ABSTRACT

The Effects of a 12-week Resistance Training Program Combined with Casein or Whey Protein Supplementation on Body Composition, Muscle Strength, and Markers of Satellite Cell Activation in Older Males

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Resistance training and protein supplementation have been shown to increase muscle mass and strength, and thus may be an effective method to combat muscle wasting (sarcopenia) that occurs with aging. Thirty-six males (62.5 yrs, 177 cm and 95.25 kg) were randomly assigned to ingest either whey isolate protein, casein protein or carbohydrate while participating in a 12-week high intensity resistance training program. Resistance training increased both upper and lower body strength, body mass and lean muscle mass while decreasing body fat percentage. However, no effects from training were observed on serum anabolic hormones and indirect markers of satellite cell activation. A trend towards significantly higher lower body strength was observed in the whey isolate (p=0.053) and casein (p=0.086) groups, respectively, compared to carbohydrate placebo group. The present study suggests protein supplementation in conjunction with high intensity resistance training may be important in combating sarcopenia.

The Effects of a 12-week Resistance Training Program Combined with Casein or Whey Protein Supplementaion on Body Composition, Muscle Strength, and Markers of Satellite Cell Activation in Older Males

by

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A Thesis

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LIST OF ABBREVIATIONS

1RM-1 repetition maximum

CAS-casein

CHO-carbohydrate

c-Met-cMet receptor

DEXA-Dual Energy x-ray absorptiometry

DHEA-dehydroxyandrosterone

DNA-deoxyribonucleic acid

ELISA-enzyme linked immunoabsorbent assay

HGF-hepatocyte growth factor

Hsp-heat shock protein

IGF-1-insulin like growth factor 1

IL-6-interleukin 6

MHC-myosin heavy chain

MNF-myonuclear factor

MRF-myogenic regulatory factory

Pcr-phosphocreatine

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Last, but certainly not least, I would like to thank my Lord and Savior Jesus Christ. My purpose in life is to love and serve you. Thank you for giving me this opportunity and guiding me in the right direction for my future.

DEDICATION

To my husband, Justin Bane

Thank you for putting your life on hold to help me achieve my dreams and goals. Thank you for all of your help and support. Thank you for being my rock through this and encouraging me through the tough times. I am so lucky to have someone who cares so much about me and would do anything to make sure my dreams come true. I love you so much and thank you for all that you have done and will continue to do. I can't wait to see what God has in store for us! I love you so much.

CHAPTER ONE

Introduction

Aging is a slow-developing, continuous process that compromises the normal function of various organs and organs systems. Skeletal muscle comprises a large portion of the human body, and senescent changes to this tissue create numerous problems for older adults. Older adults typically experience age-related declines in skeletal muscle mass and strength; a condition known as sarcopenia. Sarcopenia has been hypothesized to be a progressive atrophy and loss of muscle fibers as well as a reduction in muscle "quality" due to the infiltration of fat and other non-contractile material such as connective tissue (Brooks and Faulkner, 1994; McNeil, Doherty, Stashuk and Rice, 2005). These changes also lead to a number of additional complications which include: increased prevalence of falls, decreased metabolism, loss of appetite, increased fractures, loss of independence, and ultimately, a diminished quality of life. It is evident that half the population over 65 years of age suffer from complications associated with sarcopenia (Drummond, Dreyer, Pennings, Fry, Dhanani, Dillon, Sheffield-Moore, Volpi and Rasmussen, 2008) and more than half have a diagnosed disability (Manton 2001). This statistic is alarming because recent demographics show that the world's population aged 60 years and older will triple within 50 years, from 600 million in the year 2000 to more than 2 billion by 2050. Elderly people make up two thirds of the population in the developed world. Due to greater longevity, the subpopulation of elderly people aged 80 years and over is presently the fastest growing subpopulation in the developed world

(WHO. Ageing (Online). [http://www.who.int.ezproxy.baylor.edu/topics/ageing/en [2008]).

Healthy adults typically synthesize and degrade approximately 300g of protein per day (Mitch and Goldberg, 1996), and thus a small, but significant, changes in either will have an impact on either protein synthesis or degradation, and thus can have a significant impact on muscle mass (Franch and Price, 2005). At the whole body level, regulation of skeletal muscle protein turnover is affected by key factors such as nutrition and exercise (or lack thereof in each case). These factors then contribute to the levels of hormones and cytokines within the systemic microenvironment that determine whether signaling for hypertrophy or atrophy will be favored within the muscle cell via the stimulation or inhibition of protein synthesis and protein degradation (Guttridge, 2004). Despite the complex nature of sarcopenia, it is clear from the literature that maintaining quality and quantity of muscle protein comes down to a balance that exists between muscle protein anabolism and catabolism (Koopman and van Loon, 2009). This remodeling process, known as "protein turnover" is essentially controlled by the initiation of protein synthesis and degradation (Hasten, Pak-Loduca, Obert and Yarasheski, 2000). Promoting a net positive protein balance though the stimulation of protein synthesis and inhibtion of protein breakdown would not only increase muscle mass and size, but more importantly delay the progression of, or possibly reverse the effects of sarcopenia.

In the past decade strength training has been investigated extensively as a means of reversing the muscle mass loss that occurs with aging (McCartney, Hicks, Martin and Webber, 1995; Hunter, McCarthy and Bamman, 2004). High-intensity resistance training (HIRT) has led to increased protein synthesis, along with muscle hypertrophy measured

at the whole body, whole muscle, and muscle fiber levels, in older adults (Hunter, McCarthy and Bamman, 2004). However, adaptations (i.e. muscle hypertrophy, strength gains, etc) are lower in older individuals compared to younger individuals. (Welle and Thornton, 1998). RT has shown the most promise in reducing/reversing effects of sarcopenia, although the optimum regime specific for older adults remains unclear (Kosek, Kim, Petrella, Cross and Bamman, 2006).

Though resistance training can increase protein synthesis and thus promote muscle hypertrophy in young and older populations, without feeding, such changes are minimal. Specifically, dietary supplements such as whey protein and casein have been previously shown to enhance muscle adaptions from resistance training (Farnfield, Carey and Cameron-Smith, 2005). There is evidence to suggest that compared to regular protein supplements, whey protein isolate is more effective at increasing blood amino acids and protein synthesis due to its different absorption kinetics and amino acid profile (Mahe *et al*., 1996; Bucci and Unlu, 2000). Hydrolyzed whey proteins are rapidly absorbed in the upper jejunum (Mahe *et al*., 1996) and contain the highest concentration of the essential amino acids, including BCAA's (Bucci and Unlu, 2000). Furthermore, whey protein exhibits the highest biological value (BV) of any known protein (Renner, 1983). Whey protein has a BV score of 104 whereas casein, another milk protein, beef and fish have BV scores of 77, 75, and 75, respectively (Renner, 1983). Biological value is the measure of the protein's ability to retain nitrogen in the muscle (Colgan, 1993), and a positive nitrogen balance is associated with muscle anabolism (protein synthesis). Previously, Cribb *et al.,* (2006) demonstrated that whey protein significantly enhanced the effects of resistance training on muscle strength and performance in humans. In support of these

effects, Tipton and colleagues (2004) showed that following acute ingestion of whey protein supplementation after exercise, muscle protein synthesis was increased, resulting in net muscle protein synthesis. In contrast to whey protein isolate, casein protein forms a gel or clot in the stomach, and therefore is slowly absorbed providing a sustained gradual release of amino acids into the blood stream, sometimes lasting for several hours. Recently, Tang and colleagues (2009) demonstrated that the feeding-induced simulation of muscle protein synthesis (MPS) in young men is greater after whey hydrolysate consumption than casein both at rest and after resistance exercise; moreover, despite both being fast proteins, whey hydrolysate stimulated MPS to a greater degree than casein after resistance exercise. These differences may be related to how quickly the proteins are digested (i.e., fast vs. slow) or possibly to small differences in leucine content of each protein. Though acute changes in MPS following whey hydrolsate and casein protein supplementation are evident in younger individuals, further research is needed to determine whether each milk protein exerts the same effect in older individuals. A recent finding suggests that muscle protein synthesis is possibly delayed in older compared to younger individuals following resistance exercise (Drummond, *et al.*, 2008). Since, whey protein ingestion provides a rapid, but short lived increase in blood AA compared to casein, it could be suggested that a more prolonged elevation of AA in the blood following casein supplementation may be more effective at stimulating MPS following resistance exercise in older individuals due to the delay in MPS. However, such differences influence long term adaptations in muscle growth and development is unclear. Given the link between sarcopenia and disability among elderly men and women and the expanding number of elderly people in the United States; sarcopenia is becoming an

increasing health issue in the western world. Therefore, the need for continued research into the development of the most effective interventions to prevent or at least partially reverse sarcopenia, including the role of resistance exercise and other novel pharmacological and nutritional interventions is clearly needed.

Purpose of the Study

The overall purpose of this study was to determine if whey isolate protein and casein protein ingestion, when combined with a 12-weeks resistance training program would provide greater enhancement of body composition, muscle strength and systemic and cellular regulators of satellite cell activation, compared to carbohydrate only ingestion.

Aim 1: To determine the chronic effects of whey isolate protein and casein protein ingestion on lean muscle mass, fat mass and body fat percentage when combined with a fully supervised resistance training program in older males.

Aim 2: To determine the chronic effects of whey isolate protein and casein protein ingestion on upper and lower body muscle strength when combined with a fully supervised resistance training program in older males.

Aim 3: To determine the mechanisms by which whey isolate protein and/or casein protein are eliciting their positive effects within the skeletal muscle by firstly examining anabolic hormone levels testosterone and IGF-1 within the serum, and secondly, examining indirect markers of satellite cell activation, HGF and c-MET, within the serum and muscle, respectively.

Hypotheses

H1: Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant differences in upper and lower body strength in older males when compared to placebo

H2: Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in lean body mass in older males when compared to placebo

H3: Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in fat mass in older males when compared to placebo.

H4: Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in body fat percentage in older males when compared to placebo.

H5: Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in IGF-1 expression when compared to placebo.

H6: Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in HGF expression when compared to placebo.

H7: Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in serum free testosterone levels when compared to placebo.

H8: Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in c-Met receptor expression when compared to placebo.

Delimitations

- Subjects consisted of men between the ages of 54-76. Subjects were deemed medically capable of engaging in an exercise program. This was determined by their primary care physician before enrolling in the study. Subjects with any of the following conditions were excluded from the study if they had, unless otherwise approved by their doctor,: 1) any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypongonadism; 2) history of pulmonary disease, hypertension, liver or kidney disease, musculoskeletal disorders, neuromuscular or neurological diseases, autoimmune disease, cancer, peptic ulcers, or anemia; 3) taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic, psychotropic, neuromuscular/neurological, or androgenic medications; 4) any bleeding disorders; 5) any chronic infections (e.g. HIV); 6) no concurrent participation in other physical or exercise modalities.
- Subjects were randomly assigned to one of three treatment arms: resistance exercise and 1) carb only, 2) casein + carbohydrate, 3) whey + carbohydrate.
- Baseline measurements included resting heart rate, height, weight, blood pressure, BIA, DEXA scan, blood draw, muscle biopsy (vastus lateralis), and a 1-rep max on bench press and leg press. Identical measurements were taken again at the end of weeks 4, 8, and 12.

- All exercise regimens were done at Baylor University in Russell Gymnasium .
- All sessions were guided. Guided exercise ensured homogeneity among research participants.

Limitations

- A broad spectrum of age and severity of sarcopenia could have possibly skewed the results of the study.
- The exercise prescription was not individualized (other than intensity determined by the initial and subsequent 1-rep max tests), which would possibly give the best results; however, this was simply not feasible with a large population. Also, a homogeneous population among all treatment arms is standard.
- The variation of resistance training exercises, sets/reps, and progressions was limited. The need for consistency limits the study subjects to stay at 3 sets of 10 repetitions 3 days/week for 12-weeks. Although the intensity changes, this got monotonous for subjects and the gains that they may have seen otherwise with individual training may not be as much.

Assumptions

- The assumption was made that all subjects did not perform any other resistance training exercise outside of the study.
- It was also assumed that subjects take their supplement everyday for the entirety of the study
- The assumption that, on workout days, the supplement was taken within 30 minutes after resistance training session over the 12-week study was made.

- It was also assumed that the only exercise that was done is the exercise protocol given to them.
- Assumptions were also made that all subjects truly give a valiant effort in all baseline tests and post-tests.

Definition of Terms

1. MyoD- myogenic regulatory factor active during myogenesis
2. Myogenin-myogenic regulatory factor active during myogenesis
3. MRF-4- myogenic regulatory factor 4. Also known as Herculin or myf-6. Basic helix-loop-helix (bHLH) proteins that are known to have vital roles in the regulation of muscle gene transcription during the differentiation process of developing skeletal muscle .
4. myf5- myogenic regulatory factor active during myogenesis
5. Myosin Heavy Chain- large elongated protein consisting of a long tail extending to the carboxy terminal and a globular domain at the amino terminal.
6. Satellite Cells- quiescent cells located on the surface of the myofiber but beneath the basal lamina.
7. Myofibril-cylindrical structure within muscle that contains contractile proteins. Separated from other myofibrils by the sarcoplasmic reticulum and intermyofibrillar mitochondria.
8. Hypertrophy- growth of a tissue or organ as a result of increased size of individual cells.
9. Myonuclei- nucleus within a muscle fiber

10. Sarcopenia- decreased muscle mass seen with advancing age due to decreased fiber size and number.
11. Atrophy- decreased tissue size or wasting. Often results from lack of use or specific disease process.
12. Apoptosis- cell death. Also known as programmed cell death. Occurs under normal physiological conditions.
13. Sarcolemma-plasmalemma and basement membrane of a muscle fiber.
14. Basal lamina- portion of basement membrane that lies adjacent to muscle fiber membrane. Made up of electron-lucent lamina lucida and electron-dense lamina densa.
15. Myoblast- precursor cells that will become muscle cells by fusion to form myotubes that evolve into muscle cells.
16. IGF-1-Insulin-like growth factor.

CHAPTER TWO

Review of Literature

Aging is a simple fact of life that affects all of us differently. One inevitable consequence of aging in which most of the population has to face is the deterioration of muscle mass known as sarcopenia. In fact, roughly 45% of the U.S. population is sarcopenic (Janssen, Shepard, Katzmarzyk and Roubenoff, 2004). Sarcopenia can be the foundation upon which many age-related aliments are built. Such aliments that are associated with aging and loss of muscle mass are: osteoporosis, diabetes, unwanted weight gain, an increased susceptibiity to illness, falls and related injuries.

Sarcopenia is considered an important public health problem due to its primary (metabolic alterations) and secondary consequences (strength loss, decreased autonomy) (Rieu, *et al*., 2006). In the United States alone, $18.5 billion of total direct health care costs in 2000 were attributable to sarcopenia (Janssen, *et al*., 2004) and this will undoubtedly increase due to the world's population living longer. One of the most costly issues that comes from the aformentioned result of sarcopenia are those that arise from falls. More than 400 potential risk factors for falling have been identified, but a consistent finding is the link with sarcopenia. Although multifactorial in origin, several factors have been attributed to the emergence of sarcopenia. Among these are inadequate nutrition (i.e. calorie low protein intake [Vellas, Hunt, Romero, Koehler, Baumgartne and Garry, 1997]); decreased production of anabolic hormones (e.g., testosterone, DHEA, growth hormone, IGF-1 [Bhasin, 2003]), low grade inflammation (Schaap, Pluigm, Deeg and Visser, 2006) and lack of physical activity (Deschenes, 2004). An in depth review of

all factors contributing to sarcopenia is beyond the scope of this thesis, therefore, the current chapter will only review those primary causes that influence muscle mass and quality.

Sarcopenia

Sarcopenia is a disease in which the aging population will inevitably have to face. Typically, at the age of 30, individuals lose ~5% of their lean muscle mass per decade (Holloszy, 2000). At the age of 50, this number increases to 1-2% of lean muscle mass loss per year (Shel and Yates, 2001).

Lean muscle mass generally contributes up to ~50% of total body weight in young adults but declines with aging to 25% when reaching an age of 75–80 yr (Short, Vittone, Bigelow, Proctor, and Nair, 2004). The loss of muscle mass is typically offset by gains in fat mass. Cross-sectional data suggests that the average adult can expect to gain approximately 1 pound of fat every year between the ages of 30-60, and lose about a helf pound of muscle per year; a change that is equivalent to a 15 pound loss of muscle and a 30 pound gain in fat (Cribb, 2006). This undesirable gain can result in a variety of health related and metabolically challenging issues. In fact, the absolute decline in muscle mass and muscle oxidative capacity, in combination with a greater fat mass, contributes to the greater risk of developing insulin resistance and/or type 2 diabetes due to the reduced capacity for blood glucose disposal and a greater likelihood of excess lipid deposition in liver and skeletal muscle tissue. The latter will also lead to hyperlipidemia, hypertension, and cardiovascular comorbidities (Koopman and van Loon, 2009). Also, the knee extensors (e.g., vastus lateralis), which are important for ambulation and weight-bearing

function, demonstrate a 30% decrease in whole muscle size between the ages of 50 and 80 yr (Deschenes, 2004).

Essentially, sarcopenia is a result of dysfunction in the regulatory mechanisms that maintain the size of human muscle mass. The quality and quantity of muscle protein is maintained through a continuous remodeling process (known as protein turnover) that involves continuous protein synthesis and breakdown (Cribb, 2006). A number of regulators affect muscle protein turnover within muscle. These include, but not limited to, decreased production of anabolic hormones testosterone and IGF-1 (Bhasin, 2003), decreased total caloric and protein intake (Vellas, *et al*., 1997), low grade inflammation (Schaap, *et al*., 2006), and reduced physical activity (Deschenes, 2004). Each of these regulators exerts their effects via the stimulation or inhibition of protein synthesis and protein degradation. Sarcopenia may be the result of increased or reduced expression of some or all of these regulators.

Protein Turnover

Regulation of whole body protein metabolism seems to have a direct link to sarcopenia, with muscle tissue having an integral role in the regulation of whole body amino acid metabolism (Cribb, 2006). The regulation of whole body protein metabolism involves a regulatory system between muscle, blood and liver metabolism of amino acids (AA). Specifically, plasma cysteine is an important regulator of whole body protein metabolism that ultimately influences changes in body composition (Holm *et al*.,1997). The amount of this "non-essential" AA in plasma regulates whole body protein metabolism via the hepatic catabolism of cyst(e)ine (i.e., both cysteine and its disulphide twin, cystine) into sulphate (SO42-) and protons (H+), an essential process that inhibits

the rate limiting step in hepatic urea production (the end product of protein degradation) and shifts whole body nitrogen disposal toward glutamine biosynthesis. The most important function of this regulatory circuit is to ensure that any time urea production is too high and plasma AA are accordingly too low, controlled muscle catabolism is triggered. This leads to export of cyst(e)ine from muscle, an increase in plasma cyst(e)ine and a down regulation of hepatic urea production (and preservation of LBM). However, a few studies have suggested that in cachectic conditions (illnesses that promote muscle wasting such as HIV and various forms of cancer), but also aging, this regulatory process appears to be disturbed. That is, a failure to conserve muscle proteins and convert abnormally large amounts of AA into glucose, and release large amounts of nitrogen as urea. The availability of cyst(e)ine in the blood stream could determine the threshold at which other AA are converted into other forms of chemical energy, which in turn may influence body whole body protein metabolism, and thus maintenance of muscle mass and size (Hack *et al*., 1996;1997;1998; Kinscherf *et al*.,1996; Holm *et al*.,1997).

Within the muscle, quality and quantity of lean muscle mass is maintained through protein turnover that involves not only protein breakdown, but more importantly, continuous protein synthesis of muscle protein turnover and their ability to stimulate or inhibit protein synthesis and degradation. This process is controlled by the initiation of protein synthesis and degradation (Hasten, Pak-Loduca, Obert *and* Yarasheski, 2000). The regulation of protein turnover within the muscle is multifaceted, but this process is basically controlled by the initiation of protein synthesis (P13K-mTOR signaling pathway) and degradation (via proteolytic pathways) (Rennie *et al*., 2004). Whilst a

comprehensive review of these pathways is beyond the scope of this thesis (for review see Bolster *et al.,* 2004.), they need to be mentioned, as certain factors (hormones, exercise and amino acids) appear to activate these pathways, and thus up-regulate protein synthesis or minimize protein breakdown. Furthermore, activation of these pathways may assist in explanation of results described in later chapters.

Protein Synthesis

The regulation of protein synthesis includes initiation, elongation and termination (Bergmann and Lodish, 1979). However, compared to the latter two potential points of control, initiation appears to be the rate-limiting step in the overall regulation of protein synthesis (Sonenberg, 1994). The rate of initiation of protein synthesis can be regulated directly or indirectly through a number of multiple signal transduction pathways.

Skeletal muscle protein synthesis is closely regulated *in vivo*, and the phosphatidylinositol 3-kinase (PI3-knase)/mammalian target of rapamycin (mTOR) pathway has been implicated as having a pivotal role in this process. Within this pathway, protein kinase B (or Akt), eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (2), and ribosomal protein S6 kinase (p70S6K) are several key intermediates involved in the regulation of translation initiation and protein synthesis (Proud *and* Denton, 1997). In addition, activation of Akt/PKB controls glycogen synthase kinase (GSK), which in turn causes activation of eukaryotic initiation factor (eIF)2B, and thus increased global protein synthesis, and cell growth. Basically, Akt/PKB serves as a branch point in the P13K-mTOR signaling pathway which in turn leads to rapid phoshorylation of 4E-BP1 and p70S6K, and thus increased translation of mRNA encoding specific proteins (Bolster *et al.,* 2004). Phosphorylation of 4E-BP1

frees eIF4E, which can then associate with eIF4G to form the preinitiation complex and initiate protein synthesis (Kleijn, Scheper, Voorma, Thomas, 1998). Phosphorylation of p70S6K increases the phosphorylation of ribosomal protein S6 and facilitates the synthesis of some ribosomal proteins, initiation factors, and elongation factors that play important roles in protein synthesis (Pearson, 1995).

Drummond and colleagues (2008) found that muscle protein synthesis may in fact be delayed in older individuals. In fact, they found that in young populations (29.7±1.7), muscle protein synthesis occurs rapidly after a bout of exercise and ingestion of BCAA. This however, is not the case with older individuals (70.0±2.1). Muscle response was actually be delayed (up to 6 hours) in older individuals (Drummond, *et al*., 2008). In this study, when older individuals took the same BCAA after a single bout of resistance training, (leucine enriched supplementation) the older men's muscle protein synthesis peaked at 6 hours, vs. 2 hours in the younger population (Drummond, *et al*., 2008). There are several factors that may play a role in this observed delay and degradation of muscle protein synthesis in older individuals. mTOR is a serine/threonine protein kinase that regulates cell growth, proliferation, motility, survival, protein synthesis, and transcription. In younger individuals, a bout of resistance training increases activation of mTOR and subsequent downstream targets which ultimately lead to protein synthesis. In older individuals, this response is delayed. Although authors do not fully understand the mechanism behind this delay, it has been suggested that increased activation of adenosine monophosphate kinase (AMPK) may play a role. AMPK negatively regulates protein synthesis through the inhibition of the mTOR signaling pathway. In younger populations, AMPK levels remain low following resistance training. However, in older

individuals, AMPK levels are increased. Regardless of the cause, it is clear that reduced activation of mTOR signaling pathway will lead to reduced protein synthesis, activation of satellite cells and subsequent growth.

