any other unusual or adverse effects?			
Rate the ***severity*** of the following symptoms according to the scale where: 0 = none 1 = minimal 2 = slight 3 = moderate 4 = severe 5 = very severe			
Dizziness?			
Nausea and/or upset stomach?			
Headache?			
Fast or racing heart rate?			
Heart skipping or palpitations?			
Shortness of breath?			
Nervousness?			
Blurred Vision?			
Any other unusual or adverse effects?			

PLEASE REPORT ANY SIDE EFFECTS IMMEDIATELY TO EITHER DR. WILLOUGHBY OR MELYN GALBREATH, THE ESNL RESEARCH NURSE.

Directions: If necessary, please contact either Darryn Willoughby, Ph.D. at 254-710-3504 or Melyn Galbreath, R.N. at 254-710-7277. You may also e-mail either at Darryn_Willoughby@baylor.edu or Melyn_Galbreath@baylor.edu.
Thanks for your participation!

APPENDIX H

Perceive Muscle Soreness Rating

Baylor University

Exercise and Biochemical Nutrition Laboratory

Perceive Muscle Soreness Rating

Directions:

Considering the overall severity of soreness in your thigh muscle upon movements such as sitting and standing, draw an intersecting line across the continuum line extending from 0-10. This mark will indicate your level of soreness (0 = no soreness, 10 = extreme soreness). The distance of each mark will be measured from zero and the measurement utilized as the perceived soreness level.

Testing Session: _____ Date: _____

0 ——————————————————— **10**

APPENDIX I

Nutritional Handouts

Helpful Tips to Ge ű ¸ ƻ dzNJ ά a ƛ f ʅ έ C ƛ

There are lots of ways to enjoy ά Ƴ ƛLJNJ ƻ ŘdzŎ ű ă Φ ² Ƙ Ŝ ű Ƙ ŜNJ ƛ ű ƛ foİ ƎNJ ŀ ō ō ƛ ƴ
adding ά Ƴ ƛ űf ƻ ʅ έ è ƻ dzNJ Ŧ ŀ Ø ƻ NJ ƛ ű Ŝ NJ Ŝ Ŏ ƛ LJ Ŝ Σ ƛ ű Ω ă ƴ Ŝ Ø Ŝ NJ ō Ŝ Ŝ ƴ
Ŏ ƻ ƴ Ø Ŝ ƴ ƛ Ŝ ƴ ű Ƴ Ŝ ű Ƙ ƻ Ř ă ű ƻ Ǝ Ŝ ű è ƻ dzNJ ά Ƴ ƛ f ʅ έ Ŧ ƛ Ȅ Φ

Pour ά Ƴ ƛ on your cereal. There's no better way to start your day.

Make a smoothie with fruit and ά Ƴ ƛ. f ʅ έ

Substitute ά Ƴ ƛ for water when you cook oatmeal or other hot cereals.

Add ά Ƴ ƛ to your coffee in the morning instead of creamer.

Enjoying ά Ƴ ƛ is now easier and than ever before. Check out the following recipes to help you get your three cups per day.

Straw-Nana Smoothie

Ingredients
1 cup ñmilkò
1/2 cup diced, frozen strawberries
1 sliced, frozen banana
2 Tbsp. sweetener of choice (optional)
1/8 teaspoon vanilla (optional)

Preparation
In blender combine ñmilkò, fruit, sweetener and vanilla.

Servings
2

Choco-nana Smoothie

Ingredients
1 cup ñmilkò
1 sliced, frozen banana
2 Tbsp. sweetener of choice (optional)

2 Tbsp. chocolate syrup

Preparation
In blender, combine ñmilkò, banana, syrup and sweetener.

Servings
2

Mexican Hot Chocolate

Ingredients
2 cups ñmilkò
6 Squares Mexican chocolate

Preparation
Melt the chocolate in a double boiler on low heat. When it is completel
on low, stirring periodically. When chocolate and ñmilkò
with cinnamon or chocolate sprinkles.

Servings
2

Silky Peanut Butter Cup

Ingredients
12 oz. ñmilkò
1 Tbsp. peanut butter
1 banana
3-4 Tbsp. chocolate baking powder
2-3 scoops vanilla ice cream

Preparation
Combine all ingredients in blender.
Blend until smooth. Insert straw if you're patient, spoon if you're hungry.