Muscle Satellite Cells

Adult muscle cells contain a population of quiescent undifferentiated monocuclear myogenic cells located between the sarcolemma and basal lamina of the muscle fiber (Petrella, *et al*., 2008). Since their first description in the early 1960s, the discovery of muscle satellite cells have confirmed the existence of population of proliferative cells that contribute to postnatal growth, repair of damaged fibers and maintenance of adult skeletal muscle (Mauro, 1961). Satellite cells are present in all skeletal muscles; however, satellite cell population is dependent on species, fiber type composition and age (Hawke and Garry, 2001).

It has been debated in muscle biology that whether myonuclear addition is required during skeletal muscle hypertrophy. Findings strongly suggest myonuclear addition via SC recruitment may be required to achieve substantial myofiber hypertrophy in humans. In skeletal muscle tissue, SCs are essential for myofiber repair, maintenance, and growth. As such, an age-related decline in SC number and/or function could have an important role in the etiology of sarcopenia. Previous studies have either shown a similar or lower SC content in older adults when compared with young adults (Sinha-Hikim, Cornford, Gaytan, Lee *and* Bhasin, 2006). Individuals with a greater basal presence of SCs demonstrated, with training, a remarkable ability to expand the SC pool, incorporate new nuclei, and achieve robust growth (Petrella, *et al*., 2008). During postnatal development, SCs proliferate and differentiate into new myonuclei to support the

increasing needs of the growing myofibers. In mature muscle fibers, SC are typically quiescent but can become activated in existing myofibers. Upon damage (such as that caused by resistance training) they fuse with each other forming new myofibers, replacing damaged fibers and/or myonucei (Hawke and Garry, 2001, see Figure 1). Therefore, ongoing muscle repair is needed for muscle fiber maintenance, and requires a continuous supply of functional SC throughout one's lifetime. In addition to SC content, proliferative capacity of SC may also be diminished. Rodent models show a significant decrease in the satellite cell population from 4.6% of all cells at 8 months of age to 2.4% at 30 months of age, has been found in the murine soleus (Snow, 1977).

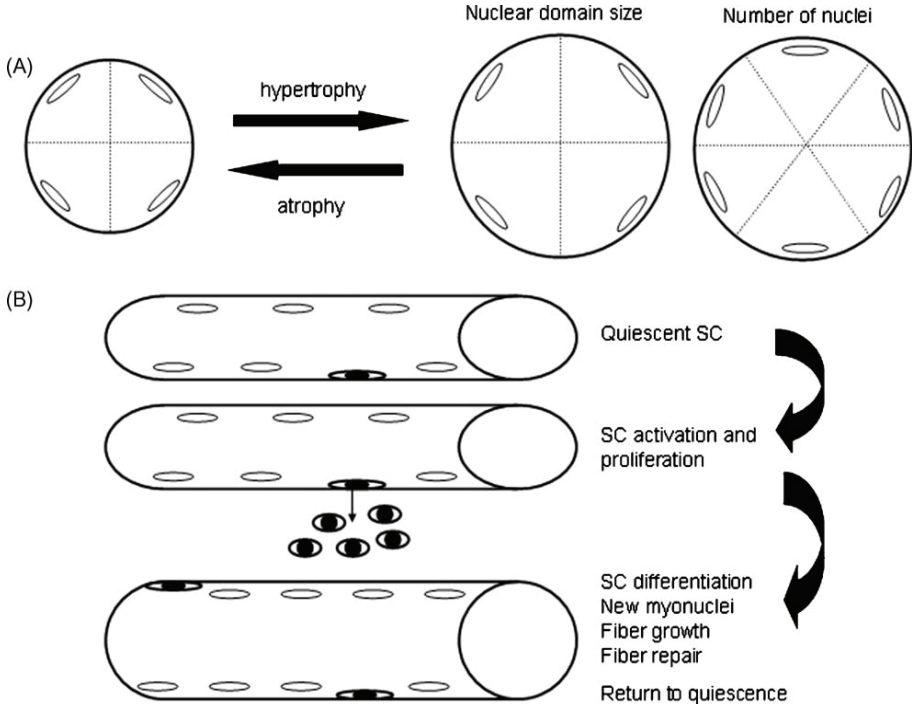


Figure 1: Myonuclei and satellite calls in the skeletal muscle adaptive response. (from Sniders, Verdijk*and* van Loon, 2009).

Human skeletal muscle, however, does not demonstrate the same decline in satellite cell numbers with increasing age. In addition, humans fail to demonstrate a difference in the proliferative potential of satellite cells derived from children aged 9

years and those isolated from adults greater than 60 years of age. Thus, it is apparent that the impaired skeletal muscle regenerative response seen with aging in humans likely results from more factors than just declining satellite cell numbers and proliferative capacity (Jejurikar and Kuzon, Jr., 2003). It is clear that decline in the number of SC and their inability to become active and proliferate in response to anabolic stimuli may contribute to the age-related loss of muscle mass and strength (Verdijk, *et al*., 2007).

In vitro studies have implicated an extensive number of trophic factors involved in maintaining a balance between growth and differentiation of satellite cells to restore normal muscle architecture (for a review see Wozniak *et al.,* 2005). These include members of the fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) families, insulin-like growth factor (IGF), hepatocyte growth factor (HGF), tumour necrosis factor-α (TNF-α), IL-6 family of cytokines, neural derived factors, nitric oxide (NO) and ATP.

HGF, C-Met and Satellite Cell Activation

Hepatocyte growth factor is one of the primary effectors of satellite cell activation via the c-Met receptor (Allen *et al.,* 1995). HGF is made by fibers and stored or sequestered in the surrounding extracellular matrix (Tatsumi *et al.,* 1998). C-Met is the HGF receptor and often indicates increases in satellite cell activation. The binding of HGF to c-Met initiates numerous signalling cascades including the mitogen-activated protein kinase (MAPK) and phosphatidyl inositol 3 kinase (PI-3K) pathways (Furge *et al.,* 2000), all of which positively influence the transcription of genes required for growth and cell division (Vermeulen *et al.,* 2003).

It has been shown that HGF can stimulate activation and early division of adult satellite cells in culture, and that the action of HGF is similar to the action of the unidentified satellite cell activator found in extracts of crushed muscle (Allen, Temm-Grove, Sheehan and Rice, 1997). Evidence also demonstrates that hepatocyte growth factor is present in uninjured adult rat skeletal muscle and that the activating factor in crushed muscle extract is hepatocyte growth factor (Tatsumi, Anderson, Nevoret, Halevy and Allen, 1998). In the aforementioned study, immunoblots of crushed muscle extract demonstrate the presence of hepatocyte growth factor in rats after muscle damage was induced. Also, c-Met was found to be localized to putative satellite cells. In muscle, hepatocyte growth factor/scatter factor and c-Met are co-localized in activated satellite cells in regions of muscle repair. This study concluded that with direct injection of hepatocyte growth factor into uninjured tibialis anterior muscle of 12-month-old rats stimulated satellite cell activation. These experiments demonstrate that hepatocyte growth factor/scatter factor is present in muscle, can be released upon injury, and has the ability to activate quiescent satellite cells *in vivo*. In one study, the human skin fibroblast from old donors (over 80 years) produced more than those from young and middle-aged donors (Miyazakia, Gohdab, Kajic, Nambaa, 1998). From these findings, it is likely that HGF production of interleukin-1 (IL-1a) by human fibroblasts increases in the process of aging *in vivo* as well as *in vitro*. In addition, expression of the HGF receptor gene c-Met also increased in these cells with aging in culture. This may be due to increases HGF over expression in these cells.

Myogenic Regulatory Factors

In addition to trophic factors that indirectly mediate satellite cell activity through specific signaling pathways (PI-3K pathway; Coolican *et al.,* 1997), myogenic regulatory factors (MRFs), directly control myogenic cell differentiation and proliferation due to their ability to directly bind to DNA, hence initiating DNA transcription and regulating gene expression. MRFs, which include Myo-D, myogenin, MRF-4, and Myf5, are members of a family of basic helix-loop-helix proteins. In general, Myo-D and Myf5 are involved in the determination of myoblasts during proliferation, whilst myogenin and MRF-4 are involved in the later stages of adult fiber differentiation. MRFs also regulate satellite cell differentiation and induce transcription of skeletal muscle-specific genes such as creatine kinase, myosin light chains, myosin heavy chains (MHC), troponin I, and desmin (Braun and Arnold, 1991).

Repair/regeneration and growth of skeletal muscle is largely dependent on the addition of myonuclei to existing, terminally differentiated myofibers, by activation of satellite cells (Holterman and Rudnick, 2005). As stated earlier, with injury or stress to muscle fibers, satellite cells are induced to proliferate and differentiate, ending with their incorporation into mature myofibers as myonuclei. These events are regulated by several local processes that appear to respond in a load-dependent manner. Expression of the "early" MRFs, myogenic factor (myf)-5 and myogenic differentiation factor D (MyoD), commit somatic cells to the myogenic lineage, whereas the "late" MRFs, myf-6 and myogenin, terminally differentiate proliferative myoblasts toward formation of multinucleated myotubes in developing muscle (Sabourin and Rudnicki, 2000) or into nuclear donors to developed myofibers for repair/regeneration and growth. Myogenin

and MyoD are also implicated in regulating MHC transitions (Mozdziak, Greaser *and* Schultz, 1998). Basal levels of some MRFs are upregulated in aging, sarcopenic muscle and, based on recent findings in rodents, the magnitude of upregulation appears to be directly linked to the degree of sarcopenia (Drummond, *et al*., 2008), suggesting that sarcopenic muscles remain in a state of failing compensatory effort in an attempt to stave off degeneration and atrophy

As stated earlier, sarcopenic muscles have been found to remain in a state of failing compensatory effort in an attempt to stop muscle atrophy. The ability for the muscle to regulate its time in the catabolic and anabolic states determines whether or not muscle growth is achieved. If the aging muscle is in fact, in the catabolic state as a protective mechanism, then the ability for the muscle to be broken down further and then regenerate is compromised. Muscle proteolysis has been studied to determine whether or not this is truly a factor of the deterioration of muscle mass with age.

Muscle Proteolysis

Increased muscle degradation may occur due to several factors in aging muscle. Skeletal muscles, like other mammalian tissues, contain several proteolytic systems. Muscles also contain two major cytosolic proteolytic pathways, Ca2+-dependent and ATP-ubiquitin-dependent (Dardevet, Sornet, Taillandier, Savary, Attaix, Grizard, 1995). The Ca2+-dependent proteinases do not contribute significantly to increased skeletal muscle proteolysis in many muscle wasting conditions and do not play a major role in the degradation of myofibrillar proteins (Furuno, Goodman, Goldberg, 1990). By contrast, the ATP-ubiquitin-dependent proteolytic pathway, which was previously widely believed to degrade abnormal and short-lived proteins, have been shown to play a role in the

degradation of myofibrillar proteins (Ciechanover, 1994). In this pathway, ubiquitin first covalently binds to protein substrates in a multistep process requiring ATP, and serves as a signal for degradation. Ubiquitin-protein conjugates are then preferentially degraded by a very large 1,500-kD (26 S) proteolytic complex that also requires ATP for activation and substrate hydrolysis (Dardevet, 1995).The 26 S complex consists of the 20 S proteasome core, a multicatalytic proteinase composed of 14 different subunits, plus regulatory components containing several proteins including ATPases (Ciechanover, 1994). Experiments have shown that glucocorticoids regulate the ATP-ubiquitin dependent proteolytic pathway in muscles from starved and acidotic rats, apparently by increasing the expression of ubiquitin and of 20 Sproteasome subunits (Wings and Goldberg, 1993). To our knowledge, the effects of glucocorticoids on gene expression of lysosomal or Ca2+- dependent proteinases are unknown in skeletal muscle. With aging, the ubiquitin-proteoysis process is upregulated. This means more cells are "tagged" for elimination from the body. Increased expression of genes in the ubiquitin-proteasome pathway might be related to impairment of proteasome function caused by accumulation of lipofuscin in old muscle (Welle , Brooks , Delehanty , Needler,Thornton, 1993).

Degradation of myofibrillar proteins is regulated through the ubiquitin-proteasome system (UPS) and key regulatory proteins are consistently increased in studies showing skeletal muscle atrophy (Jagoe and Goldberg, 2001; Lecker *et al*., 2004 and Price, 2003). Two genes, atrogin-1 (muscle atrophy F-box) and muscle ring finger 1 (MuRF1) encoding the E3 ubiquitin ligases have been identified (Bodine *et al*., 2001). They are specifically expressed in cardiac and skeletal muscles and are dramatically up-

regulated in multiple atrophy models (Bodine, *et al*., 2001). Furthermore, when under normal conditions MuRF1 and atrogin-1 mice appear to be phenotypically identical to wild-type littermates, after muscle denervation these animals display a partial protection against muscle wasting (Bodine *et al*., 2001). In one study, it was observed that during the early stage of sarcopenia (24 months of a Sprague–Dawley rats which have a maximal life expectancy of 28 months), the loss of muscle mass in rat *Tibilalis Anterior* was associated with elevated levels of ubiquitinated proteins (Clavel, Coldefy, Kurkdjian, Salles, Margaritis, Derijard, 2006). These results suggest that ubiquitin conjugation is higher in aged muscles. This result is supported by a previous study which shows a large increase in ubiquitin protein level in old rat and human muscles (Cai, Lee, Li, Tahnk and Chan, 2004).

It is evident that regulators of muscle protein turnover work specifically by stimulation (acceleration) or inhibition of protein synthesis and protein degradation. The regulation of protein turnover is multifaceted. Cortisol, cytokines and ubiquitin proteins activate protein degradation whereas insulin, amino acids, mechanical loading and the anabolic hormones (testosterone, growth hormone, IGF-1, and mechno-growth factor) all stimulate protein synthesis (Cribbs, 2006). Whilst a comprehensive review of these factors is beyond the scope of this thesis, it is clear that anabolic hormones such as testosterone and IGF-1, mechanical loading and protein/amino acids all stimulate muscle protein synthesis to varying degrees, and thus are critical in maintaining and promoting muscle development and growth throughout a person's life.

Hormonal Factors: Testosterone & IGF-1

Testosterone is a steroid hormone from the androgen group. In mammals, testosterone is primarily secreted in the testes of males and the ovaries of females, although small amounts are also secreted by the adrenal glands. It is the principle male sex hormone and an anabolic steroid. In men, testosterone plays a key role in the development of male reproductive tissues such as the testes and prostate as well as promoting secondary sexual characteristics such as increased muscle and bone mass and hair growth. In addition, testosterone is essential for health and well-being as well as preventing osteoporosis. On average, an adult human male body produces about ten times more testosterone than an adult human female body.

As stated earlier, there are several hormonal explanations for the loss of muscle mass with age. Most notably, the loss of testosterone with age has been linked with the loss and inability to gain muscle mass when compared with younger counterparts. It has been demonstrated that myofiber hypertrophy with resistance training is superior in young men vs. young women and older adults (Kosek, *et al*., 2006). One obvious difference in these two populations is the amount of free-testosterone in each population. It is common knowledge that a steady decrease in testosterone occurs over time in men (Mitchell and Harman, 2001). In one study, it was shown that free testosterone was 59% higher in young men (27.0±1 yr) when compared to older men (63.7±1 yr **)** (Petrella, Kim, Cross, Kosek,Bamman, 2006). Cross-sectional studies show that at age 80 yr the total serum testosterone concentration is approximately 75%, and the free testosterone concentration is approximately 50% of that at age 20 yr (Snyder, Peachey, Hannoush, Berlin, Loh, Lenrow, Holmes, Dlewati, Santanna, Rosen, Strom, 1999). In young men,

testosterone has been shown to decrease fat mass, increase lean mass and increase muscle strength.

Insulin-like growth factor (IGF-1) is a trophic factor required for the proliferation of myoblasts, the porliferation of myogenic differentiation and subsequent growth and hypertrophy of myofibers. Age-related decrease in plasma IGF-1 concentration is well established and has been linked to muscle wasting (Renganathan, Messi and Delbono, 1998). Reduced IGF-1 singnalling is involved in muscle atrophy and results from decreased muscle exercise, reduced growth hormone and insulin levels, reduced vitamin D and treatment with drugs like corticosteroids, dexamethasone and cyclosorin. In addition, elevated leves of infammatory cytokines like TNF-α and IL-6 can cause muscle wasting (Grounds, 2002). TNF-α may also act by inhibiting IGF-1 signalling. Excitation-contraction uncoupling has been identified as a mechanism underlying skeletal muscle weakness in aging mammals. The basic mechanism for this uncoupling is a larger number of ryanodine receptors (RyR1) uncoupled to dihydropyridine receptors (DHPRs) (Delbono, O'Rourke and Ettinger, 1995). In one study, results of using transgenic mice oversxpressing IGF-1showed that IGF-1 dependent prevention of age-related decline in DHPR expression is associated with stronger muscle contration in older transgenic mice (Renganathan, Messi and Delbono, 1998). IGF-1 stimulates Ca^{2+} influx by activating a Ca^{2+} permeable cation channel via the IGF-1 receptor, and Ca^{2+} influx may play a cricital role in the mitogenic action of IGF-1. The age-related decrease in DHPR expression leads to a significant impairment in the sarcolemmal action potential transduction into Ca^{2+} signal and consequenty in a dimished mechanical response (Delbono, et. al., 1995).

IGF-1 also inhibits degradation via the ubiquitin pathway. Studies performed both on cell culture and rodents have suggested that the transcriptional regulation of atrogin-1 and MuRF1 is controlled by an IGF-1/Akt dependent signalling pathway (Sandri *et al*., 2004 and Stitt *et al*., 2004). In addition, it has been demonstrated that both the proinflammatory cytokine Tumor Necrosis Factor α (TNFα) and reactive oxygen species (ROS) induce transcriptional up-regulation of MuRF1 and atrogin-1 (Clavel, *et al*., 2006). It has been speculated that up-regulation of atrogin-1 could be involved in the decline of myogenesis and regeneration capacities of the aged muscle through MyoD degradation and thereby participates in the etiology of sarcopenia (Clavel, *et al*., 2006). Also, the stimulation of atrogin-1 in aging *via* the downregulation of the IGF-1/Akt pathways is in agreement with data showing that overexpression of a locally acting isoform of IGF-1 expressed in skeletal muscle preserves muscle mass and regenerative potential (Musaro, McCullagh, Paul, Houghton, Dobrowolny, Molinaro, Barton, Sweeney, Rosenthal, 2001). Despite age related declines in testosterone and IGF-1, it has been well documented that exercise, in particular, resistance exercise can influence their levels.

Exercise and the Aging Muscle

Resistance exercise has been an increasingly recognized as an important component for the maintnance and growth of muscle mass. An overwhelming amount of literature demonstrates that resistance training can benefit older individuals in a variety of ways ([Chrusch and Chilibeck, 2001], [Esmarck, *et al*., 2001], [Hasten, *et al*., 2000], [Izquierdo and Hakkinen, 2001], [Kosek, *et al*, 2006], [Yarasheski, *et al*., 1993]). Hypertrophy, gains in strength and increased quality of life have all been demonstrated.

The literature does not dispute that resistance training is a health benefit for older individuals. The questions that remain are about the mechanisms in which muscle mass deterioration occurs, the reasons younger individuals respond differently than older individuals and how we can slow the process of muscle wasting and make the response to exercise a more beneficial adaptation in older individuals.

Disability prior to death is not an inevitable part of a long life but may be prevented by moderate physical activity (Leveille, Guralnik, Ferrucci *and* Langlois, 1999). Specifically, it has been shown that muscle mass and function are improved in the elderly during resistance exercise training. These improvements must result from alterations in the rates of muscle protein synthesis and breakdown (Yarasheski, Zachwieja and Bier, 1993). High-resistance weight training leads to significant gains in muscle strength, size, and functional mobility among frail residents of nursing homes up to 96 years of age (Fiatarone, *et al*., 1990).

Despite improvements in muscle performance and reduction in physical frailty, the increase in muscle cross-sectional area seems to be modest in older populations (Yarasheski, *et al*., 1999). This suggests that muscle fibers of physically frail elders have a reduced capacity to hypertrophy in response to weight-lifting exercise training. Muscle protein synthesis adaptations are lower in healthy older men and women than in young adults (Welle, *et al*., 1995). Whether this results from relative disuse rather than aging alone has been debated among health care providers and researchers. Data, however, suggests that the slower myofibrillar synthesis rate in older subjects cannot be explained by disuse (Welle, *et al*., 1995). The underlying mechanism behind this phenomenon remains to be determined. In one study, the results showed that 3 days/wk training led to

more robust hypertrophy in young vs. old particularly among men. However, this differential hypertrophy adaptation was not explained by age variation in (muscle regulatory factor) MRF expression (Kosek, *et al*., 2006). In other studies, it has been observed that there are substantially lower basal mixed, myofibrillar, and/or mitochondrial muscle protein synthesis rates in the elderly vs. the young (Balagopal, *et al*., 1997). A decline in the synthesis rate of myosin heavy chain implies a decreased ability to remodel this important muscle contractile protein and likely contributes to the declining muscle mass and contractile function in the elderly. In a 16 week resistance training regimen, an increase in strength and type II muscle fibers was observed in young and older populations, however, the growth response was less compared to young. Drummond and colleagues (2008) found that the rate of MPS was comparable in the older population to the younger population (the onset of MPS was simply delayed in older individuals). The muscle protein synthesis in older populations did not change at all 1-3 hours after a singe bout of resistance training (Drummond, *et al*., 2008). It was demonstrated in the same study, however, that muscle protein synthesis may be delayed up to 6 hours in older individuals. The rate at which muscle protein synthesis occurred (despite time differences) was comparable to the younger individuals in the study.

Acute bouts of resistance training have been shown to increase muscle protein synthesis in older and younger individuals with the aid of supplementation (Yarasheski, Zachwieja and Bier, 1993; Hasten, *et al*., 2000; Drummond, *et al*., 2008). A recent study found for the first time in human muscle a positive correlation between extent of phosphorylation of p70s6K and MPS, albeit only in the young subjects (and not in older individuals) (Kumar, *et al*., 2009). This strengthens the support for a major role for

p70s6K in stimulating MPS after exercise, and short-term changes in both predict the longer term changes. The lack of such a correlation in the older subjects is consonant with a blunted response of MPS to exercise, and reports that muscle hypertrophy after resistance exercise training is less in older men (Kosek *et al*. 2006).

Resistance training without supplementation and long-term adaptations have been studied. Frontera *et al*. (1990) showed that older men responded to a 12-week progressive resistance training program (80% of the 1 repetition maximum, 3 sets of 8 repetition of the knee extensor and flexors, 3 days per week) by more than doubling extensor strength and more than tripling of flexor strength. Also, in longer resistance trained studies *without* supplementation, it has been shown that older men do gain strength, but body composition and fiber type remain unchanged (Verdijk, *et al*., 2009). In the aforementioned study, it was observed that skeletal muscle hypertrophy is shown to be specific for the Type II muscle fibers and accompanied by a specific increase in Type II muscle fiber SC content. In young adults, however, it has been shown that SC content is similar in Type I and Type II muscle fibers, even after resistance training. These data might suggest a causal relationship between a decline in myonuclear and/or SC content and muscle fiber type–specific atrophy. The lower number of SCs might be insufficient to support myofiber maintenance and, as such, contribute to the gradual loss of muscle mass with aging. Reduced neuromuscular activity (due to a more sedentary lifestyle and/or functional disability) and/or impaired neuronal input might represent important factors contributing to the age-related decline in myonuclear and SC number and activity and the associated reduction in muscle fiber size. In one study, MHC and mixed protein synthetic rates increased in the younger (88 and 121%) and older participants (105 and 182%; $P <$

0.001 vs. baseline) (Hasten, *et al*., 2000). These findings indicate that MHC and mixed protein synthesis rates are reduced more than actin in advanced age. Similar to that of 23-32 yr olds, the vastus lateralis muscle in 78-84 yr olds retains the capacity to increase MHC and mixed protein synthesis rates in response to short-term resistance exercise.

Considering the large interindividual variability in the hypertrophy response to resistance training and the relations between the changes in myofiber size, SC content, and myonuclear content, it is interesting to speculate which factors might be responsible for the apparent differential responses. Previous research implicated that the myogenic regulatory factors (myogenin, MyoD, myf-5, and MRF4) and various growth agents such as insulin-like growth factor-I, mechano growth factor, and hepatocyte growth factor have a role in the (load-mediated) hypertrophy and in SC activation and/or proliferation and differentiation (Dhawan and Rando, 2005). However, most data are derived from in vitro and acute in vivo studies and, as such, little is known on the potential role these agents have in the response to prolonged exercise training. Although acute bouts of resistance training followed by supplementation have been studied, long-term adaptations still need to be assessed. This will help to serve as another building block for this area of research and to provide applicable information for aging adults. Before focusing on the effects that supplemental intervention may have on older individuals, one must examine the diet of older individuals as a whole and how this may, in fact, diminish responses to resistance exercise and the ability to maintain or gain muscle mass.

Diet Concerns

The fact that elderly people may require more dietary protein than the RDA has important implications. Surveys of the nutrition status of older people have shown that a

large number (up to 30%) consume the RDA or less each day. Inadequate intake of protein over a prolonged period of time results in a decrease in the rate of whole body protein turnover and an accelerated loss of muscle mass in elderly people (Castaneda, *et al*., 1995). The reduction of whole body protein turnover implies that there may be a selective loss in the ability of skeletal muscle to efficiently use exogenous amino acids for protein anabolism. A recent study, however, found that a moderate serving of high-quality protein (beef=30g protein) maximally stimulates skeletal muscle protein synthesis in elderly subjects when compared to 90g of protein (Symons, Sheffield-Moore, Wolfe, Paddon-Jones, 2009). This indicates that older individuals might want to consider ingestion of multiple moderate-sized servings of high-quality protein-rich foods over the course of a day instead of a single, large protein-rich meal.

The etiology of this increased need for dietary protein is not well understood. While some have demonstrated that the rate muscle protein synthesis is reduced in elderly people, it appears that basal rate of muscle protein synthesis is not depressed in this population compared to that of young people. Koopman, 2009, argues that more sensitive methods should be developed to assess both basal muscle protein synthesis and breakdown rates in vivo humans. There is evidence that post-prandial rise in amino acids results in a greater rise in muscle protein synthesis in young people compared to old. There is also a greater extraction of essential amino acid during the first pass through the liver in older individuals. This may potentially diminish the amount of amino acids presented to muscle. In addition, glucose intolerance may also play an important role in a reduced post-prandial increase in muscle protein synthesis in elderly people (Evans,

2004). Therefore, when considering supplementation for an individual, it is vital that the post-prandial response to the supplement be taken into account.

In the postprandial period, the main regulators of protein metabolism are insulin and amino acid (AA) availability. Insulin acts through inhibition of proteolysis and stimulation of muscle protein synthesis. The availability of AAs, which is reflected by plasma aminoacidaemia, is the major regulating factor of protein synthesis and oxidation *(see figure 2)* (Dangin, *et al*., 2003). Since AA availability is affected by the protein digestion rate, this might explain the effects of nitrogen sources differing by their kinetics on postprandial protein gain (Boirie *et al*. 1997a; Dangin *et al*. 2001). Evidence to explain the differences between young and old digestion rates for protein remain lacking. Koopman, 2009 states that there are restrictions set by the methodology that has been used to assess the appearance rate of amino acids from the digestive system into circulation.

The relevance of the results for elderly nutrition is unclear. Indeed, in elderly subjects, indirect evidence suggests that the response of protein metabolism to AA availability is disturbed. First, the alteration of muscle protein synthesis in response to feeding, which has been detected both in old rats (Mosoni *et al*. 1995; Dardevet *et al*. 2002) and in elderly subjects (Welle *et al*. 1994), can be reversed by strong hyperaminoacidaemia (Volpi *et al*. 1999). Second, the specific anabolic response of muscle to leucine, observed in young rats, is blunted in older animals (Dardevet *et al*. 2000, 2002). Third, in elderly women, a 'pulse feeding' pattern (i.e. 80 % of the daily dietary protein consumed at noon) induces a higher nitrogen balance than a 'spread feeding' pattern (i.e. a daily protein intake evenly distributed over four meals; Arnal *et al*.

1999). In contrast, in young women, the spread diet tends to induce a better balance (Arnal *et al*. 2000). Since the pulse-feeding pattern probably induces a higher hyperaminoacidaemia than the spread-feeding pattern (Wolever, 1994; Dangin *et al*. 2001), a different sensitivity of elderly and young subjects to AAs might partly explain these results. Taken together, these data suggest that in elderly subjects, protein synthesis could be resistant to AA availability and that high hyperaminoacidaemia (or leucinaemia) improves postprandial protein gain.

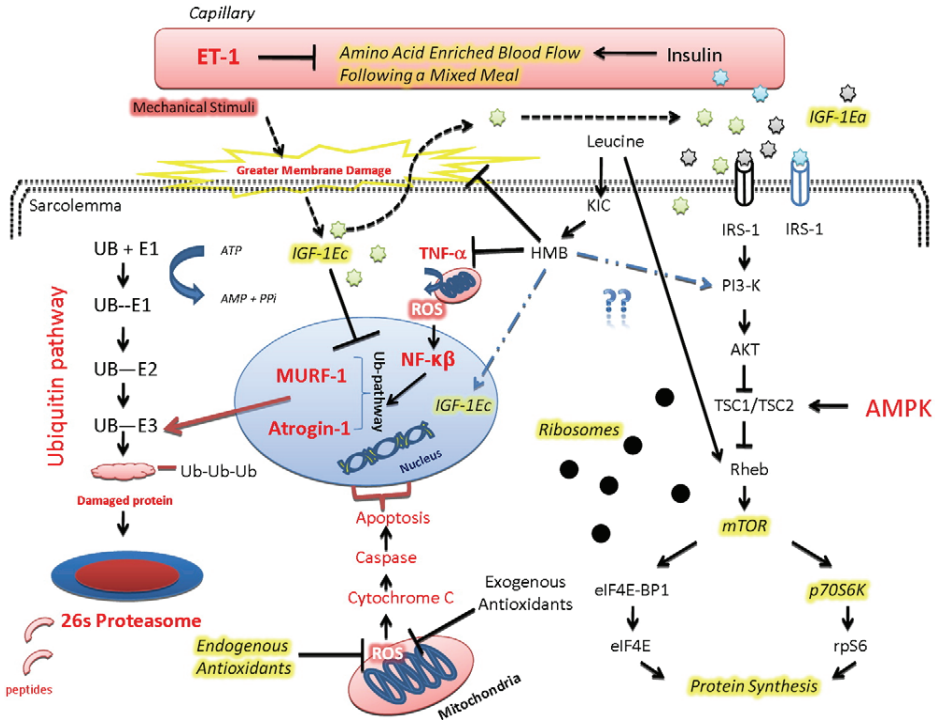


Figure 2: Potential mechanisms by which nutritional interventions may influence protein metabolism and apoptosis in sarcopenic muscle (Kim, Wilson and Lee, 2008).

Another possibility is that the relatively increased importance of the splanchnic tissues in the regulation of protein turnover in the elderly could limit the flow and the availability of alimentary amino acids to the peripheral tissues (Volpi, *et al*., 1999). The splanchnic tissues are responsible for absorption of the alimentary amino acids and their

release to the peripheral tissues. If the splanchnic tissues utilize more amino acids in the elderly, less amino acids will be available for the other tissues. This hypothesis was corroborated by the observation of Boirie *et al*. (1997) that the first-pass splanchnic uptake of leucine increases with age. Therefore, if the increase in first-pass splanchnic extraction reduces the availability of the extracted amino acids for the peripheral tissues, the response of muscle protein anabolism to a given dose of oral amino acids would be blunted in the elderly. Despite this prediction, Volpi (1999) concluded that although the splanchnic first-pass extraction of oral amino acids increases with age, it does not prevent the alimentary amino acids from reaching the systemic circulation and stimulating net muscle protein anabolism in the elderly.

Due to the alarming differences in MPS and the rate in which it occurs, even the older individuals with a adequate diet still may need supplementation post-exercise to further augment MPS. Post-exercise net protein balance remains negative in the absence of food intake. Therefore, providing amino acids immediately following exercise will help promote a net postivie protein balance. Two such dietary supplements that could promote a posivite protein balane are whey protein isolate and and casein

Casein

In young men, proteins that are digested slowly, such as casein (CAS), induce a lower but more prolonged hyperaminoacidaemia and a higher postprandial leucine deposition than a rapidly digested protein fraction, such as whey protein. This effect was shown to be independent of the AA composition of the dietary proteins (Dangin *et al*. 2001). In older men, few studies have examined the long-term effects of CAS on muscle protein synthesis. Koopman and colleagues (2009) determined that dietary protein

digestion and absorption rates and the subsequent post prandial muscle protein synthetic response do not differ between young and elderly men when consuming 35g of CAS (Koopman, Watrand, Beelen, Gijsen, Kies, Boirie, Saris, van Loon, 2009). This suggests that absorption rates of protein supplement may not be delayed in older individuals and that there may be another cause for the delay in muscle protein synethesis found by Drummond et. al, 2008. Dangin (2001) showed that casein exhibited a greater phenylalanine balanance (~35%) than the whey group (but this was not statistically significant). The phenylalanine balance remained elevated for a longer period in the casein group. Whey protein did show to have a greater leucine content, however. Leucine taken up acress the leg can be oxidized or used for protein synthesis, while muscle does not oxideze phenylalanine. Therefore, phenylalanine may be a better marker of MPS. Demling and DeSanti (2000) examined the effects of casein or whey on body composition and strength during 12-weeks of resistance training. The casein group, in particular, showed a greater degree of strength gain in the chest, shoulder and legs. Therefore, one might suggest that the use of casein during weight loss and lean body mass gain (with resistance training) leads to more gain strength when compared to casein.

The long-term effects of casein and their ability to stimulate MPS in older individuals is limited. The slow absorption of casein has been shown to have a positive effect of strength markers and levels of phenylalanine. Whether this can exhibit a positive correlation between muscular adaptations to resistance training in older individuals over a long-period of time is yet to be determined.

Whey Protein

Whey protein is the collective term for the soluble protein fractions extracted from dairy milk. In supplement form, whey proteins (concentrates and isolates) generally contain a higher concentration of EAA (45-55mgs/100gms) than other protein sources (Bucci and Unlu) and therefore score highly on most evaluations of protein quality (Fox 1992). The acute response to a single dose of WP is a higher (but transient) blood AA peak and stimulation of protein synthesis when compared to other high quality protein sources such as casein (the other major bovine milk protein) (Biorie *et al*., 1997; Dangin *et al*., 2001; 2003). More importantly, the consumption of WP in mixed macronutrient meals provides a high stimulation of MPS and high net gain in whole body protein in young and older adults in comparison to isonitrogenous casein meals (Dangin *et al*., 2003). Due to its favorable effect on protein metabolism and LBM accretion, supplementation with WP may also enhance adaptations from resistance training. During 6 weeks of RE-training, WP supplementation (1.2gms/kg/day) in RE-trained individuals resulted in an almost two fold higher gain (2.1 vs. 1.2kgs) in LBM compared to a carbohydrate control group (Burke *et al*., 2001). In a double-blinded study that involved two groups of matched, resistance trained young men, our laboratory reported a significantly greater gain in LBM and strength in the group provided a hydrolyzed WP isolate (1.5gms/kg/day) compared to the group given an equivalent dose of casein (Cribb *et al*., 2005). The results of others studies using rodents (Bouthagourd *et al*., 2002) and healthy young adults (Lands *et al*., 1999) that have directly compared the effects of WP to other high quality proteins such as casein suggest that WP has a greater capability to

improve body composition (i.e., an increase in LBM and/or a decrease in fat mass) during exercise.

Aside from general differences in EAA concentrations and absorption kinetics, WP is a rich rare source of cysteine; an amino acid thought to play a key role in the regulation of whole body protein metabolism and LBM (Hack *et al*., 1997; Hildebrandt *et al*., 2004). WP generally contain a 3 to 4 fold higher concentration of cyst(e)ine (cysteine and its disulfide twin cystine) compared other protein sources (Bucci and Unlu 2000). An abundant supply of cyst(e)ine in the blood is necessary for hepatic catabolism of cyst(e)ine into protons and sulfate; a process that inhibits carbamoylphosphate synthesis (the first and rate limiting step of urea biosynthesis) (Drogeand Holm). This process down-regulates urea production, promotes glutathione synthesis and shifts whole body nitrogen disposal in favor of preserving the muscle amino acid pool (Hack *et al*., 1997). In humans, supplementation with WP (20grams/day or 1gm/kg/body weight/day) is shown to augment this pathway (Lands *et al*., 1999; Middleton *et al*., 2004). In rodents, WP is shown to augment this pathway of protein metabolism in a dose-dependant manner (Marriotti *et al*., 2004). Due to its high concentration of EAA, cysteine and ability to promote MPS, the incorporation of WP into the diet during RE-training may assist with promoting gains in LBM strength and muscle hypertrophy. WP has become an increasingly popular sport supplement; the estimated total supply market was valued at $470 million in the United States in 2003 and this figure is expected to increase each year. In line with this, dairy protein supplementation in combination with RE has received an increasing amount of attention in recent years (Cribb *et al*., 2005; Antonio *et al*., 2001; Burke *et al*., 2001; Demling and DeSanti 2000; Fry *et al*., 2003; Chromiak *et al*.,. 2004).

However, in older populations, very few studies have examined the effects of protein supplementation on muscle characteristics during RE and no studies have examined whey protein's influence on muscle morphology during RE training.

In a recent study with fifteen elderly subjects aged between 60-85yrs (comparing whey to a supplement with equal amounts of EAA) results indicated that whey ingestion improves skeletal muscle protein accrual through mechanisms that are beyond those attributed to its EAA content (Katsanos, Chinkes, Paddon-Jones, Zhang, Aarsland, Wolfe, 2008). This means that MPS is stimulated, perhaps, through mechanisms beyond those attributed to EAA. One of the most notable differences among the two groups mentioned above was the post-absorptive arterial blood phenylalanine concentration. Increased post-absorptive blood phenylalanine means that more amino acids were absorbed to be utilized for MPS in the whey group when compared to the EAA group. The whey group was higher when compared to the EAA group which paralleled with a greater improvement in muscle protein balance. An addition benefit of whey protein supplementation may not lie within the muscle, but externally. As mentioned previous, the regulation of whole body protein metabolism involves regulation between muscle, blood and liver metabolism of AA. The conversion of AA into glucose is linked to the rate at which ammonium ions in the liver are converted into either urea or glutamine. Therefore, hepatic cysteine catabolism serves to retain the AA reservoir in muscle. Whey protein is a rich source of cysteine which can directly be correlated with an increase in plasma cysteine levels. Studies have shown that plasma cysteine levels show a strong age-dependent change of AA in adults ages 28-70 (Hack *et al*, 1997, 1998; Holm *et al*., 1997; Kinscherf *et al*., 1996). Also, older adults (60+ year) exhibit significantly lower

glutamine exchange rates and glutamine/cysteine ratios than younger adults (Hack *et al*., 1997). Since the liver of an older person with a given plasma cysteine level converts less AA to glutamine than a young, healthy individual. A low glutamine level, in turn, is correlated with increased body fat, which is seen in older individuals. Because muscle stores are relied upon increasingly with advancing age to meet the metabolic demands for glutamine, aggressive catabolism of muscle tissue throughout the lifespan occurs (Hack *et al*. 1997, 1998). Thus, increasing plasma cysteine levels through supplementation may delay this breakdown of muscle protein for glutamine synthesis.

Comparison of Whey & Casein Supplementation

The main difference between the fast and slow absorption has to do with protein synthesis and with amino acid oxidation. Stimulation of synthesis is dramatic and immediate after the fast meals, whereas it seems to be absent after the slow meals. Such unique responses to the fast and slow meals are most likely due to different levels of amino acid availability. Indeed "fast proteins," which strongly increased amino acid availability (as reflected by plasma aminoacidemia), induce a stimulation of protein synthesis. By contrast, "slow proteins," which modified plasma amino acid concentration to a much slower extent, were not associated with a stimulation of protein synthesis (Dangin, *et al*., 2001). In young men, CAS and whey have been compared in regard to muscle protein synthesis. In one study, MPS after young men ingested supplement at rest, consumption of whey was ~93% higher than casein (Tang, Moore, Kujbida, Tarnopolsky, Phillips, 2009). Post exercise, MPS in the whey group was ~122% higher in whey vs. casein. This effect has been attributed to the amino acids acting as the stimulus of MPS. Whey protein is acid soluble and thus is digested quickly and results in a pronounced

aminoacidemia. Data obtained at the whole body level show that whey induces a transient rise in whole body protein synthesis and leucine oxidation at rest (Dangin, *et al*., 2001). The increase in extracellular leucine concentration has been proposed to represent an important nutritional signal that drives the post-prandial increase in muscle protein synthesis. Therefore, it has been suggested that ingestion of additional leucine during post-exercise recovery could further accelerate post-exercise muscle protein synthesis rates (Koopman and van Loon, 2009). Casein has a modest effect on whole body protein synthesis but instead inhibits whole body protein breakdown. This process may help preserve lean muscle mass in aging individuals. Although this point is alarming, skeletal muscle only contributes ~25-30% to whole body protein synthesis and its turnover is much lower than that of more rapidly-turning over gut and plasma proteins (Nakshabendi, McKee, Downie, Russell, Rennie, 1999). This means that protein turnover measured at the whole body level may or may not be a proper representation of what is occurring at the molecular level in skeletal muscle.

In the aforementioned study, comparing casein to a supplement containing amino acids in the same amount as native CAS, and comparing whey to that same dose spread out over 13 small feedings every 20 minutes. All the feedings were composed of 30g of protein. The single whey feeding and casein amino acids group mimicked the previous results of whey protein. In the frequent whey feeding, the net lecucine balance was 87μmol/kg^{-1} and in the casein feeding it was 38 μmol/kg^{-1}. Therefore, it appears that casein protein, and its slow absorption rate, leads to a greater positive net whole-body protein state than single feedins of whey protein or amino acids at rest. Also, small

feedings of whey proteins lead to the greatest net leucine balance when compared to casein.

When resistance or endurance training is not a factor, the use of casein supplementation has been shown to induce higher protein gains when compared to whey protein (Dangin, *et al*., 2003). In young men ingesting protein meals, CAS induced a higher protein gain than whey protein. The effects of meals containing either CAS or two different amounts of whey protein (whey protein-iN: isonitrogenous with CAS, or whey protein-iL: providing the same amount of leucine as CAS) on protein metabolism was compared in nine healthy, elderly (age 72 +/- 1 years) and six young men (24 +/- 1 years). In both age groups, whey protein-iL and whey protein-iN were digested faster than CAS ($P < 0.001$). Proteolysis was inhibited similarly whatever the meal and age groups.Protein synthesis was higher with whey protein-iN than with CAS or whey protein-iL ($P < 0.01$), irrespective of age. An age-related effect ($P < 0.05$) was found with postprandial leucine balance. Leucine balance was higher with CAS than with whey protein-iL ($P < 0.01$) in young men, but not in elderly subjects.In isonitrogenous conditions, leucine balance was higher with whey protein-iN than with CAS ($P < 0.001$) in both age groups, but the magnitude of the differences was higher in the elderly men ($P = 0.05$). Therefore, it was concluded that, during aging, protein gain was greater with whey protein (rapidly digested protein), and lower with CAS (slowly digested protein). This suggests that a 'fast' protein might be more beneficial than a 'slow' one to limit protein losses during aging. This, however, has not been the case for those who are on a long-term training regimen. This may be attributed to the proposed anabolic resistance of the muscle protein synthetic machinery to become activated in elderly muscle. It is

important to note that an exercise intervention was not done in this experiment and it was an acute study (one feeding). The reasoning behind the initial rationale with this study was, with respect to the kinetics of digestion of dietary proteins, a 'fast' protein might induce higher postprandial protein retention than a 'slow' one in elderly subjects by increasing AA availability.

The debate between the two different types of supplementation raises several questions. First, is the post-prandial response and increase in glutathione to whey protein the key to helping older individuals utilize nutrients from their diet to help augment muscle protein synthesis? Secondly, will the slow absorption effects of casein help promote muscle protein synthesis due to the recently discovered delay in aging muscle after resistance exercise? The long-term adaptation to these different supplements with resistance-training in older individuals is still unclear.