Servings
2

Green Tea Chai

Ingredients
2 cups water
6 1/4-inch slices fresh ginger root
6 green cardamom pods
2 bags green tea
1 cup ñmilkò
Ground cinnamon

Preparation
Place the water, ginger and cardamom in a small stockpot. Bring to a simmer, cover and cook for 10 minutes. Add green tea and let steep for 4 minutes. Remove tea bags, ginger and cardamom with a slotted spoon. Add ñmilkò and heat to almost boiling. Serve hot

Servings
3-4

Smoothie Colada

Ingredients
8 oz. ñmilkò
6 oz. pineapple juice
2 Tbsp. coconut cream
1/2 cup frozen pineapple, diced
6 ice cubes

Preparation
Dice pineapple and freeze overnight.
Combine ingredients in blender and cover. Blend until smooth. Pour into a couple of glasses and garnish with any fruit that has funny name.

Servings
2

Rush Hour Raspberry Shake

Ingredients
4 oz. ñmilkò
6 oz. raspberry yogurt
1 cup sweetened frozen raspberries
1/2 banana
1 Tbsp. wheat germ

Preparation
Combine ingredients in blender and cover. Blend until smooth. Pour into a couple of glasses and top with ¼ tsp of almond extract (or more to taste).

Servings
2

Old Fashioned ñMilkò Sha

Ingredients
12 oz. ñmilkò
3 scoops vanilla ice cream
1 Tbsp. vanilla extract
2 tsp. sugar

Preparation
Combine ingredients in blender and cover. Blend until smooth. Add malt or chocolate powder as desired.

Servings
2

W h a t i s a ñ D a i r y S e r v i n g

For six weeks of this study (2 week "washout" and 4 week supplementation period), you will be asked to eliminate dairy milk from your diet. In addition, throughout this six week period, you will be asked to limit your daily dairy intake to two servings per day.

1 serving equals	**Visual Comparison**
1 cup of yogurt	1 cup = softball
½ cup of macaroni and cheese	
½ cup of ice cream	½ cup = ½ baseball
½ cup of cottage cheese	
1.0 oz. of regular and cream cheese	1 oz = 4 stacked dice

Isoflavone

Fact Sheet

Isoflavone Contents of Food: Soyfoods to Avoid

Below are isoflavones contents (total isoflavones, daidzein and genistein) of some foods. These isoflavones values have been taken from USDA database. Values of isoflavones are expressed in mg per 100g.

Food product	Total Isoflavones	Daidzein	Genistein
Soy flour, full-fat	177.89	71.19	96.83
Soy flour, textured	148.61	59.62	78.90
Soy flour, defatted	131.19	57.47	71.21
Soybeans	128.34	46.46	73.76
Soy protein concentrate, aqueous washed	102.07	43.04	55.59
Soy protein isolate	97.43	33.59	59.62
Natto	58.93	21.85	29.04
Soybean chips	54.16	26.71	27.45
Tofu, fried	48.35	17.83	28.00
Tempeh	43.52	17.59	24.85
Miso	42.55	16.13	24.56
Soybean sprouts	40.71	19.12	21.60
Tofu, soft	29.24	8.59	20.65
Tofu, silken	27.91	11.13	15.58
Soy infant formula, powder	25.00	7.23	14.75
Tofu, firm	22.70	8.00	12.75
Soy hot dog	15.00	3.40	8.20
Okara	13.51	5.39	6.48
Soy protein concentrate, alcohol extracted	12.47	6.83	5.33
Bacon, meatless	12.10	2.80	6.90
Soy milk	9.65	4.45	6.06
Vegetarian burger	9.30	2.95	5.28
Soy cheese, Mozzarella	7.70	1.10	3.60
Soy cheese, Cheddar	7.15	1.80	2.25
Soy drink	7.01	2.41	4.60

Isoflavone

Fact Sheet

Isoflavone Contents of Food: Acceptable Soyfoods

Below are isoflavones contents (total isoflavones, daidzein and genistein) of some foods. These isoflavones values have been taken from USDA database. Values of isoflavones are expressed in mg per 100g.