CHAPTER THREE

Methods

Participants

A total of 35 healthy elderly men (aged 55–75 years) will volunteered to participate in a 12-week resistance-training and supplementation intervention program (three sessions per week). Subjects were deemed medically capable of engaging in an exercise program. This was determined and consented by their primary care physician before enrolling in the study. Subjects with any of the following conditions were excluded from the study if they had, unless otherwise approved by their doctor,: 1) any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypongonadism; 2) history of pulmonary disease, hypertension, liver or kidney disease, musculoskeletal disorders, neuromuscular or neurological diseases, autoimmune disease, cancer, peptic ulcers, or anemia; 3) taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic, psychotropic, neuromuscular/neurological, or androgenic medications; 4) any bleeding disorders; 5) any chronic infections (e.g. HIV); 6) no concurrent participation in other physical or exercise modalities.

All participants were informed of the nature and possible risks of the experimental procedures, before written informed consents were obtained. All procedures were performed according to the Declaration of Helsinki, and the study was approved by the Baylor University Institutional Review Board.

Study Design

All supervised testing and analysis was conducted in the Exercise and Sport Nutrition Laboratory (ESNL) and/or the Exercise Physiology Biochemistry Lab (EBNL) in the Department of Health, Human Performance, and Recreation at Baylor University. Training sessions were completed at Baylor University under the qualified supervision of a faculty/staff/student of the Health, Human Performance and Recreation Department at Baylor University.

Independent and Dependent Variables

The independent variables are the nutritional supplement [Whey Protein + Carbohydrate (WP-CHO), Casein + Carbohydrate (CAS-CHO) and Carbohydrate only (CHO)), the resistance training program and sampling time points. Dependent variables will include body composition measurement (whole body DEXA scans, body weight), fasting whole blood and serum analysis, myofibrillar protein content, HGF, cMet, IGF-1, and muscle strength (bench press, leg press) as determined via a one repetition maximal test.

Familiarization and Entry

Participants who inquired about the study were interviewed on the phone to determine if they qualified to participate in the study. Participants who met eligibility criteria and received an exercise approval from from their doctor were invited to attend an entry/familiarization session in the ESNL at Baylor University. During this session, participants thoroughly read, review and sign an informed consent and complete a medical history questionnaire. Participants meeting entry criteria were familiarized to the study protocol via a verbal and written explanation outlining the study design. This

included explaining supplementation protocol, familiarizing the participants to the tests to be performed, and practicing the bench press, and leg press. Participants will then be randomly assigned one of the three groups.

Dietary Intake

Dietary intake will be recorded for four days (one weekend day and three weekdays) prior to the first and last testing session to detect any abnormal changes from baseline to the end of the study. On all testing days, participants will be asked to fast overnight. The dietary logs were evaluated with Food Processor dietary assessment software (ESHA Nutrition Research, Salem, OR) to determine the average total Calorie consumption and macronutrient composition in their diet.

Strength Assessment

Maximum strength was assessed at baseline (week 0), week 4, 8, and 12 by means of one-repetition maximum (1RM) strength tests on a isotonic 45 degree regular leg press (Nebula Fitness, Inc., Versailles, OH) and bench press machine (Nebula Fitness, Inc., Versailles, OH). The 1RM test was repeated following month one, two, and three of exercise training. These tests were performed on the initial lab visit, and at the end of month one, two, and three of exercise training. Participants began the 1-RM leg and bench press by warming up with 5 to 10 repetitions at approximately 50%. Following a two minute rest, three to five repetitions were performed at a slightly higher intensity (70%). The weight was then gradually increased until an official 1-RM is reached. The rest between each set of repetitions was two minutes.

Muscle Biopsies and Venous Blood Sampling

Percutaneous muscle biopsies were performed at baseline (week 0), week 4, 8, and12. Approximately 50 mg was 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 1 and 2 cm using a 5 mm Bergstrom needle. All biopsies were performed under sterile procedure and local anesthesia (1% lidocaine). For the remaining three biopsies, tissue were extracted from the same location as the initial biopsy by using the pre-biopsy scar, depth markings on the needle, and a successive incision that was made approximately 0.5 cm to the former from medial to lateral. Following removal, muscle samples were frozen in liquid nitrogen and stored at -80°C for later analysis.

Venous blood sampling was performed at baseline (week 0), week 4, 8, and12. Participants were required to fast for at least 8-hours prior to each of the four blood draws. Blood samples were obtained by standard/sterile procedures by personnel who were experienced in phlebotomy and are qualified to do so under guidelines established by the Texas Department of Health and Human Services. Venous blood samples were extracted from the antecubital vein into 10 mL collection tubes using a standard VacutainerTM apparatus. The serum was centrifuged for 15-minutes and then frozen at -80°C; whole blood samples will not be taken.

Anthropometric and Body Composition Analysis

Anthropometric and body composition analysis were performed at baseline (week 0), week 4, 8, and 12. Total body weight and mass was determined on a calibrated electronic scale with a precision of +0.02 kg (Detecto, Webb City, MO). Total body

water will be estimated using a Xitron 4200 Bioelectrical Impedance Analyzer (San Diego, CA) which measures bio resistance of water and body tissues based on a minute low energy, high frequency current (500 micro amps at a frequency of 50 kHz) transmitted through the body.

Body composition and bone mineral content was measured with DEXA (Hologic, Waltham, MA) by personnel with x-ray technology training. Whole-body and regional lean mass, fat mass, and bone mineral content was determined using the system's software package enCORE 2005 (version 9.15.00). The DEXA body composition test requires each participant lie in a supine position while a low dose of radiation scanned their entire body for approximately 7 minutes. The DEXA segments regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments for determination of fat, soft tissue (muscle), and bone mass. Radiation exposure from DEXA for the whole body scan is approximately 1.5 mR per scan. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation exposure was approximately 6 mR for the entire study; therefore, it is not considered to be dangerous to the subject.

Exercise Intervention Program

Supervised resistance type exercise training was performed three times a week for a period of 12 weeks for each participant. Training sessions consisted of five minutes of warming-up on a cycle ergometer or elliptical. Training sessions were done at Baylor University in Russell Gymnasium. Participants completed resistance exercises similar to those utilized by (Chrusch, *et al*., 2001). Participants completed the bench press, lat pull-down, biceps curl, triceps press down, leg press, leg extension, leg curl, and leg press.

Three sets of 10 repetitions were completed for each exercise. An intensity of 80% of 1 RM was utilized during training. Upon successful completion of 3 sets of 10 repetitions, an increase of 5% of weight used was implemented (Kosek, Kim *et al*. 2006). A rest period of 1.5 minutes was timed to separate each set. An egg timer was given to each subject to ensure homogeneity of rest time. Participants completed three training sessions per week for the duration of the study period (Chrusch, *et al*. 2001). Workload intensity was adjusted based on the 1RM tests (Weeks 4 and 8). In addition, workload was increased if more than ten repetitions could be performed in two out of the three sets. Training logs detailing the amount of weight, reps, and sets were kept for each participant.

Supplement Protocol

Research participants were randomly assigned to receive (double-blinded) one of three supplements groups 1)WP 20g + 5 g CHO, 2). CAS 20g + 5 g CHO and 3). 25g CHO only. Each group combined with a calorie equalization portion of glucose (DGC AST Sport Science, Golden, CO) that was similar in color and texture to the supplement. Participants were instructed to ingest either the supplement or the placebo everyday for the duration of the 12-week study period. On workout days, the participants were asked to ingest the supplement within 30 minutes of the completion of each workout session.

Serum IGF-1

Serum free/bioactive IGF-1 concentration were determined using an ELISA kit [Active® free IGF-1 ELISA by Diagnostic Systems Laboratories Inc. (DSL-10-9400; Webster, TX)]. According to the manufacturer, the sensitivity for this assay is 0.015

ng/ml, and does not cross-react with insulin growth factor binding proteins. This assay was an enzymatically amplified "two-step" sandwich-type immunoassay. In the first step of this assay, 50 µl of standards, controls (high and low), and serum samples will be pipetted induplicate along with 20-µl sample buffer (a protein based BSA buffer with a non-mercury preservative) into the wells (which will be coated with anti-free IGF-1). After a 1 hour incubation on the microplate shaker, the wells will be washed five times with wash buffer (buffered saline with non-ionic detergent). Following the incubation and washing, the wells will be treated with another anti-Free IGF-1 detection antibody labeled with the enzyme horseradish peroxidase. After a second 30-minute incubation and wash step (five times), the wells will be incubated with 100 µl of TMB solution for 10 minutes. Lastly, 100 µl of an acidic stopping solution (0.2 M sulfuric acid) was added to each well and the degree of enzymatic turnover of the substrate was determined by a wavelength absorbance measurement at 450nm. The absorbance measured will be directly proportional to the concentration of free IGF-1 present. A set of seven free IGF-1 standards ranging from 0 to 9 ng/ml were used to plot a standard curve of absorbances versus free IGF-1 concentration from which the free IGF-1 in the unknown serum samples were calculated using a standard curve.

Hepatocyte Growth Factor (HGF)

Serum hepatocyte growth factor concentration was determined using an ELISA kit [RayBio® Human HGF ELISA Kit, RayBiotech, Inc. (ELH-HGF-001), Norcross, GA]. According to the manufacturer, the sensitivity of this kit is typically less than 8 pg/ml. The assay utilizes an antibody specific for human HGF. In the first step of this assay, 100 µl of samples and standards were added in duplicate to each well and allowed

to incubate at 4°C for 2.5 hours on a microplate shaker. Each well was then washed 4 times with 300 µl wash buffer utilizing a multi-channel autowasher. After the incubation period and wash step, 100 µl of prepared biotin antibody was added to each well. Again, the plate was allowed to incubate at room temperature for 1 hour on a microplate shaker. This was followed by another wash step with 300 µl of wash solution. The wash was repeated 4 times. After this incubation and wash period, 100 µl of a prepared Stretavidin solution was added to each well and allowed to incubate on the microplate shaker for 45 minutes. Again, wells were washed 4 times with 300 µl of wash solution. After 45 minutes, 100 µl of TMB One-Step Substrate Reagent was added to each well. This was followed by a 30-minute incubation period at room temperature. Immediately after the 30-minute incubation period, 50 µl of a stop solution was added to each well. Plates were then immediately read at 450 nm. Concentrations of serum HGF were then calculated using a standard curve.

cMET (STAR Met)

The Upstate® colorimetric STAR kit [Upstate, Cat# 17-469, Billerica, MA] utilized for analysis of cMET receptor concentration is a solid phase sandwich enzyme linked immunoabsorbent assay for the detection of specific levels of signaling targets in whole cell extracts. In the first step of this assay, 100 µl of standards were added in duplicate to wells 1-7 of the microplate. Then, 100 µl of samples were added in duplicate to the remainder of the wells. The plate was then covered and allowed to incubate for 2.5 hours on a microplate shaker at 4°C. After incubating, plates were washed 4 times with 250 µl of wash solution using an automatic washer. After washing, 100 µl of the detection antibody was added to each well. Plates were then covered and allowed to

incubate for 1 hour as previously described. This was followed again by washing 4 times with 250 µl of wash solution. After washing, 100 µl of a 1:100 dilution of anti-Rabbit IgG HRP Conjugate was added to each well. Plates were then allowed to incubate on the microplate shaker for 45 minutes. Once again, a wash step followed. After washing, 100 µl of TMB solution was added to each well and allowed to incubate for 10 minutes on the microplate shaker. After the incubation, 100 µl of the stop solution was added to each well. Plates were then read at 450 nm for determination of phosphorylated cMET concentration. Results were compared to the standard curve for analysis.

Serum Free Testosterone

Serum free testosterone was determined using an ELISA kit [Alpha Diagnostic International, (Cat# 1885), San Antonio, Tx)]. The testosterone ELISA kit is based on competitive binding of human free testosterone from serum samples and enzym-labeled testosterone. According to the manufacturer, the sesitivity of the free testosterone ELISA kit is 0.17 pg/ml. In the first step of this assay, 25 µl of standards, controls, and samples were added to each well in duplicate. Second, 100 µl of a diluted enzyme conjugate were added to each well. The plate was then covered and allowed to incubate for one hour on a microplate shaker. After the one hour incubation period, each well of the plate was washed three times with 300 µl of wash solution using an automated plate washer. After washing, 150 µl of TMB substrate was added to each well. The plate was then covered and allowed to incubate for 15 minutes on the microplate shaker. The shaker was covered to create a dark environment. After 15 minutes of incubation, the plate was removed from the shaker and 50 µl of stop solution was added to each well. The plate

was then read at 450 nm using an ELISA reader for determination of free testosterone concentration. Results were compared to the standard curve for analysis.

Statistical Analysis

Data was analyzed by utilizing separate 3 x 4 [Group (CHO, WH, CAS)] x Test (week 0, week 4, week 8, week 12) mixed design factorial multivariate analysis of variance (MANOVA). Two sets of MANOVAs (one for anabolic hormones and one set for body composition, muscle strength, and dietary intake variables) were analyzed for this study based on dependent variables. In addition, the use of a MANOVA analysis prevented the potential for increasing of Type I error rate that might result with the use of repeated univariate procedures. ANOVA on each dependent variable was conducted as a follow-up test to any significant MANOVA. To control for alpha inflation of the ANOVA, the Bonferroni test was utilized as a follow-up test. Post-hoc tests of any interaction effects demonstrated in the ANOVA were investigated via an independent samples t-test. In addition to reporting probability values, an index of effect size was reported to reflect the magnitude of the observed effect. The index of effect size utilized was the partial Eta squared ($\eta 2$), which estimates the proportion of variance in the dependent variable that can be explained by the independent variable. Partial Eta squared effects sizes were determined to be: weak = 0.17, medium = 0.24, strong = 0.51, very strong = > 0.70 as previously described by O'Connor, *et al* (2007). All statistical procedures will be performed using SPSS 17.0 software (Chicago, IL) and an alpha probability level of <0.05 was adopted throughout. In addition, for all statistical analyses not meeting the sphericity assumption for the within-participants analyses, a Huynh-Feldt

correction factor was applied to the degrees of freedom in order to adjust (increase) the critical F-value to a level that would prevent the likelihood of committing a type I error.

CHAPTER FOUR

Results

A total of thirty-six apparently healthy male participants began the study. One subject was unable to complete all study requirements due to time conflicts. Baseline demographic data for the 35 participants who completed the study are presented in Table 1. At baseline, there were no significant differences between groups with regards to age (p=0.535), body mass (p=0.916), and height (p=0.318).

Table 1. *Participant Demographics*

Physical Characteristics	*WP*	*CAS*	*CHO*	*Significance*
Height (cm)	177.88±7.45	178.53±6.58	174.64±4.93	0.318
Mass (kg)	96.46±15.81	95.73±20.13	93.56±14.76	0.916
Age (years)	61.58 ±6.50	64.33±8.05	61.55±5.82	0.535

Note: Data are presented as means ± standard deviations for WP, CAS and CHO groups at baseline (week 0).

Confounding Variables

Prior to data collection, certain variables were identified as potential confounding variables. These variables included dietary intake and the resistance training program (i.e., total training volume). To account for dietary intake, participants completed a four-day dietary food record prior to each testing session. Resistance training workout logs were recorded weekly by the study appointed individual who led the guided exercise sessions to determine total workout volume performed by each participant.

Nutritional Intake Analysis

All participants were instructed to consume their usual diet during the 12-week course of the study. Dietary analysis does not include information related to the supplement ingested. Independent sample *t*-tests were utilized to analyze all relevant dietary variables prior to commencement of supplementation and the resistance training program. Table 2 illustrates that at baseline (week 0), there were no significant differences between groups for total daily caloric intake, macronutrient intake of protein,

Table 2. *Comparison of Nutritional Intake Variables between WP, CAS and CHO Groups*

Variable	*Time*	*WP*	*CAS*	*CHO*	*Significance*
Energy Intake ($kcal \cdot d^{-1}$)	Week 0 Week 12	1879±552 1936±545	1932±418 1946±258	1844±563 2211±726	Time=0.073 Group=0.829 GxT=0.168
CHO Intake ($g \cdot d^{-1}$)	Week 0 Week 12	208±82 207±87	226±67 228±49	195±71 229±137	Time=0.324 Group=0.811 GxT=0.410
Protein Intake ($g \cdot d^{-1}$)	Week 0 Week 12	76±30 80±18	86±24 85±28	77±23 103±71	Time=0.215 Group=0.586 GxT=0.356
Fat Intake ($g \cdot d^{-1}$)	Week 0 Week 12	82±30 85±22	79±20 75±19	76±28 111±35*¥†	Time=0.024 Group=0.222 GxT=0.006

*Note: Data is presented as mean ± standard deviation for WP, CAS and CHO groups. *Significantly different from week 0 ($p<0.05$).¥ Significantly different from WP group ($p<0.05$). †Significantly different from CAS group ($p<0.05$)*

fat or carbohydrate. Four 3 x 2 [group (WP, CAS, CHO) x time (0 and 12 weeks)] repeated measures ANOVA was used to determine any differences in total daily caloric intake and macronutrient content between supplementation groups prior to, and following the resistance training program. A significant main effect for time was detected for dietary fat intake (p= 0.024), with subsequent pairwise comparison revealing a significant

increase from week 0 to week 4 (p=0.024, η^2=0.153, *power*=5.611). A significant Group x Time interaction was also revealed for dietary fat intake (p=0.006 η^2 =.279, *power*=6.010) with independent samples *t*-tests revealing significantly higher dietary fat intake for the CHO compared to WP (p=0.05) and CAS group (p=0.006) at week 12.

Total Lifting Volume Analysis

For each exercise, the number of repetitions and weight lifted at each training session was recorded. Total lifting volume for each group reflects the total number of repetitions multiplied by the total weight lifted for each exercise (see Table 3). A 3 x 3 [group (WP, CAS, CHO) x time (4, 8 and 12 weeks)] repeated measures ANOVA was used to determine any differences in total lifting volume between supplementation groups over the course of the study. Univariate analysis revealed a significant main effect for time (F=31.805, p<0.001, η^2=0.694), with subsequent pairwise analysis indicating a significant increase in total lifting volume from 4 to 8 weeks (p<0.001), and 12 weeks (p<0.001) and from 8 to 12 weeks(p=0.001). No main effect for group or group by time interaction were observed indicating all groups increased their total volume lifted over the course of the study.

Table 3. *Comparison of Total Lifting Volume for WP, CAS and CHO Groups*

Week	*WP*	*CAS*	*CHO*	*Significance*
4	126,816 ±25,259	114,296 ±38,834	125803 ±32,918	*p=0.610*
8	147,703 ±30,489	141,766 ±47,750	144,425 ±38558	*p=0.939*
12	159,729 ±43,867	155,877 ±48,172	154730 ±39,437	*p=0.934*

Note. Data is presented as means ± standard deviations.

Muscle Strength Variables

Upper and Lower Body Strength Analysis

Data for 1RM bench press and leg press are represented in figures 3 and 4. A 3 x 4 [group (WP, CAS, CHO) x time (0, 4, 8 and 12 weeks)] repeated measures MANOVA was used to determine any differences in upper and lower body strength between supplementation groups over the course of the study

Mulitivariate analysis indicated a significant main effect for time (*Wilk's Lambda*=0.102, *F*=33.670, *p*<.001, *η2*=0.898, *power*=1.00), with univariate analysis revealing a significant time effect for 1 RM bench press (F=40.570, *p*<.001, η^2=0.592), and 1 RM leg press (F=76.605, *p*<.001, η^2=.732). Subsequent pairwise for 1RM bench press indicated a significant increase from baseline (week 0) to week 4 (*p*<0.001), week 8 (*p*<0.001), week 12, (*p*<0.001), and from week 4 to week 8 (*p*<0.001), and week 12 (*p*<0.001) and from week 8 to week 12 (*p*=0.005) (see figure 3)). Pairwise analysis for 1RM leg press indicated a significant increase from baseline (week 0) to week 4, week 8 and week 12 (*p*<0.001), and from week 4 to week 8 and week 12 (*p*<0.001) and from week 8 to week 12 (*p*<0.001).

A significant Group x Time interaction was also observed (*Wilk's Lambda*=0.428, *F*=2.024, *p*=0.044, *η2*=0.346, *power*=0.858), with univariate analysis revealing a significant interaction for 1RM leg press (p = 0.001). Subsequent independent samples *t*-tests demonstrated a trend towards significantly higher 1RM leg press strength in the WP (*p*=0.053) and CAS (*p*=0.086) groups compared to the CHO group at 12 weeks (see figure 4). No other main effects or interactions were detected. Hypothesis one states that: "Supplementation with whey isolate protein and casein protein for 12 weeks, when

combined with resistance exercise, will result in no significant differences in upper and lower body strength in older males when compared to placebo," thus hypothesis one failed to be rejected.

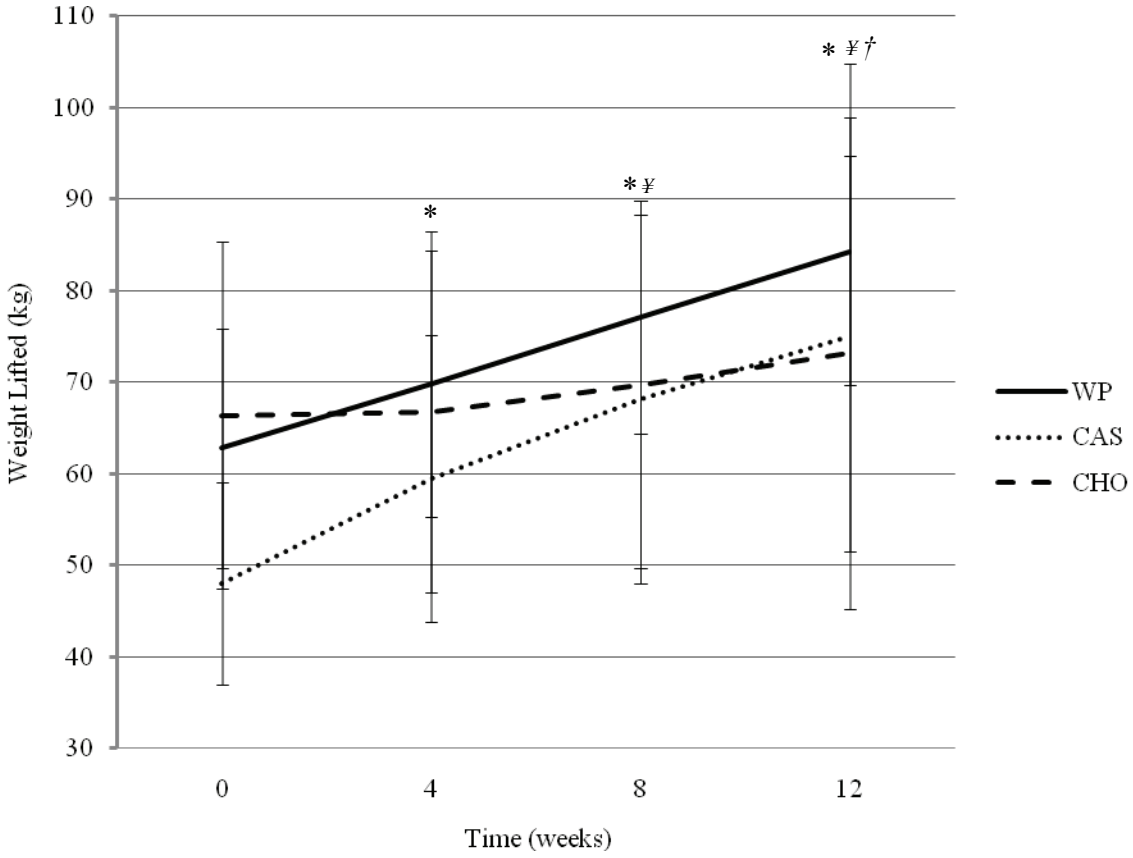


Figure 3. Time Course for 1RM Bench Press. Data (mean ± SD) represents 1RM bench press for WP, CAS and CHO groups following 12 weeks of resistance training *Significantly different from baseline (week 0) ($p<0.05$) . ¥ Significantly different from week 4 ($p<0.05$). †Significantly different from week 8 ($p<0.05$).

Body Composition Analysis

Data for body fat percentage (BF%), fat mass (FM) and fat-free mass (FFM) are presented in figures 5, 6 and 7 respectively. Three 3 x 4 [group (WP, CAS, CHO) x time (0, 4, 8 and 12 weeks)] repeated measures ANOVA was used to determine any differences in FM, FFM in BF% percentage between supplementation groups over the

course of the study. Univariate analysis revealed a significant main effect for time for BF% (F=3.602, p=.016, η^2=.101), with subsequent pairwise comparison revealing a trend

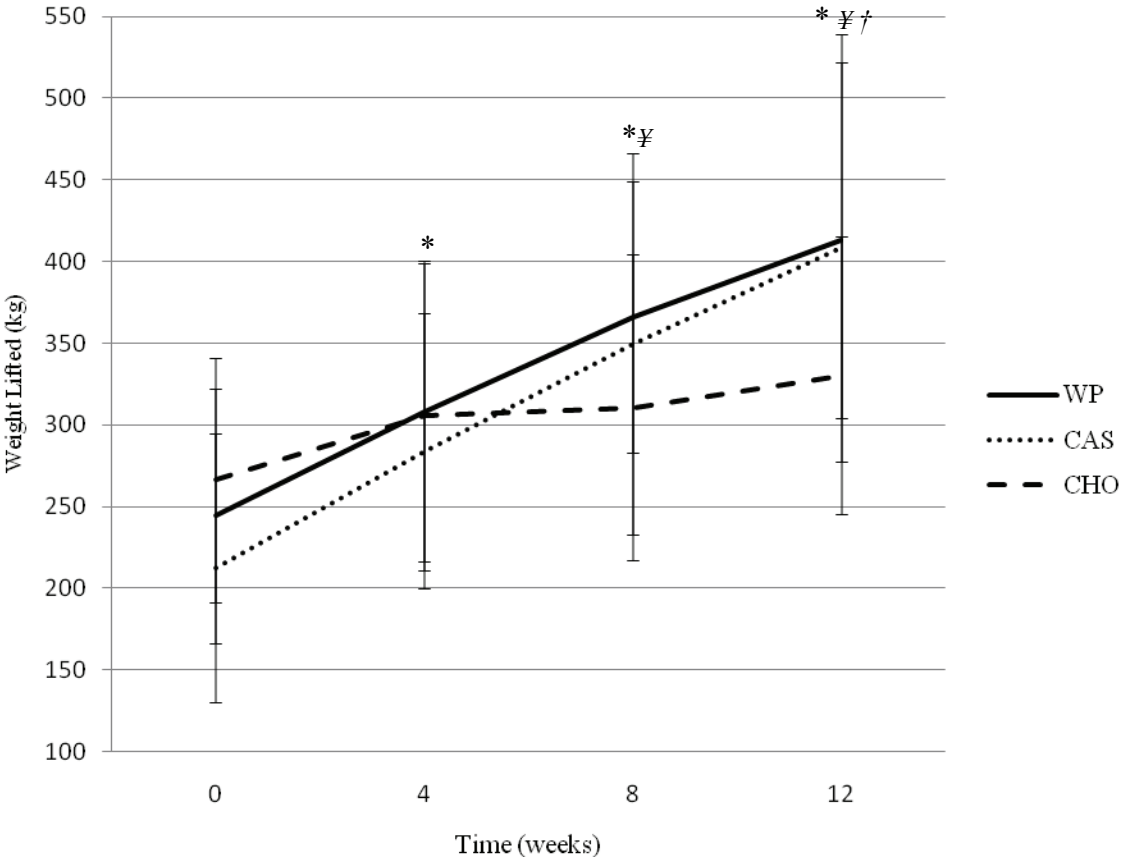


Figure 4. Time Course for 1RM Leg Press. Data (mean ± SD) represents 1RM leg press for WP, CAS and CHO groups following 12 weeks of resistance training *Significantly different from baseline (week 0) (p<0.05). ¥ Significantly different from week 4 (p<0.05). †Significantly different from week 8 (p<0.05).

towards significantly lower BF at 12 weeks compared to 0 weeks (p=0.080). A significant main effect for time for FFM was also observed (F=5.478, p=.002, η^2=.146), with subsequent pairwise comparison indicating a significant increase in FFM from 0 weeks to 8 (p=0.022) and 12 weeks (p=0.024) indicating that all groups increased in lean body mass following the resistance training program. No other main effects for time, group or group by time interactions were observed for BF%, FFM and FM (see figures 5, 6 and 7).

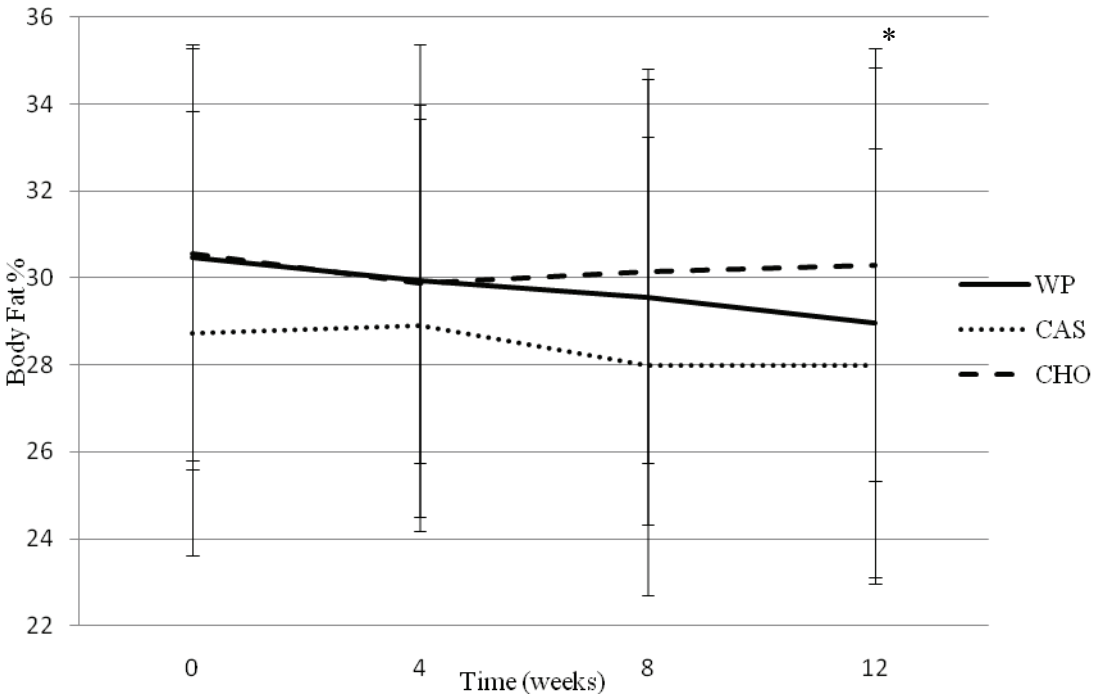


Figure 5 . Time Course for BF%. Data (mean ± SD) represents BF% for WP, CAS and CHO groups following 12 weeks of resistance training. *Trend toward significance from baseline (week 0) (p=0.08).

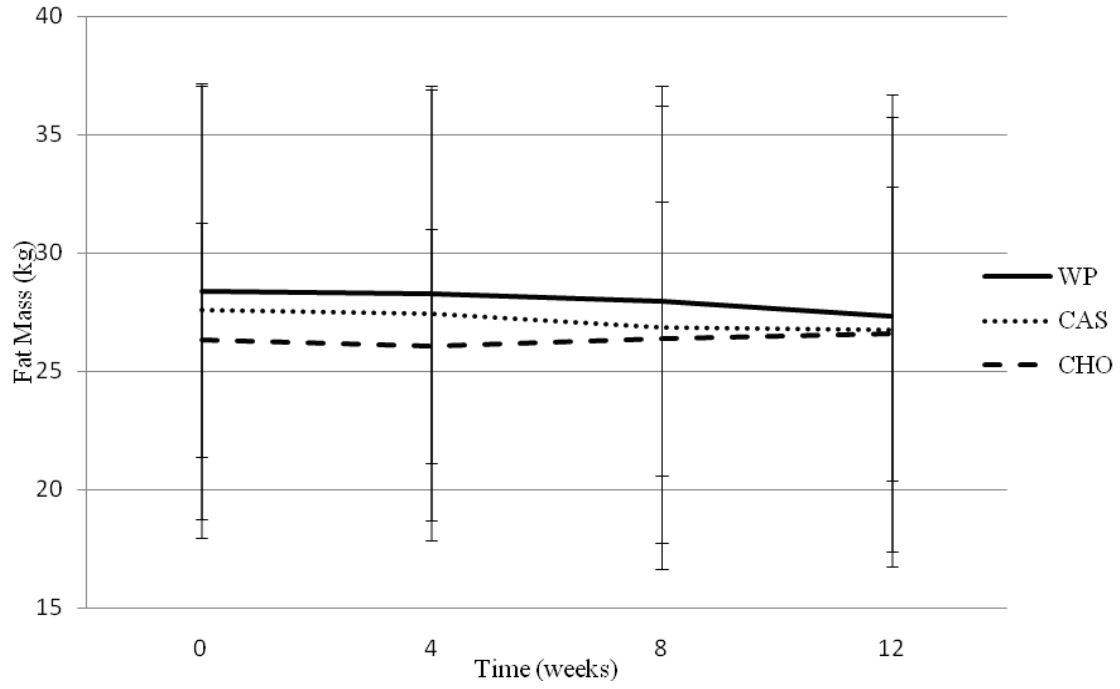


Figure 6. Time Course for FM. Data (mean ± SD) represents FM for WP, CAS and CHO groups following 12 weeks of resistance training.

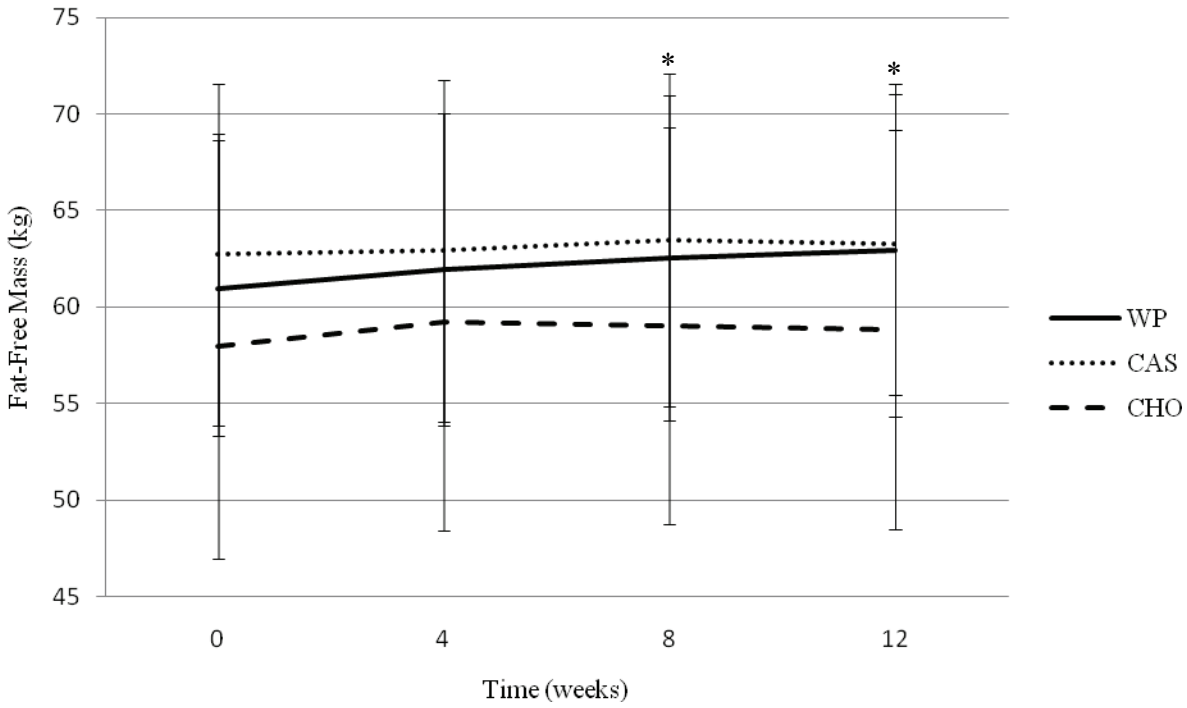


Figure 7. Time Course for FFM. Data (mean ± SD) represents FFM (kg) for WP, CAS and CHO groups following 12 weeks of resistance training. *Significantly different from baseline (week 0) ($p<0.05$).

Hypothesis two states that: "Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in lean body mass in older males when compared to placebo", and thus we failed to reject. Hypothesis three states that: "Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in fat mass in older males when compared to placebo," and thus we failed to reject. Hypothesis four states that: "Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in body fat percentage in older males when compared to placebo," and thus we failed to reject.

Serum Variables

Data for serum HGF, IGF-1, and free testosterone are represented in table 4. A three 3 x 4 [group (WP, CAS, CHO) x time (0, 4, 8 and 12 weeks)] repeated measures MANOVA was used to determine any differences serum HGF, IGF-1 and free testosterone between supplementation groups over the course of the study. A significant group by time interaction was revealed (*Wilk's Lambda*=0.307, *F*=2.150, *p*=0.018, η^2=0.446, *power*=0.949) with univariate analysis indicating a moderate trend towards a significant interaction for HGF (p = 0.092). A significant main effect for group (*Wilk's Lambda*=0.662*, F*=2.292, *p*=0.047, η^2=0.186, *power*=0.755) was also detected, with univariate follow up indicating a significant group effect for IGF-1 (*p*=0.002).

Table 4: Serum Variables for WP, CAS and CHO Groups

Variable	Week 0	Week 4	Week 8	Week 12	Significance
IGF-1 (pg/mL)					
WP	0.66±.39	0.70±.35*	0.63±.34*	0.74±.47*	*Group*=0.013
CAS	1.02±.52	0.91±.42*	0.87±.40*	0.90±.40*	*Time*=0.225
CHO	0.42±.23	0.31±.27	0.34±.25	0.34±.33	*G x T*=0.492
HGF (pg/mL)					
WP	282.72±533.39	250.75±364.61	359.70±525.34	272.95±408.32	*Group*=0.934
CAS	381.93±865.95	374.27±810.43	343.78±768.78	340.09±740.88	*Time*=0.014
CHO	346.45±384.59	254.53±343.78	324.07±405.67	193.44±235.19	G x T=0.092
Testosterone(pg/mL)					
WP	14.10±8.54	17.64±14.16	17.90±14.33	18.05±14.86	*Group*=0.991
CAS	14.41±11.27	19.58±16.74	17.46±14.39	15.96±11.65	*Time*=0.214
CHO	18.10±13.67	17.49±17.60	14.05±8.05	15.43±15.76	*G x T*=0.163

*Data are presented as mean ± standard deviation for WP, CAS and CHO groups. * Significantly different compared to CHO group.*

Pairwise comparison revealed significantly higher serum IGF-1 levels in both the CAS ($p<0.001$) and WP ($p=0.030$) group compared to CHO group. A moderate trend for

significantly higher serum IGF-1 levels in the CAS group compared to the WP group (p=0.099) was also observed.

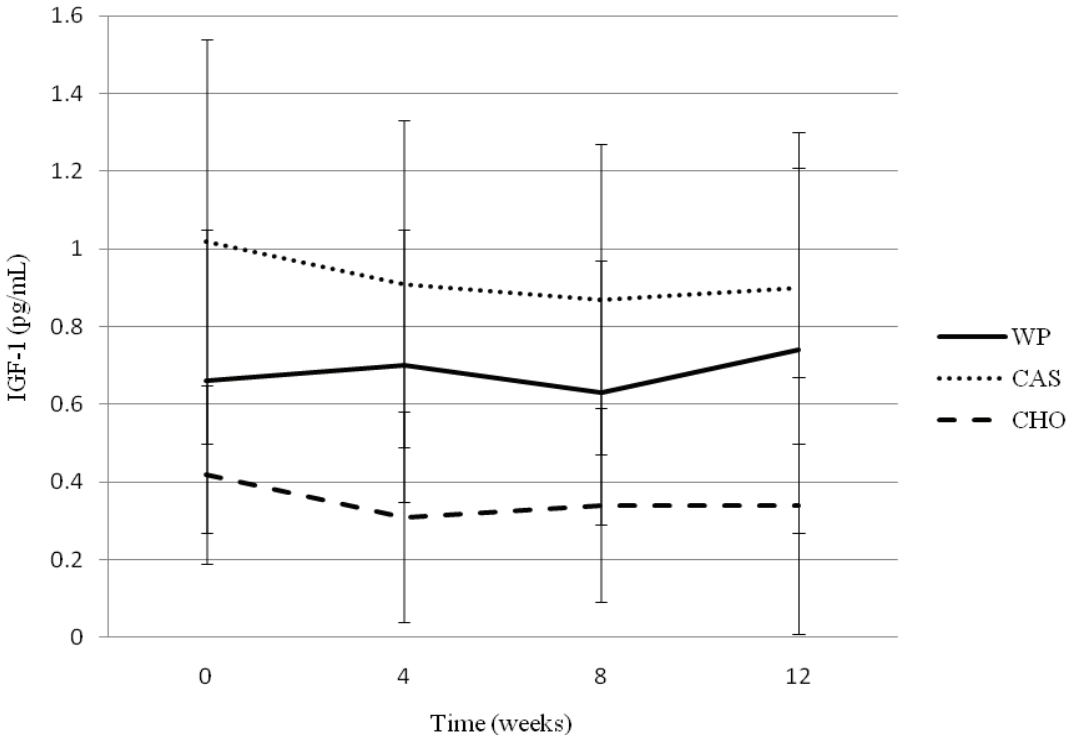


Figure 8. Time Course for IGF-1. Data (mean ± SD) represents IGF-1 for WP, CAS and CHO groups following 12-weeks of resistance training.

Hypothesis five states that: "Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in IGF-1 expression when compared to placebo," thus the hypothesis was rejected. Hypothesis six states that: "Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in HGF expression when compared to placebo," thus we failed to reject. Hypothesis seven states: "Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in

no significant difference in serum free testosterone levels when compared to placebo," thus we failed to reject.

Skeletal Muscle Variables

Data for skeletal muscle phosphorylated c-Met receptor concentration is represented in figure 9. A 3 x 4 [group (WP, CAS, CHO) x time (0, 4, 8 and 12 weeks)] repeated measures ANOVA was used to determine any differences in serum c-Met receptor concentration between supplementation groups over the course of the study. A significant main effect for group (p=0.001) and group by time interaction (p=0.017) were revealed. Subsequent pairwise analysis for group effect indicated significantly higher c-Met receptor concentration in the CHO group compared to WP (p=0.001) and CAS (p=0.001) groups.

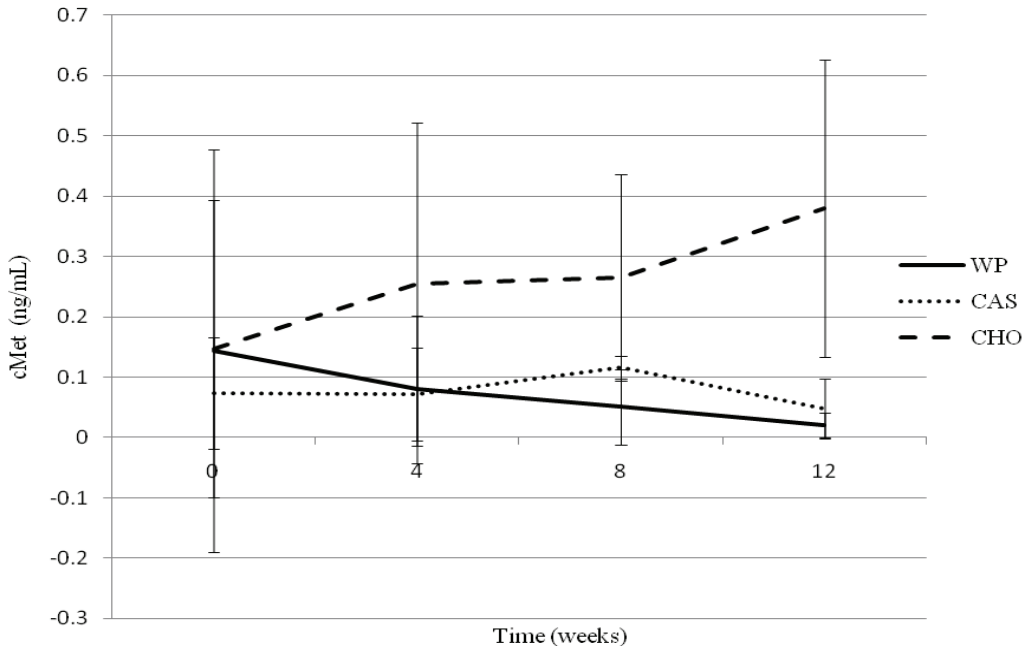


Figure 9. Time course of phosphorylated cMET receptor concentration. Data (mean ± SD) represents fat-free mass for WP, CAS and CHO groups after 12-weeks of resistance training. * CHO Significantly different from WP group (p<0.001). # CHO Significantly different from CAS group (p<0.05).

Independent samples *t*-tests revealed significantly higher serum c-Met levels in the CHO group compared to the WP group at 8 ($p<0.001$) and 12 weeks ($p<0.001$), and CAS group at 4 ($p=0.033$) and 12 weeks ($p<0.001$, see figure 9). Hypothesis eight states that: "Supplementation with whey isolate protein and casein protein for 12 weeks, when combined with resistance exercise, will result in no significant difference in c-Met receptor expression when compared to placebo," thus the hypothesis was rejected.