Food product	Total Isoflavones	Daidzein	Genistein
Split peas	2.42	2.42	0.00
Shoyu	1.64	0.93	0.82
Pigeon peas	0.56	0.02	0.54
Clover sprouts	0.35	0.00	0.35
Peanuts	0.26	0.03	0.24
Navy beans	0.21	0.01	0.20
Mung beans	0.19	0.01	0.18
Granola bars	0.13	0.05	0.08
Chickpeas	0.10	0.04	0.06
Green tea	0.05	0.01	0.04
Broadbeans	0.03	0.02	0.00
Cowpeas	0.03	0.01	0.02
Lima beans	0.03	0.02	0.01
Lentils	0.01	0.00	0.00
Alfalaval seeds, sprouted	0.00	0.00	0.00
Black beans	0.00	0.00	0.00
Flax seed	0.00	0.00	0.00
Rye bread	0.00	0.00	0.00

APPENDIX J

International Physical Activity Questionnaire

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (October 2002)
LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT
FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on healthï related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective.* Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport. Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

   ☐ Yes

   ☐ No → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

   _____ **days per week**

   ☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

   _____ **hours per day**
   _____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

   _____ **days per week**

   ☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No bicycling from place to place → ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**

☐ No moderate activity in garden or yard → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

☐ No moderate activity inside home → ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

☐ No vigorous activity in leisure time → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?
_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

APPENDIX K

Medical Release Form

B A Y L O R

U N I V E R S I T Y

Dear Medical Provider:

One of your patient's would like to participate in the Effects of Four Weeks of Daily Soy Milk or Dairy Milk Ingestion on the Exercise-Induced Inflammatory and Proteolytic Responses in Plasma and Skeletal Muscle in a Post-Menopausal Female Population" that is being conducted by Darryn Willoughby, Ph.D. (254-710-3504; Darryn_Willoughby@baylor) through the Exercise & Biochemical Nutrition Laboratory at Baylor University. In order to do so, she must meet the entrance criteria described below and have approval from her personal physician to participate in the study. The study will involve apparently health, physically active post-menopausal females who have not been undergoing hormone replacement therapy for three months prior to the study orally ingesting a cup of soy milk and dairy milk three times per day for four weeks. They also will be subjected to a treadmill protocol which involves running downhill for 45 minutes. Assessments will include obtaining fasting blood and muscle samples from the thigh. In addition, each participant will be asked to attempt to avoid consuming foods with elevated levels of isoflavones and to limit dairy intake for two weeks. If you feel she meets the entrance criteria and/or any existing medical condition that she may have is under control and would not be a limitation for her to participate in the study, please sign the medical clearance below. If you feel the need to contact me, please feel free to do so.

Subjects

Forty-five apparently healthy, physically active, but not trained (not engaged in an exercise program involving either resistance or endurance training at least thrice weekly for one year) post-menopausal women will be recruited to participate in this study. I understand that in order to participate in this study, a trained individual will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if: 1) I do not have physician's approval; 2) I have been on hormone re have hypertension, glucose-intolerance, type II diabetes, thyroid conditions, hyperlipidemia and/or other medical conditions, controlled or uncontrolled, that my physician feels would affect my participation in this study; 4.) I have taken nutritional supplements that may affect my antioxidant status (e.g., Vitamins C and E, EPA/DHA, EGCG) for three months prior to the study 5.) I report any unusual adverse events associated with this study that in consultation with the supervising physician recommends removal from the study.

Medical Clearance

I medically clear _____ participate in this study.

Physician's Name _____

Physician's Signature _____

APPENDIX L

Compliance Log

Complianc e Log (Subject:_____)

Week 1 (total cups/week=___)

Day	Cups Consumed
1	
2	
3	
4	
5	
6	
7	

Week 2 (total cups/week=___)

Day	Cups Consumed
1	
2	
3	
4	
5	
6	
7	

Week 3 (total cups/week=___)

Day	Cups Consumed
1	
2	
3	
4	
5	
6	
7	

Week 4 (total cups/week=___)

Day	Cups Consumed
1	
2	
3	
4	
5	
6	
7	

*Remember, you must drink 3 cups per day___

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