CHAPTER FIVE

Discussion

Introduction

The primary purpose of this study was to determine if the consumption of two dietary proteins; casein and/or whey isolate would result in greater enhancement of body composition, muscle strength, and biochemical mechanisms regulating skeletal muscle hypertrophy compared to carbohydrate ingestion, when combined with a 12-week resistance training program in older males. Research into dairy protein supplementation as an ergogenic aid has received an increasing amount of attention in recent years (Demling and DeSanti, 2000; Antonio *et al.*, 2001; Burke *et al.*, 2001; Fry *et al.*, 2003; Chromiak *et al.*, 2004; Tipton *et al.*, 2004; Anderson *et al.*, 2005; Cribb *et al.*, 2006; Phillips *et al.*, 2005). The ability of whey protein and casein to stimulate muscle protein synthesis and enhance skeletal muscle hypertrophy from resistance training has been well documented in younger populations (Tang *et al.*, 2009; Tipton *et al.*, 2004**)**. However, in older populations, the few studies that have examined the effects of whey protein and/or casein supplementation on mixed muscle protein synthesis with or without resistance exercise have shown inconsistent findings (Farnfield *et al.*, 2005; Katsanos *et al.*, 2008; Koopman *et al.*, 2008)**.** Further, no studies to the author's knowledge have compared the chronic effects of whey protein and casein supplementation in conjunction with resistance training on systemic and cellular regulators of muscle growth and development. In the current study, whey isolate protein and casein protein ingestion resulted in higher lower body strength compared to carbohydrate only ingestion

following a 12-week resistance training program. Furthermore, both groups displayed higher serum IGF-1 levels during the course of the study. Notwithstanding, it was evident that 12 weeks of resistance training alone increased both upper and lower body strength, body mass and lean muscle mass while decreasing body fat percentage. However, no effects from training were observed on serum anabolic hormones (i.e. testosterone and IGF-1) and indirect markers of satellite cell activation (HGF and phosphorylated C-Met). Despite few differences between treatment groups, the present study does provide further information to the limited body of literature examining the chronic effects of whey isolate protein and casein protein supplementation and resistance training on pathways involved in skeletal muscle cell growth and development in older individuals.

Body Composition and Muscle Strength

The 12-week resistance training program utilized in the current study increased both upper and lower body strength as assessed by 1-RM bench press and leg press, respectively, in all three groups. However, a strong and moderate trend towards significantly higher 1RM leg press strength was observed in the whey isolate ($p=0.053$) and casein ($p=0.086$) groups, respectively, compared to carbohydrate placebo group. Recent studies have confirmed that resistance exercise stimulates an increase in myofibrillar and sarcoplasmic proteins (Louis *et al.*, 2003; Mittendorfer *et al.*, 2005) as well as connective tissue proteins (Miller *et al.*, 2005). A single bout of resistance exercise results in the acute stimulation of muscle protein synthesis (up to 50-100% above basal values) that peaks within 3-24 hours, and can remain elevated, although at a diminishing rate, for up to 48 hours post-exercise (Chesley *et al.*, 1992; Biolo et al., 1995;

Phillips *et al.*, 1997). Studies that have assessed both the rate of muscle protein breakdown and synthesis in response to a bout of resistance exercise have demonstrated that in a fasted state (Biolo *et al.*, 1995; Phillips *et al.*, 1997) the net muscle protein balance remains slightly negative. It is suggested that recovery of muscle protein synthesis, and hence a positive protein balance after exercise would require intake of protein or amino acids (Anthony *et al.*, 1999). In the present study, oral ingestion of whey isolate protein and/or casein protein after each resistance exercise session possibly increased delivery of amino acids to the muscle, thus, augmenting muscle protein synthesis and minimising protein degradation; creating a positive protein balance.

Although muscle protein synthesis and breakdown rates were not measured in the present study, previous studies have shown that providing exogenous amino acids, especially within the first 4 hours after resistance exercise (as implemented in the present study), enhances protein synthesis, reduces protein breakdown, and maintains a positive protein balance (Tipton *et al.*, 1999; Biolo *et al.*, 2003). Although these results were not observed following ingestion of whey protein, a more recent study has confirmed the positive impact from whey protein supplementation, in addition to casein (another milk protein) on protein metabolism after resistance training exercise (Tipton *et al.*, 2004). Participants were randomly assigned to either a whey, casein or placebo group. Results showed that a 20g bolus dose of whey or casein provided a positive effect on muscle protein balance after resistance training. While both proteins had different effects on blood amino acid responses, it was clear that whey protein (and casein) were able to increase muscle protein net balance following resistance training.

Thus, in the present study, the higher lower body strength values in the whey isolate protein- and casein protein-supplemented groups compared to carbohydrate placebo group following the resistance training program, may be reflecting not only an increases in protein synthesis, but equally as important it may be indicating reduced protein breakdown, and thus lessening the amount of muscle degeneration and loss of muscle mass that can be associated with eccentric exercise. Moreover, since satellite cell proliferation requires a positive net muscle protein balance (Evans 2001), muscle fiber regeneration is likely to be enhanced following whey protein and/or casein supplementation. Recent studies in humans (Cuthbertson *et al.*, 2005) have confirmed in vitro (Christie *et al.*, 2002) and in vivo (Yoshizawa *et al.*, 1997; 2004) that amino acids stimulate muscle protein synthesis directly via activation of the Raptor- mTOR complex (Hara *et al.*, 1998) and increase phosphorylation of the p70S6k and eIF4-BP1 complexes (Liu *et al*., 2002). The large (289 kDa) Raptor- mTOR complex is expressed more in muscle than other tissues (Kim *et al.*, 2002), is nutrient sensitive, and contains multiple binding sites. Taken together, these acute effects on muscle protein synthesis and breakdown can translate into more chronic adaptations such as increased fiber cross sectional area and mass, and thus, higher force output. However, this can only be speculated as single fiber analysis was not performed in the current study.

Similar to changes in muscle strength, the resistance training program also had a significant impact on body composition. A significant increase in FFM with a concomitant trend for lower BF% ($p=0.080$) was observed following the 12 weeks resistance training program. However, no differences were observed between groups. Results from the current study support previous research that show increases in FFM with

resistance training in elderly individuals (Chrusch *et al.*, 2001;Drummond *et al.*, 2006; Esmark *et al.*, 2001). RE induces mechanical stress (tensile, compressive and shear) and transient changes in sarcoplasmic calcium concentrations, energy substrate levels, the re-dox state as well as increases the availability of hormones and cytokines. Sufficient changes to any (or all) of these variables are thought to activate a network of signal transduction pathways that transfer the mechanical stimuli into specific chemical signaling within the cell. Once activated, these pathways activate transcription factors that alter the expression of muscle genes within the nucleus. Active, nuclear transcription together with receptor binding of muscle growth-factors, androgens and glucocorticoids change the expression of the major muscle growth regulators such as IGF-1, myostatin and other muscle genes. Eventually leading to increased proliferation, differentiation and fusion of satellite cells to the muscle fiber; a process that is considered integral to muscle hypertrophy. Despite no effects from training on serum IGF-1 and indirect markers of satellite cell activation HGF and phosphorylated C-Met; a limitation in the present study was that muscle and blood sampling times were 48 hours following the last bout of resistance training and hence any transient increases may have been missed. This concept will be discussed further in later sections. Interestingly, both whey isolate- and casein protein-supplemented groups demonstrated higher lower body strength compared to carbohydrate placebo group even though similar increases in LBM (i.e. increased muscle mass) was observed between groups. It could be speculated that although no differences in LBM were observed between groups, both protein supplemented groups may have had higher proportion of contractile and sarcoplasmic proteins compared to total protein, increased sensitivity of the contractile apparatus to calcium and/or greater hypertrophy of

fast twitch fibers; all which would translate into higher force output with no significant changes in LBM (Akima *et al.*, 1999). However, this is highly speculative and requires further investigation.

Serum IGF-1 and Free Testosterone

Insulin-like Growth Factor 1 (IGF-1) is an anabolic hormone that is capable of stimulating muscle hypertrophy. Increased transcriptional expression of IGF-1 can occur as a result of muscle stretch or overload as seen with resistance training (Borer 2003). Previous studies have examined the acute responses of IGF-1 and GH in response to resistance exercise in younger populations (Kraemer and Ratamess, 2005). However, few studies have examined the long term physiological effects of resistance training on serum IGF-1 levels in older adults, but moreover, effects of whey isolate and casein supplementation IGF-1 responses following resistance exercise.

In the current study, both whey isolate protein- and casein protein-supplemented groups displayed higher serum IGF-1 levels compared to carbohydrate placebo group. However, no significant changes were observed over time. Therefore, since no changes over time (time effect) or interaction were observed, it is likely that both whey isolate and casein groups had higher basal IGF-1 levels and thus maintained such levels throughout the study. The results from the current study are in agreement with Nicklas and colleagues (Nicklas *et al*., 1995). In the aforementioned study, it was demonstrated that 16 weeks of progressive resistance training in males older than 55 years of age had no significant influence on baseline serum IGF-1 concentration, in addition to serum testosterone concentration and growth hormone concentrations. In contrast, Bodine *et al*. (2001) showed that Akt and mTOR were activated with resistance training, suggesting

that an increase in IGF-1 led to increased PI3K signaling. This would correlate with an increase in FFM due to the upregulation of MPS (which did not occur in the current study). Reardon and colleagues (2001) interpreted the increase of IGF-1 as a compensatory mechanism attempting to restore muscle mass. Therefore, the inability to show a significant increase in IGF-1 over time could help to conclude that IGF-1 did not act as a compensatory mechanism striving to restore muscle mass in all groups. Age may help to explain the inability to increase IGF-1 in response to resistance training. Grounds (2002), concludes that reduced IGF-1 signaling in aging muscle is involved in muscle atrophy and results from decreased muscle exercise, reduced growth hormone and insulin levels and other age-related ailments that are beyond the scope of this study. The reduced signaling can be assumed across all groups in the current study because, despite 12-weeks of high-intensity resistance training and assumed muscle damage, IGF-1 failed to increase.

In addition, it could be suggested that since blood samples are obtained 48 hours following the last bout of exercise, it is likely that serum IGF-1 concentrations returned to baseline levels. Indeed, previous studies examining the short term response to serum IGF-1 concentrations following an acute bout of resistance exercise have demonstrated an increase shortly within the recovery period (12-48 hrs), with serum IGF-1 levels normally returning to baseline within 48 hours (Florini, Ewton *et al.* 1996). With this in mind, given the significant increases in FFM and upper and lower body strength in the current study, it could be speculated that transient increases in serum IGF-1 shortly following the resistance exercise session are still eliciting their anabolic effects within the

muscle. However, whether whey isolate or casein can provide a greater enhancement of serum IGF-1 is still unclear and requires further examination.

Similar to IGF-1, testosterone is also an anabolic hormone because of its role in increasing muscle mass through changes in muscle gene expression. It is common knowledge that as a man ages, his testosterone levels drop. In the older population, this is a concern due to the fact that lower testosterone levels have been associated with decreases in bone mineral density, lean body and muscle mass, muscle strength, aerobic capacity and increases in total and abdominal body fat (Mitchell, Harman *et al.* 2001). Hakkinen and Pakarinen (1993) found that after a bout of fatiguing resistance training, that serum free testosterone levels were significantly lowered on the first and second rest days after the testing session. In the current study, serum free testosterone concentration was unaffected (by time or group) by the resistance training program. These findings are in agreement with previous studies that have shown no change in serum free testosterone following chronic resistance exercise (Izquierdo, Hakkinen *et al.*, 2001). Izquierdo and colleagues (2001) utilized two groups of men; middle-aged group with a mean age of 46 and an older group with a mean age of 62. Both groups participated in a 16-week resistance training program. Results showed no significant change in serum free testosterone following the resistance training program in both groups. Due to the increase in upper and lower body strength in the aforementioned and current study, it can be speculated that these hormones are still exerting their effects within the muscle as evidenced by significant increases in FFM and upper and lower body strength. Conversely, Hakkinen *et al.* (2000) demonstrated that following 6 months of heavy resistance training in both middle aged and elderly men, serum free testosterone was

significantly increased at each testing session. Moreover, unlike the current study and others (Izquierdo *et al.*, 2001), blood was sampled directly after the resistance training session and thus, serum free testosterone was measured within the acute (5-15 minute) window following resistance exercise. Therefore, similar to observations with serum IGF-1 concentration, it is possible that by sampling at 48 hours post-exercise, we are missing the window in which these anabolic hormones are elevated. But as mentioned previously, it can be speculated that these hormones are still exerting their effects within the muscle as evidenced by significant increases in FFM and upper and lower body strength.

HGF and cMET

As stated earlier, hepatocyte Growth Factor (HGF) is one of two factors that have been shown to activate quiescent satellite cells (SC). After muscle injury, HGF associates with the injured muscle fibers. Binding of HGF to its receptor, c-Met, phosphorylates c-Met which in turn leads to RAS activation and also prolonged activity of MAPK. Binding of HGF to c-Met is also capable of activating the PI3K pathway. Results from the present study show that the resistance training program and/or dietary supplementation had no significant effect of serum HGF levels. In contrast, phosphorylated cMET receptor concentration was significantly higher in the carbohydrate group compared to whey isolate protein group at 8 and 12 weeks and the casein protein group at 4 and 12 weeks. Since HGF and cMET are strong indicators of satellite cell proliferation, one would expect an increase in these markers due to the increase in FFM and strength markers in all groups. In fact, a decline in the number of SC and their inability to become active and proliferate in response to anabolic stimuli has

been speculated to contribute to the age-related loss of muscle mass and strength (for a thorough review see Snijders, Verdijk and van Loon, 2009). It is not readily apparent as to why a significant increase in phosphorylated cMET receptor concentration was observed in the carbohydrate group compared to the whey isolate protein and casein protein groups. No studies to the author's knowledge have examined the chronic effects of resistance exercise on serum HGF and skeletal muscle phosphorylated c-Met concentration in older individuals. Further, limited research has used human models, with a majority of research using animal or cell-culture models. In contrast to older individuals utilized in the current study, previous unpublished research from our laboratory (Shelmadine *et al.*, 2009) examined the effects of 28 days of high intensity resistance exercise on serum HGF and skeletal muscle phosphorylated c-Met concentration in younger males ages 18-35. Results showed that following 28 days of resistance exercise, serum HGF was decreased (8.71%), whereas phosphorylated c-Met concentration was increased (8.55%). It is clear that this concept requires further investigation, specifically in older populations.

Nutritional Intake

Dietary analysis revealed participants in the whey isolate protein –, casein protein - and carbohydrate – supplemented groups were similar in caloric energy, carbohydrate and fat intake, but more importantly protein consumption. Although supplements were not matched for carbohydrate and/or fat content, the caloric effect of both supplements were the same as isocaloric supplements were used. The consumption of diets that include sources of high-quality protein and total protein intakes that are similar to, or moderately above the recommended dietary allowance (RDA) of 0.8 g x kg(-1) x d(-1)

has been recommended in older adults, especially those who are regularly perform resistance exercises (Campbell *et al.*, 2007). In addition, others have suggested higher protein intakes of 1.0-1.3 g x kg(-1) x d(-1) may be required to maintain nitrogen balance and offset a potentially lower energy intake, decreased protein synthetic efficiency, and impaired insulin action in elderly individuals (Morais, Chevalier and Gougeon, 2006). Conversely, recent literature suggests increasing protein intake above recommended daily allowances through protein-enriched nutritional supplements does not influence training-induced improvements when adequate dietary protein is consumed (Timmerman *et al.*, 2008).

In the current study, all three groups consumed the current RDA for protein over the course of study period. Moreover, since improvements in strength were only observed in the whey isolate- and/or casein-supplemented groups compared to carbohydrate only group, it could be suggested that such observations can be attributed to the extra protein provided by the protein supplements. Interestingly, the carbohydrate group [~1.1 g x kg (-1) x d(-1)] was consuming a higher RDA compared to whey isolate [0.88g x kg(-1) x d(-1)] and casein group [0.83g x kg(-1) x d(-1), although such differences were not statistically significant.

Summary and Future Direction

In conclusion, the results of the present study suggest that whey isolate protein and casein protein ingestion may increase muscle strength to a greater extent than carbohydrate only ingestion following 12 weeks of resistance training in older individuals. Furthermore, both groups displayed higher serum IGF-1 levels during the course of the study. Notwithstanding, it was evident that 12 weeks of resistance training

alone increased both upper and lower body strength, body mass and lean muscle mass while decreasing body fat percentage. However, no effects from training were observed on serum anabolic hormones (i.e. testosterone and IGF-1) and indirect markers of satellite cell activation (HGF and phosphorylated C-Met). Despite few differences between treatment groups, it is clear from past and emerging literature that high intensity resistance training can significantly off-set the detrimental side-effects associated with muscle deterioration with aging.

The current study appreciably contributes to the scientific literature, as it is one of a few studies have examined the long-term effects of whey isolate and casein protein supplementation in older individuals. Further, no studies to the authors' knowledge have examined changes in HGF and phosphorylated c-MET receptor content in order to determine SC proliferation following long-term casein or whey isolate supplementation and/or high intensity resistance training within older populations. Also, it is important to note that all subjects were guided through their exercise regimens; therefore, exercise homogeneity among groups is assumed to be very high.

Future studies should examine and/or compare the timing and absorption of whey isolate and casein in older populations. A study by Tang *et al.*, (2009) examined the effects whey hydrolysate, casein, or soy protein isolate on mixed muscle protein synthesis at rest and following resistance exercise in young men. Results showed that feeding-induced simulation of MPS in young men was greater after whey hydrolysate (~21g) consumption compared to casein (~21g) both at rest and after resistance exercise. These differences may have related to how quickly the proteins were digested (i.e., fast vs. slow) or possibly to small differences in leucine content of each protein. What was most

interesting is that the differences in skeletal MPS that was observed may have implications for populations with compromised nutrient sensitivity (such as the elderly). It has been found that the protein digestibility paradigm observed in young individuals is actually "reversed" in the old with respect to whole body protein metabolism, and that "fast" protein ingestion is associated with a greater whole body leucine balance. If the leucine "trigger" concept is correct, then we would speculate that these results may reflect the inability of casein to increase blood EAA, BCAA, or leucine concentration high enough to turn on MPS in older persons who appear to have a reduced sensitivity to amino acids or an "anabolic resistance. Considering protein ingestion after exercise appears critical to enhance skeletal muscle hypertrophy with resistance training in the elderly, it could be proposed that elderly individuals would likely obtain the greatest benefit with respect to stimulating MPS and likely muscle protein accretion by consuming a "fast" leucine-rich dietary protein such as whey both at rest and after resistance exercise compared to slow absorbing casein protein. Indeed, in the current study, whey isolate group showed greater gains in lower body strength compared to casein group, although this was not statistically significant. Whether this was due to greater protein stimulation due to the fast acting proteins versus slow acting proteins is unknown and clearly warrants further investigation.

Obtaining blood draws prior to 24-hrs post-exercise may also help to better determine HGF and cMET's role in aged muscle and its association with satellite cell proliferation and differentiation. Determining the benefit of "guided exercise" versus "on your own" exercise may also be an applicable and conducive study to older individuals in determining the importance (or non-importance) of supervised exercise sessions.

The current study helps bring light the long-term effects of casein and whey supplemental intervention. Acute studies that have shown increases in MPS in older individuals following resistance training accompanied with some sort of nutritional intervention (Yarasheski, Zachwieja, & Bier, 1993; Hasten *et al.*, 2000; Drummond *et al.*, 2008). The delay in MPS observed by Drummond *et al.* (2008) may help guide further research studies in regard to nutrient-timing when striving to enhance MPS in older individuals following resistance training.

The importance of exercise has been confirmed numerous times in the aging community. The ability to make this knowledge obtained from research applicable to the wider community is crucial in making a difference in slowing the deterioration of muscle mass and all the negative implications that accompany it. Also, researchers must strive to determine precise nutritional intervention to help enhance the positive adaptations of resistance training in older, sarcopenic individuals.

APPENDICES

APPENDIX A

BAYLOR UNIVERSITY COMMITTEE
FOR PROTECTION OF HUMAN SUBJECTS IN RESEARCH
(Baylor IRB)

ANNUAL REVIEW FORM

Note: Projects may be considered complete when no further data will be collected, provided that the approved process for dissemination of data will be followed.

Please print or type - complete all items requested:

IRB Project#:*200708148* Academic Year Proposal Submitted: *Spring 2008* Due Date: *5/7/09*

Principal Investigator: *Matthew Cooke* Date of initial approval: 5/7/2008

Project Title: *The Effects of Combining a Creatine-Protein-Carbohydrate Supplement and Resistance Training on Body Composition, Muscle Strength, and Markers of Satellite Cell Activation in Older Males*

Department: HHPR Phone #: 254-710-4025

Mailing Address: HHPR Department, One Bear Place #97313

1. Do you wish to keep this project open? YES X NO
   If no, you must complete the **Notice of Completion/Termination Form** instead of this one.
   If yes, what is the anticipated completion date? July 2010

2. Informed Consent:
   A. Informed consent was obtained on ALL human subjects? YES X NO
   B. **Attach a copy of the consent form used.**
   Note: If any changes need to be made to the form that will be used in the future, submit two copies of the revised form in addition to the one previously used with an explanation of the changes. Be sure the form is formatted exactly as it will be used (on letterhead, etc.) Highlight the necessary changes on one copy; the other, without the highlights, will be stamped with the IRB approval stamp, if approved.

3. Have you altered the previously approved methodology or procedure used in this protocol **IN ANY WAY?** YES NO X

   If "YES", were the changes submitted to the IRB for approval prior to initiation? YES NO

Signature - Investigator — Date: 5-4-09

Signature - Faculty Advisor — Date:

Signature - Departmental Chair — Date: 5-4-09

Please send completed form to:
Baylor University IRB
One Bear Place #97334
Waco, Texas 76798-7334

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

Part 1: Signature Page

1. Name Matthew Cooke, Ph.D.,
2. Email Address (optional) Matt_Cooke@baylor.edu
3. Complete Mailing Address P.O. Box 97313
4. Position Assistant Professor
5. Faculty Advisor (if researcher is Graduate Student) _____
6. Department/School HHPR/SOE
7. Telephone # x3504 FAX # x3527
8. Are you using participants in research (***Y*** or N) or in teaching exercises (Y or ***N***)?
9. Title of the research project/teaching exercise:

The Effects of Combining a CrM-Protein-Carbohydrate Supplement and Resistance Training on Body Composition, Muscle Strength, and Markers of Satellite Cell Activation in Older Males

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 Participants in Research; Dr. Matt Stanford, Chairman, 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 participants in research, contact Dr. Stanford at extension 2961.

Signature of Principal Investigator — Date: 03/24/08

_____
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 progressive loss of neuromuscular function that often leads to progressive disability and loss of independence. The term sarcopenia is now commonly used to describe the loss of skeletal muscle mass and strength that occurs in concert with biological aging (Roth, Ferrell et al. 2000; Bales and Ritchie 2002; Janssen, Heymsfield et al. 2002; Borst 2004; Beccafico, Puglielli et al. 2007). By the seventh and eighth decade of life, muscle strength can be decreased, on average, by 20-40% for both men and women (Doherty 2003). Although age-associated decreases in strength per unit muscle mass, or muscle quality, may play a role, the majority of strength loss can be accounted for by decreased muscle mass (Doherty 2003). Multiple factors lead to the development of sarcopenia and the associated impact on function. Loss of skeletal muscle fibers secondary to decreased numbers of motor neurons appears to be a major contributing influence, but other factors, including decreased physical activity, altered hormonal status, decreased total caloric and protein intake, inflammatory mediators, and factors leading to altered protein synthesis, must also be considered (Thompson 2007). Given the link between sarcopenia and disability among elderly men and women and the expanding number of elderly people in the United States; sarcopenia is becoming an increasing health issue in the western world (Zacker 2006). Therefore, the need for continued research into the development of the most effective interventions to prevent or at least partially reverse sarcopenia, including the role of resistance exercise and other novel pharmacological and nutritional interventions is clearly needed.

In the past decade strength training has been investigated extensively as a means of reversing the muscle mass loss that occurs with aging (McCartney, Hicks et al. 1995; Hunter, McCarthy et al. 2004). High intensity resistance training (HIRT) has led to increased protein synthesis, along with muscle hypertrophy measured at the whole body, whole muscle, and muscle fiber levels, in older adults (Hunter, McCarthy et al. 2004). However, recent literature has demonstrated that muscle loss may still occur in older adults, even though weight bearing exercises are performed, suggesting nutrition is also an important component to combating sarcopenia (Campbell and Leidy 2007). Two nutritional ergogenic aids that have shown to enhance the effects of resistance training in elderly populations are CrM (CrM) and protein (in particular branch chain amino acids). CrM (CrM) as a nutritional supplement and ergogenic aid for athletes has been extensively studied over the past 20 years, and its effects on high intensity, short term exercise are well documented (Williams and Branch 1998; Kreider 2003; Rawson and Volek 2003). In older

populations, emerging literature suggests that CrM supplementation is capable of increasing muscle accretion during resistance training through the up-regulation of myogenic transcription factors and muscle specific-genes such as myosin heavy chain possibly leading to muscle hypertrophy (Candow and Chilibeck 2007). Indeed, Chrusch and colleagues (Chrusch, Chilibeck et al. 2001) showed greater increases in lean body mass, 1RM muscle strength and muscle endurance (leg press and isokinetic knee extension) following CrM supplementation when combined with 12 weeks of resistance training in older men (mean age 70yrs) compared to carbohydrate placebo. Recently, Olsen and colleagues (Olsen, Aagaard et al. 2006) demonstrated for the first time that CrM supplementation in combination with strength training amplifies the training-induced increase in satellite cell number and myonuclei concentration in human skeletal muscle fibers, thereby allowing an enhanced muscle fiber growth in response to strength training. Although this was observed in younger individuals, such effects in older populations need to be investigated.

The consumption of diets that include sources of high-quality protein and total protein intakes that are moderately above the recommended dietary allowance of 0.8 g x kg(-1) x d(-1) while regularly performing resistance exercises can help older people retain or increase whole-body fat-free mass and muscle mass (Campbell 2007; Campbell and Leidy 2007). However, recent literature suggests protein-enriched nutritional supplements do not influence training-induced improvements when adequate dietary protein is consumed (Timmerman and Volpi 2008). With such conflicting data, further research is needed to determine the effects of protein supplementation on training-induced adaptations in older populations.

Though it is evident that both supplements are capable of exerting individual effects within the body; whether combining both supplements will have an additive effect is unclear. Recently, Cribb et al., (Cribb, Williams et al. 2007) in a double-blind, randomized protocol, examined the effects of 3 supplement groups; protein only (PRO; 1.5 g x kg(-1) body weight x d(-1), protein-carbohydrate (PRO-CHO;1.5 g x kg(-1) body weight x d(-1) PRO, 1.5 g x kg(-1) body weight x d(-1) CHO), and a CrM-protein-carbohydrate (CrM-PRO-CHO; 1.5 g x kg(-1) body weight x d(-1) PRO, 0.1 g x kg(-1) body weight x d(-1) CrM, 1.5 g x kg(-1) body weight x d(-1) CHO) on body composition, muscle strength and hypertrophy in resistance trained males. Assessments were completed one week prior to, and following a 10-wk structured, supervised resistance

training program. Results showed that CrM-PRO-CHO provided greater improvements in 1RM strength, lean body mass, fiber cross-sectional area, and contractile protein compared with PRO and PRO-CHO groups (Cribb, Williams et al. 2007). Similarly, Burke and colleagues (Burke, Chilibeck et al. 2001) examined the effects of whey protein (WP;1.2 g/kg/day), whey protein and CrM (W-CrM; 0.1 g/kg/day), and placebo (P; 1.2 g/kg/day maltodextrin) on body composition and muscle strength. Results showed greater increases in lean tissue mass and bench press following W-CrM supplementation compared to those who supplemented with only WP or P (Burke, Chilibeck et al. 2001).

Therefore, while it is evident that additive effects of combining CrM and PRO appear to be effective in young individuals; whether such additive effects occur in older populations is yet to be determined. Furthermore, the mechanisms by which such supplements (alone or combined) exert their effects within the muscle is also unclear.

The primary purpose of this proposed clinically-controlled, double-blind study is to determine if a CrM-PRO-CHO supplement will provide greater benefits (i.e., lean body mass, muscle strength and hypertrophy) compared with a PRO-CHO, CrM-CHO or placebo, when combined with a partially supervised resistance training program in older males.

Part 3: Methodology

Methods & Participants

Sixty apparently healthy, recreationally active, but non-resistance trained [no regular, consistent resistance training (i.e. thrice weekly) for at least one year prior to the onset of the study], males between the ages of 55-75 will volunteer to participate in the double-blind study. Only participants considered as low risk for cardiovascular disease and with no contraindications to exercise as outlined by the American College of Sports Medicine (ACSM) and who have not consumed any nutritional supplements (excluding multi-vitamins) 3 months prior to the study will be allowed to participate. All participants must provide written informed consent and be cleared for participation by passing a mandatory medical screening. All eligible participants will sign university-approved informed consent documents and approval will be granted by the Institutional Review Board for Human Participants. Additionally, all experimental procedures involved in the study will conform to the ethical consideration of the Helsinki Code.

Study Site

All supervised testing and supplement assignment will be conducted in the Exercise & Sport Nutrition Laboratory (ESNL) at Baylor University. All sample analyses will be completed in the Exercise and Biochemical Nutrition Laboratory (EBNL) at Baylor University.

Independent and Dependent Variables

The independent variables will be the supplement (CrM-PRO-CHO, CrM-CHO, PRO-CHO, Placebo), resistance exercise protocol, and number of blood sampling and muscle biopsy times during the course of the study. Dependent variables will include resistance training adaptations (body composition, muscle strength, muscle mass, myofibrillar protein content, myosin heavy chain protein expression and muscle fiber type and area), serum testosterone, IGF-1, and markers of satellite cell activation [hepatocyte growth factor (HGF), c-met tyrosine kinase (c-met)], proliferation (Myo-D, myf-5), and differentiation (myogenin, MRF-4, total DNA content). In addition, the safety profile of 12 weeks of supplementation will be assessed by way of evaluating various serum and urinary clinical chemistry markers.

Entry and 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 will then be invited to attend an entry/familiarization session. Prior to entry/familiarization session, participants will be required to obtain written consent from their doctor to participate in the proposed study. Participants receiving confirmation from their doctor will report to the lab and also complete a medical history questionnaire and undergo a general physical examination to confirm their eligibility. Participants meeting entry criteria will be familiarized to the study protocol via a verbal and written explanation outlining the study design and will then be given an appointment time to perform baseline/pre-supplementation assessments. At this time, participants will be instructed to refrain from exercise for 48 hours, fast for 8-hours, and record their dietary intake for 4 days prior to each testing sessions occurring at weeks 0, 4, 8 and 12 weeks.

Muscle Biopsies and Venous Blood Sampling

Percutaneous muscle biopsies (50-70 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 1 and 2 cm. For the remaining four biopsies, attempts will be made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy scar, depth markings on the needle, and a successive incision 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 will be immediately frozen in liquid nitrogen and then stored at -70°C for later analysis. A total of four muscle samples will be obtained. Muscle samples will be obtained prior to the first dose of supplement and beginning of the resistance-training program (week 0) and then on day 29 (week 4), day 57 (week 8) and day 85 (week 12 of supplementation and resistance training program).

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. The serum will be removed and frozen at -70°C for later analysis. A total of blood samples will be obtained. Blood samples will be obtained prior to the first dose of supplement and beginning of the resistance-training program (week 0) and then on day 29 (week 4), day 57 (week 8) and day 85 (week 12 of supplementation and resistance training program).

Using commercially available enzyme-linked immunoabsorbent assay (ELISA) kits (Diagnostic Systems Laboratories, Webster, TX; Biosource, Camarillo, CA), the concentrations of serum testosterone, IGF-1 and HGF will be determined with a microplate reader (Wallac Victor 1420, Perkin Elmer, Boston MA). Total DNA content, Myofibrillar protein concentration and total muscle CrM concentration will be determined spectrophotometrically. Using ELISA analysis, muscle tissue samples will be analyzed to determine c-met, Myo-D, myogenin, MRF-4, and myf5 expression. Myosin heavy chain isoform protein expression will be determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Using a Dade Dimension clinical chemistry analyzer and an Abbott Cell Dyne 3500 hematology analyzer, blood and serum samples will be assayed for general clinical chemistry markers (i.e.,

glucose, total protein, blood urea nitrogen, creatinine, BUN/creatinine ratio, uric acid, AST, ALT, CK, LDH, GGT, albumin, globulin, sodium, chloride, calcium, carbon dioxide, total bilirubin, alkaline phosphatase, triglycerides, cholesterol, HDL, LDL) while whole blood samples will be assayed for standard cell blood counts with percentage differentials (i.e., hemoglobin, hematocrit, red blood cell counts, MCV, MCH, MCHC, RDW, white blood cell counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils). These assays will help evaluate the effects of the various supplementation regimens on general markers of tissue degradation, immune function, and clinical safety.

Anthropometric and Body Composition Testing Procedures

Total body mass (kg) will be determined on a standard dual beam balance scale (Detecto). Total body water (total, intracellular, and extracellular) will be determined with bioelectrical impedance (BIA) while percent body fat, fat mass, and fat-free mass, will be determined using DEXA. The participants will undergo total body mass, BIA, and DEXA at each testing session when blood and muscle samples are obtained, and these time points are prior to the first dose of supplement and beginning of the resistance-training program (week 0) and then on day 29 (week 4), day 57 (week 8) and day 85 (week 12 of supplementation and resistance training program).

Dietary Analysis

Participants will be required to record their dietary intake for 4 days prior to each of the four testing sessions at weeks 0, 4, 8 and 12 where blood and muscle samples are obtained. The participants' diets will not be standardized and participants will be asked not to change their dietary habits during the course of the study. The 4-day dietary recalls will be evaluated with the Food Processor dietary assessment software program to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet for the duration of the study.

Supplementation Protocol

Participants will be matched according to age and total muscle strength (both upper and lower body strength) and be assigned a 12-week supplementation protocol, in double-blind fashion, consisting of the oral ingestion of either a 1). CrM-Protein-Carbohydrate (10% CrM; 43% Whey Protein and 47% Maltodextrose); 2). CrM-Carbohydrate (10% CrM; and 90% Maltodextrose); 3).

Protein-Carbohydrate (50% Whey Protein and 50% Maltodextrose) or 4). Isocaloric Placebo (Crsytal Light®, a flavored non-energetic beverage). Each supplement will be similar in caloric content. The participants will be asked to consume 1.5 grams per kilogram body weight of their supplement in three equal servings throughout the day (described with measuring scoops provided). For example, one serving will be consumed midmorning, another soon after the workout in the afternoon (or similar time on non-training days), and the final serving in the evening before sleep. Participants will return empty containers of their supplement and also complete a supplement compliance questionnaire.

Training Protocol

Participants will participate in a partially supervised, periodized 3-day per week resistance-training program exercising both upper and lower extremity for a total of 12-weeks. Prior to beginning the resistance-training program, participants will be familiarized with the exercise equipment and 1RM for each exercise equipment will be determined indirectly. An indirect method (i.e. using a specific calculation to estimate 1RM) will be used to determine 1RM for each exercise equipment (excluding bench press and leg press) to lessen the potential risk of injury when attempting a 1RM. Prior to the workout, participants will perform a standardized series of stretching exercises. Participants will complete the bench press, lat pull-down, biceps curl, triceps press down, leg press, leg extension, leg curl, and leg press. Three sets of 10 repetitions will be completed for each exercise. An intensity of 80% of 1 RM will be utilized during training. Upon successful completion of 3 sets of 10 repetitions, an increase of 5% of weight utilized will take place. A rest period of one minute will separate each set. Participants will complete three training sessions per week for the duration of the study period. Participants will keep training logs detailing amount of weight utilized, number of repetitions completed, and number of sets completed. Training will be conducted at the Student Life Center (SLC) at Baylor University.

Strength Assessment

In order to determine possible effects of the supplement on muscular strength, participants will perform four one-repetition maximum (1-RM) tests on the bench press and leg press sled exercises prior to the first dose of supplement and beginning of the resistance training program (week 0), on day 29 (week 4), on day 57 (week 8) and on day 85 (week 12 of supplementation

and resistance training). Participants will perform a standardized series of stretching exercises. Participants will warm up by completing 5 to 10 repetitions at approximately 50% of the estimated 1-RM. The participant will rest for 1 minute, and then complete 3 to 5 repetitions at approximately 70% of the estimated 1-RM. The weight will then be increased conservatively, and the participant will attempt to lift the weight for one repetition. If the lift is successful, the participant will rest for 2 minutes before attempting the next weight increment. This procedure will be continued until the participant fails to complete the lift. The 1-RM will be recorded as the maximum weight that the participant is able to lift for one repetition.

Assessment of Serum Testosterone, IGF-1 and HGF (Growth Factor) Levels

From the four blood samples obtained at weeks 0, 4, 8 and 12, serum testosterone, IGF-1 and HGF levels will be determined using enzyme linked immunoabsorbent assay (ELISA).

Assessment of Skeletal Muscle Protein Expression

From the four muscle tissue samples obtained at weeks 0, 4, 8, and 12, assessment of c-met, Myo-D, myogenin, MRF-4, and myf5 expression will be determined using (ELISA). The expression of MHC protein isoforms will be determined by SDS-PAGE, and the content of myofibrillar protein, total DNA and total muscle CrM concentration will be determined spectrophotometrically.

Assessment of Skeletal Muscle Fiber Type and Area

Transverse sections of muscle tissue obtained from weeks 0, 4, 8, and 12 will undergo histochemical analysis to classify muscle fiber types I, IIa, and IIx on the basis of the stability of their ATPase activity, as previously described [19]. Muscle cross sectional area and fiber type will be determined using an inverted microscope (VWR Vista Visia Microscope, Model 82026-630) and analytical imaging package.

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

At each of the four testing sessions where blood and muscle samples are obtained at weeks 0, 4, 8, and 12, participants will undergo assessment of heart rate and blood pressure. Heart rate will be determined by palpation of the radial artery using standard procedures. Blood pressure will be

assessed in the supine position after resting for 5-min using a mercurial sphygmomanometer using standard procedures.

Reported Side Effects from Supplements

After weeks 4 and 12, participants will report by questionnaire whether they tolerated the supplement, supplementation protocol, as well as report any medical problems/symptoms they may have encountered throughout the protocol of the study.

Statistical Analyses

Data will be analyzed by utilizing separate 4 x 4 [Group (CrM/PRO/CHO, CrM/CHO, PRO/CHO, Placebo)] x Test (week 0, week 4, week 8, week 12) mixed design factorial multivariate analysis of variance (MANOVA). Three sets of MANOVAs (one set for skeletal muscle variables, and one set for blood clinical chemistry variables, and one set for body composition, muscle strength, and dietary intake variables) will be analyzed for this study based on dependent variables that are likely to be related to one another. In addition, the use of a MANOVA analysis also prevents the increasing of Type I errors that would result with the use of repeated univariate procedures. The MANOVA will be used to control for alpha inflation of the subsequent univariate analysis of variance (ANOVA). ANOVA on each dependent variable will be conducted as follow-up tests to the MANOVA. To control for alpha inflation of the ANOVA, the Bonferroni test will be utilized. Post-hoc tests of any interaction effects demonstrated in the ANOVA will be investigated via an independent samples t-test. In addition to reporting probability values, an index of effect size will be reported to reflect the magnitude of the observed effect. All statistical procedures will be performed using SPSS 15.0 software (Chicago, IL) and a probability level of <0.05 adopted throughout. However, to protect against Type I error, the conservative Hunyh-Feldt Epsilon correction factor will be used to evaluate observed within-group F-ratios.

Research Team

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

Paul LaBounty, Ph.D. Dr. LaBounty is an Assistant Professor of Anatomy, Physiology and Nutrition in the Department of Health, Human Performance, & Recreation at Baylor University. Dr. LaBounty will assist in data collection and performing laboratory analyses.

Brian Brabham, M.S. Mr. Brabham is an exercise physiologist pursuing his Ph.D. in Exercise, Nutrition, and Preventative Health. His primary research focus is aging, nutrition and resistance training. He will provide assistance with participant recruitment, day-to-day scheduling and testing, data collection and analysis.

Thomas Buford, M.S. Mr. Buford is an exercise physiologist pursuing his Ph.D. in Exercise, Nutrition, and Preventative Health and serves as a research assistant in the EBNL. His primary research focus is exercise immunology/inflammation and molecular signaling in skeletal muscle. He will provide assistance with participant recruitment, day-to-day scheduling and testing, data collection and analysis.

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

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 co-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.

Mike Greenwood, PhD, FACSM, FISSN, CSCS*D. Dr. Greenwood is a Professor and Research and Graduate Coordinator in the Department of Health, Human Performance, and Recreation at Baylor University. Dr. Greenwood is internationally recognized in the field of strength and conditioning and has conducted a vast amount of research focusing on the role of exercise and nutrition. Dr. Greenwood will provide direct administrative oversight for the study.

Richard B. Kreider, PhD, FACSM. Dr. Kreider is Professor and Chair of the Department of Health, Human Performance and Recreation at Baylor University. Dr. Kreider is the director of the Exercise and Sport Nutrition Laboratory and is an internationally recognized exercise scientist. He has conducted a vast amount of research primarily in the areas of sport nutrition and the role of exercise and nutrition on health and disease. Dr. Kreider will provide direct administrative oversight for the study.

Procedures

Medical Monitoring. Interested participants will first need to obtain medical clearance from their doctor. Once clearance is obtained, participants will be invited to a familiarization session. During this time, participants will sign consent forms and complete medical history information. Participants will then undergo a general exam by the research nurse to confirm eligibility obtained from their doctor. 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. 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., Dr. Matthew Cooke Ph.D., or Dr. Lori Greenwood. If clinically significant side effects are reported, the participants will be referred to discuss the problem with Dr. Lori Greenwood, and if deemed necessary Dr. Greenwood will refer the participant 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 their possible contraindications to exercise assessed by Certified Exercise Physiologists and the laboratory nurse in accordance to standard procedures described by the American College of Sports Medicine (ACSM) (*ACSM's Guidelines for Exercise Testing and Prescription, 6th ed. Williams & Wilkins Publishers, 2000)*. Only those participants considered as low 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.

Assessment of Hemodynamic Safety Markers (Heart Rate & Blood Pressure). At the four testing sessions where blood and muscle samples are obtained at weeks 0, 4, 8, and 12, participants will undergo assessment of heart rate and blood pressure. Heart rate will be determined by palpation of the radial artery using standard procedures. Blood pressure will be assessed in the supine position after resting for 5-min using a mercurial sphygmomanometer using standard procedures.

Reported Side Effects from Supplement Questionnaires. At weeks 4 and 12, participants will report by questionnaire whether they tolerated the supplement, supplementation protocol, as well as report any medical problems/symptoms they may have encountered throughout the protocol of the study.

Estimated Energy Intake/Dietary Inventories. For 4 days during weeks 0, 4, 8, and 12, participants will record all food and fluid intake on dietary record forms for dietary analysis in order to standardize nutritional intake. Participants will brings these forms with them upon each visit to the laboratory for the two testing sessions. Dietary intake will be assessed using the Food Processor IV Nutrition Software.

Body Composition Assessments. At weeks 0, 4, 8, and 12, participants will undergo body composition tests in the ESNL. Prior to each assessment, height will be measured using standard anthropometry and total body weight will be measured using a calibrated electronic scale with a precision of +/- 0.02 kg. Total body water will then be estimated using a Xitron 4200

Bioelectrical Impedance Analyzer (San Diego, CA) which measures bio-resistance of water and body tissues based on a minute low energy, high frequency current (500 micro-amps at a frequency of 50 kHz) transmitted through the body. This analyzer is commercially available and has been used in the health care/fitness industry as a means to assess body composition and body water for over 20 years. The use of this device has been approved by the Food and Drug Administration (FDA) to assess total body water and the current to be used has been deemed safe. This is measured through four electrodes placed on the body: one electrode will be placed on the posterior surface of the right wrist, in between the radial and ulna styloid processes (wrist bones), another electrode will be placed on the posterior surface of the right hand at the distal base of the second metacarpal; the third electrode will be placed on the anterior surface of the right foot at the distal end of the first metatarsal. Participants will lie on a table in the supine position and electrodes will be connected to the analyzer. After the participant is connected, age, gender, weight, height, and activity level are entered into the unit by the technician. After the unit has measured the resistance, which takes approximately 30 seconds, the unit then calculates total body water and body water percent.

Body composition/bone density will then be determined using a calibrated Hologic 4500W dual-energy x-ray absorptiometry (DEXA) by licensed personnel with limited x-ray technology training under the supervision of Richard B. Kreider, PhD, MX. The DEXA body composition test will involve having the participant lie down on their back in a standardized position in a pair of shorts/t-shirt or a gown. A low dose of radiation will then scan their entire body for approximately six (6) minutes. The DEXA segments regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments for determination of fat, soft tissue (muscle), and bone mass. Radiation exposure from DEXA for the whole body scan is approximately 1.5 mR per scan. This is similar to the amount of natural background radiation a person would receive in one month while living in Waco, TX. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation dose will be less than 5 mR for the entire study.

Strength Assessments. At weeks 0, 4, 8, and 12, all strength/exercise tests will be supervised by certified lab assistants experienced in conducting strength/anaerobic exercise tests using standard procedures. Strength testing will involve the participants performing one repetition maximum (1 RM) on the isotonic Nebula hip/leg sled and isotonic bench press. Participants will warm-up (2 sets of 8–10 repetitions at approximately 50% of anticipated maximum) on the hip/leg sled and bench press. Participants will then perform successive 1 RM lifts starting at about 70% of anticipated 1RM and increasing by 10 – 20 lbs until the participant reaches their 1RM.

Serum Testosterone, IGF-1 and HGF Levels. Serum samples will be analyzed for testosterone, IGF-1 and HGF protein content using ELISA. Samples will be processed and then placed into individual wells of a microtiter plate, previously coated with a primary antibody against each of the respective proteins, for incubation. The plates will then be washed and incubated with a conjugated secondary antibody that binds to the primary antibody. The membrane will be washed again, and then exposed to a chemical substrate that produces color when exposed to the

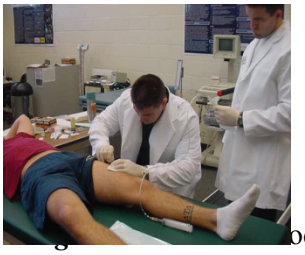
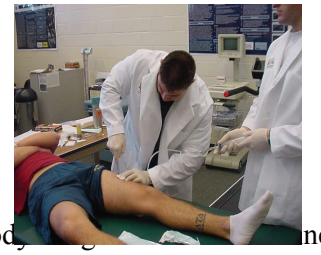
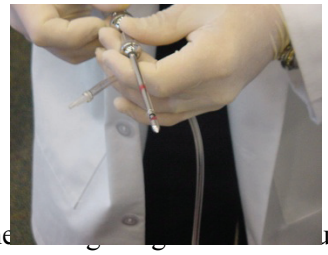
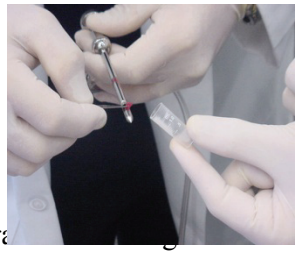
conjugated secondary antibody. Protein concentrations will be determined with a Wallac Victor-1420 micoplate reader (Perkin-Elmer Life Sciences, Boston, MA). The assays will be performed at either 405 or 450 nm wavlength, each against a known standard curve.

Myo-D, myogenin, MRF-4, myf5, and MHC Protein Expression. Muscle tissue samples will be analyzed for Myo-D, myogenin, MRF-4, and myf5 expression by ELISA. Samples will be processed and then placed into individual wells of a microtiter plate, previously coated with a primary antibody against each of the respective proteins, for incubation. The plates will then be washed and incubated with a conjugated secondary antibody that binds to the primary antibody. The membrane will be washed again, and then exposed to a chemical substrate that produces color when exposed to the conjugated secondary antibody. Protein concentrations will be determined with a Wallac Victor-1420 micoplate reader (Perkin-Elmer Life Sciences, Boston, MA). The assays will be performed at either 405 or 450 nm wavlength, each against a known standard curve. For MHC protein isoform expression, muscle tissue will be homogenized with Tri Reagent (Sigma), and the protein from the total homogenate will separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Criterion Blotter (Bio-Rad, Hercules, CA). Total DNA and total muscle CrM concentration will be determined spectrophotometrically.

Blood Samples. Participants will donate approximately 3-4 teaspoons of fasting venous blood (6 – 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., Dr. Matthew Cooke PhD., 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. Participants 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 21 gauge sterile needle attached to a plastic vacutainer holder will be inserted into the vein using standard procedures. Three serum separation vacutainer tubes (red tops) and one EDTA vacutainer tubes (purple top) 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 serum samples, transfer serum into labeled serum storage containers, and store at -20°C for later analysis.

Muscle Biopsies. Percutaneous muscle biopsies (approximately 50-70 mg) will be obtained from the vastus lateralis of each participant's thigh. Samples will be extracted under local anesthesia of 2% Xylocaine with epinephrine from the middle portion of the muscle at the midway 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 incision and depth markings on the needle. First, the participant will lie supine or assume a comfortable reclining position on a sterilized table. Once the extraction point is identified, the area will be shaved clean of leg hair, washed with an antiseptic soap and cleaned with rubbing alcohol. In addition, the biopsy site will further be cleansed by swabbing the area with Betadine (fluid antiseptic) and then draped. A small area of the skin approximately 2 cm in diameter will be anesthetized with a 1.0 mL subcutaneous injection of Xylocaine. Once anesthetized, a scalpel point will be used to produce the initial biopsy site by making an incision approximately 1 cm in length through the skin, subcutaneous fat, and fascia lata. Due to the localized effects of the anesthetic, the participant should feel no pain during this process. The biopsy needle will be advanced into the incision approximately 1 cm and during this part of the procedure the participant may feel pressure to the thigh area. Once the muscle sample has been obtained, pressure will be immediately applied and the wound will immediately be bandaged. With the incision being so small bleeding is slight; therefore, only a butterfly bandage is needed to close the incision, which is then covered with a pressure bandage. The needle and scalpel blade 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 a appropriately labeled biohazard waste receptacle. The tissue sample will be stored at -70°C for future analyses. Once the local anesthesia has taken effect (approximately 2-3 minutes) the biopsy procedure will take approximately 15-20 seconds. Written instructions for post-biopsy care will be given to the participants. The participant will be instructed to leave the bandages on for 24 hours (unless unexpected bleeding or pain occurs) and asked to report back to the lab within 24 hours to have the old bandages removed, the incision inspected and new bandages applied. The participant will be further advised to refrain from vigorous physical activity during the first 48 hours post-biopsy. These suggestions will minimize pain and possible bleeding of the area. If needed, the participant may take non-prescription analgesic medication such as Ibuprofen 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.

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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.

Mercurial Sphygmomanometer. Blood pressure will be assessed by auscultation of the brachial artery using a mercurial sphygmomanometer using standard clinical procedures.

Bioelectrical Impedance Analyzer (BIA). The Omron HBF-306 Bioelectrical Impedance Analyzer (*Omron Healthcare Inc., Vernon Hills, IL*) which measures bio-resistance and body composition based on a minute low energy, high frequency current transmitted through the body

from surface electrodes embedded in the handles of the unit. The analyzer is calibrated internally to a standard electrical current by pressing the calibration key located on the unit. A trained research assistant will perform this procedure.

Dual-Energy X-Ray Absorptiometer (DEXA). Body composition measurements will be determined by qualified personnel (in compliance with State Regulations) using a Hologic Discovery W dual energy x-ray absorptiometer (*Waltman, MA*). This system segments regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments (i.e., bone mass, fat mass, and fat-free/soft tissue mass). Quality control (QC) calibration procedures will be performed on a spine phantom (Hologic X-CALIBER Model DPA/QDR-1 anthropometric spine phantom) prior to each testing session. In addition, weekly calibration procedures will be performed on a density step calibration phantom.

Resistance Exercise Machines. Maximum strength (1-RM strength) tests will be performed on a isotonic leg/hip sled (Nebula Fitness, Inc., Versailles, OH) and a isotonic bench press (Body Masters, Inc., Rayne, LA). Equipment and testing will be contained within the ESNL.

Muscle Biopsy Needle. The muscle biopsy technique will be performed with a 5-mm Bergstrom biopsy needle (shown in photos on previous page).

Clinical Chemistry Analyzers. Blood and serum samples will be used to run clinical chemistry profiles [glucose, total protein, blood urea nitrogen, creatinine, BUN/creatinine ratio, uric acid, AST, ALT, CK, LDH, GGT, albumin, globulin, sodium, chloride, calcium, carbon dioxide, total bilirubin, alkaline phosphatase, triglycerides, cholesterol, (HDL, LDL), whole blood cell counts including hemoglobin, hematocrit, red blood cell counts, MCV, MCH, MCHC, RDW, white blood cell counts (neutrophils, lymphocytes, monocytes, eosinophils, baosphils), nitrate, and nitrite] photometrically by way of a DADE Dimension RXL clinical chemistry analyzer (Dade-Behring, Inc., Newark, DE), and an Abbott Cell Dyn 3500 hematology analyzer (Abbott Laboratories, Chicago, IL). Prior to use each system will be calibrated with standard quality assurance protocols.

Serum and Muscle Protein and Metabolite Analyses. Blood and muscle samples will also be used to assess hormone profiles and muscle metabolites spectrophotometrically using either enzyme-linked immunoabsorbent assays (ELISA) or spectrophotometrically with a Wallac Victor-1420 micoplate reader Wallac Victor-1420 micoplate reader (Perkin-Elmer Life Sciences, Boston, MA). The assays will be performed at either 405 or 450 nm wavlength against a known standard curve.

Participants

Recruitment

Sixty non-resistance-trained (no regular, consistent resistance training for at least one year) male participants between the ages 55 to 75 will participate in this study. A recruitment flyer that will be posted on campus, at area fitness centers, and on the Internet (http://www3.baylor.edu/HHPR/research/participants/) and sent via campus mail is attached.

Selection Criteria

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

1. have been involved in a habitual resistance training program (minimum of 3 hours/week for at least 1 year);
2. have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism;
3. have a history of pulmonary disease, hypertension, hepatorenal disease, musculoskeletal disorders, neuromuscular/neurological diseases, autoimmune disease, cancer, peptic ulcers, anemia, or chronic infection (e.g., HIV);
4. are taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (e.g, thyroid, insulin, etc), psychotropic, neuromuscular/neurological, or androgenic medications;
5. have taken ergogenic levels of nutritional supplements that may affect muscle mass (e.g., CrM, HMB) or anabolic/catabolic hormone levels (e.g., androstenedione, DHEA, etc) within three months prior to the start of the study.
6. have any absolute or relative contraindication for exercise testing or prescription as outlined by the American College of Sports Medicine;
7. report any unusual adverse events associated with this study that in consultation with the supervising physician recommends removal from the study.

Compensation or Incentives

Participants completing all familiarization and testing sessions as well as turning in all required materials (i.e., dietary logs, reported side effects for supplement questionnaires, and unused supplements) in the study will be paid $150. Participants may receive information regarding results of these tests if they desire.

Potential Risks

Participants who meet eligibility criteria will be exposed to a very low level of electrical current that will be passed through each participant's body using a bioelectrical impedance analyzer. This analyzer is commercially available and has been used in the health care/fitness industry as a means to assess body composition and body water for over 20 years. The use of the BIA analyzer has been shown to be safe methods of assessing body composition and total body water and is approved by the FDA. CrM and whey protein supplementation has been extensively studied for various uses, both medical and non-medical in humans. Initial research has demonstrated that oral administration of this compound is not associated with any significant medical side effects. However, the CrM and amino acid formulation has yet to be studied, even though these

supplements are currently available in over-the-counter nutritional supplements sold in United States. As with the vast majority of nutritional supplements, however, the FDA may not have evaluated the safety or marketing claims of these supplements.

Participants who meet eligibility criteria will be participanted to strength testing sessions involving dynamic muscle contractions. Participants in this study will not be experienced resistance trainers, and will be instructed to only perform the prescribed resistance training protocol throughout the duration of the study. 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 1-RM testing and the dynamic exercise protocol are possible. However, potential injury due to exercise will be minimized by ensuring that all participants adhere to correct lifting technique. In addition, only Darryn Willoughby, Ph.D., Dr. Matthew Cooke 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. However, there are minor risks of muscular pain and soreness associated with the resistance training protocol required in this study which are not uncommon to any exercise program especially for individuals who do not resistance train on a regular basis. Participants will donate 3-4 teaspoons (6-12 milliliters) of venous blood four 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 participant 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 participant does not adequately cleanse the area for approximately 48-72 hours post biopsy. While leaving the butterfly bandage in place, participants will be instructed to cleanse the biopsy area with soap and water every 4-6 hours, pat the area dry and reapply a fresh adhesive bandage. The participant will be instructed to leave the bandages on for 24 hours (unless unexpected bleeding or pain occurs) and asked to report back to the lab within 24 hours to have the old bandages removed, the incision inspected and new bandages applied. 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 participants 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. Darryn Willoughby, Ph.D. or Dr. Matthew Cooke, Ph.D., will perform all muscle biopsies. Up to this point in his professional career, Dr. Willoughby has successfully performed over 500 muscle biopsies on both male and female participants ranging from 18-85 years of age without any complications. Similarly, Dr. Matthew Cooke has performed over 200 muscle biopsies on both male and female participants ranging from 18-85 years of age 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 participant 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 these CrM and whey protein-containing nutritional supplements are effective, there is a possibility that they may gain insight into how to enhance training-induced improvements from resistance exercise, but more importantly, reduce the rate of age-related muscle loss and the decline in force output often seen in the older population. Participants may also 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 training adaptations and reduces age-related loss in muscle mass and strength. This information will be helpful to older populations who suffer from the effects of sarcopenia.

Assessment of Risk

Even though clinical data are available outlining the safety effects of many CrM-containing supplements, because they are still relatively new to the market the potential medical benefits of the different CrM supplement formulations are not yet well delineated. Although, CrM is available in a number of over the counter nutritional supplements, initial results suggest that these supplements may provide benefit at increasing muscle CrM uptake, promoting muscle accretion, and enhancing training adaptations during training. However, additional well-controlled research is necessary before conclusions can be drawn. This study will help determine whether ingesting CrM and whey protein may effectively increase muscle mass and satellite cell activation for active, untrained individuals. 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 resistance 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. Therefore, the potential benefits of participants 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 participant 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 College of Sports Medicine, International Society of Sports Nutrition, Experimental Biology, etc.) and published in a peer reviewed scientific journal (e.g., Medicine & Science in Sport and Exercise, Journal of Sport Science and Medicine, International Journal of Sport Nutrition and Exercise Metabolism, etc.).

Statement on Conflict of Interest
Partial support of this study was obtained from supplements donated by Vital Pharmaceuticals, Inc. (Davie, FL). Researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Letter to IRB Regarding Amendments of the Previous IRB Approved Study

Dear Dr. Stanford,

I have attached the IRB Annual Review Form for the continuation of the IRB #200708148.

We would like to make additional amendments to our previous submission (#200708148) entitled: "The Effects of Combining a Creatine-Protein-Carbohydrate Supplement and Resistance Training on Body Composition, Muscle Strength, and Markers of Satellite Cell Activation in Older Males"

I'd like to make a number of changes to the IRB:
1). ADD TWO (2) MORE GROUPS. Previously, we had proposed 4 groups: 1) CrM-PRO-CHO, 2) PRO(Whey)-CHO, 3) CrM-CHO and 4) Placebo (CHO). We have completed 2 groups to date and would like to add 2 more groups: 5) (PRO (Casein)-CHO and 6) Protein (Leucine)-CHO). This will increase participant number to 90 (15 each group). These 2 groups will be used for a Master's Thesis project.

References for the benefit of using these 2 new supplements in older individuals are below:

Katsanos CS, Kobayashi H, Sheffield-Moore M, Aarsland A, Wolfe RR. Effect of a leucine-enriched amino acid drink on muscle protein synthesis in the elderly. FASEB J. 2005;19:A1573

Verhoeven S, Vanschoonbeek K, Verdijk LB, Koopman R, Wodzig WK, Dendale P, van Loon LJ. Long-term leucine supplementation does not increase muscle mass or strength in healthy elderly men. Am J Clin Nutr. 2009 May;89(5):1468-75. Epub 2009 Mar 25.

Kerksick CM, Rasmussen CJ, Lancaster SL, Magu B, Smith P, Melton C, Greenwood M,Almada AL, Earnest CP, Kreider RB. The effects of protein and amino acid supplementation on performance and training adaptations during ten weeks of resistance training. J Strength Cond Res. 2006 Aug;20(3):643-53.

2). Look at more signaling pathways in tissue homogenate and blood serum that relates to muscle growth and regeneration
Insulin, IRS-1 Akt, 4EBP1, p70-S6K, MTOR, Myostatin, Follistatin, MGF

I have attached 2 IRB consent forms (original and updated version). Changes in old version highlighted in yellow. Since we didn't mention analysis on muscle and blood in the consent form, I have not included this
I was also informed that you will no longer be the Chair of the University IRB committee. I would like to thank you for all your help and work as the Chair.

Regards,
Matthew Cooke

Amendments Approved May, 2009

APPENDIX B

BAYLOR UNIVERSITY

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

Title of Investigation: The Effects of Combining a Creatine-Protein-Carbohydrate Supplement and Resistance Training on Body Composition, Muscle Strength, and Markers of Satellite Cell Activation in Older Males

Principal Investigator: Matthew Cooke, PhD.
Department of HHPR, Baylor University

Co-investigators: Darryn S. Willoughby, PhD, FACSM, FISSN, CSCS, CISSN
Department of HHPR, Baylor University

Brian Brabham, MS
Department of HHPR, Baylor University

Paul La Bounty, PhD
Department of HHPR, Baylor University

Thomas Buford, PhD
Department of HHPR, Baylor University

Ronald Wilson, MD
Department of HHPR, Baylor University

Mike Greenwood, Ph.D., FACSM, FISSN, CSCS*D
Department of HHPR, Baylor University

Richard B. Kreider, PhD, FACSM, FISSN
Chair, Department of HHPR, Baylor University

Sponsors: Baylor University

Rationale:

Aging is associated with progressive loss of neuromuscular function that often leads to progressive disability and loss of independence. The term sarcopenia is now commonly used to describe the loss of skeletal muscle mass and strength that occurs in concert with

biological aging. By the seventh and eighth decade of life, muscle strength can be decreased, on average, by 20-40% for both men and women. Although age-associated decreases in strength per unit muscle mass, or muscle quality, may play a role, the majority of strength loss can be accounted for by decreased muscle mass. Multiple factors lead to the development of sarcopenia and the associated impact on function. Loss of skeletal muscle fibers secondary to decreased numbers of motor neurons appears to be a major contributing influence, but other factors, including decreased physical activity, altered hormonal status, decreased total caloric and protein intake, inflammatory mediators, and factors leading to altered protein synthesis, must also be considered. Given the link between sarcopenia and disability among elderly men and women and the expanding number of elderly people in the United States; sarcopenia is becoming an increasing health issue in the western world. Therefore, the need for continued research into the development of the most effective interventions to prevent or at least partially reverse sarcopenia, including the role of resistance exercise and other novel pharmacological and nutritional interventions is clearly needed.

In the past decade strength training has been investigated extensively as a means of reversing the muscle mass loss that occurs with aging. High intensity resistance training (HIRT) has led to increased protein synthesis, along with muscle hypertrophy measured at the whole body, whole muscle, and muscle fiber levels, in older adults. However, recent literature has demonstrated that muscle loss may still occur in older adults, even though weight bearing exercises are performed, suggesting nutrition is also an important component to combating sarcopenia. Two nutritional ergogenic aids that have shown to enhance the effects of resistance training in elderly populations are creatine monohydrate (CrM) and protein (in particular branch chain amino acids). Creatine monohydrate (CrM) as a nutritional supplement and ergogenic aid for athletes has been extensively studied over the past 20 years, and its effects on high intensity, short term exercise are well documented. In older populations, emerging literature suggests that CrM supplementation is capable of increasing muscle accretion during resistance training through the up-regulation of myogenic transcription factors and muscle specific-genes such as myosin heavy chain possibly leading to muscle hypertrophy.

Similarly, the consumption of diets that include sources of high-quality protein and total protein intakes that are moderately above the recommended dietary allowance of 0.8 g x kg(-1) x d(-1) while regularly performing resistance exercises can also help older people retain or increase whole-body fat-free mass and muscle strength. However, recent literature suggests protein-enriched nutritional supplements do not influence training-induced improvements when adequate dietary protein is consumed. With such conflicting data, further research is needed to determine the effects of protein supplementation on training-induced adaptations in older populations.

Though few studies have examined the individual effects of creatine and/or protein supplementation in conjunction with resistance training in elderly populations; no studies have examined the combined effects of both supplements. Furthermore, limited studies

have compared this to the slow digesting protein, casein and the branch chain amino acid, leucine. Moreover, how each supplement elicits its effects within the muscle is still also unclear. Therefore, the primary purpose of this proposed clinically-controlled, double-blind study is to determine if a CrM-PRO-CHO supplement will provide greater benefits (i.e., lean body mass, muscle strength and hypertrophy) compared with a PRO(Whey)-CHO, PRO (Casein)-CHO, Protein (Leucine)-CHO, CrM-CHO or placebo, when combined with a partially supervised resistance training program in older males.

Description of the Study:

I will be one of 90 apparently healthy untrained males between the ages 55 to 75 who will participate in this study. 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. Prior to participating in this study, I also understand that I need to obtain written consent from my doctor clearing me to participate in the proposed study. If I am cleared to participate in the study, I will be familiarized to the testing procedures. This session will take approximately 30 minutes to complete. Once I complete the familiarization session, I will be scheduled for baseline testing.

Following the familiarization session, I will be instructed to refrain from exercise for 48 hours and fast for 8 hours prior to baseline testing. I will be provided with a dietary analysis form that I am to complete for 4 days prior to testing. Once I report to the lab for each testing session, I will turn in my dietary analysis form.

I understand that I will then donate about 6-12 milliliters (3-4 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 5 minutes and I understand that I will be asked to donate the same volume of blood on four separate occasions throughout the study.

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 dominant leg. The biopsy area will be shaved clean of leg hair, washed with antiseptic soap and 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 2 cm in diameter will be anesthetized with a 1.0 mL subcutaneous injection of the topical anesthetic Xylocaine. Once the local anesthesia has taken effect (approximately 2-3 minutes) the biopsy procedure will only take 15-20 seconds. Once anesthetized, I understand that a scalpel point will be used to make an incision approximately 1 cm in length through the skin. Due to the localized effects of the anesthetic, however, I should feel no pain during this process. At this point, I understand that the biopsy needle will be advanced into the incision approximately 1 cm and during

this part of the procedure I may feel pressure in my thigh area. Once the muscle sample has been obtained, pressure will be immediately applied to the incision. With the incision being so small bleeding is slight; therefore, only a butterfly bandage is needed to close the incision, which will then be covered with a pressure bandage. I understand that I will be provided verbal and written instructions for post-biopsy care. I understand that I will be instructed to leave the butterfly bandage in place for 72 hours. However, I understand that I will be asked to report back to the lab 24 hours after the biopsy (unless unexpected bleeding or pain occurs) to have the old bandage removed, the incision inspected and new bandages applied, and that I will also report back to the lab for the same reason at 48 hours after the biopsy. 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 Tylenol 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. I understand that I will be asked to undergo the muscle biopsy procedure on four separate occasions throughout the study.

I will be matched by my age and total muscle strength (upper and lower body muscle strength) and then randomly assigned to ingest in a double blind manner a supplement containing either 1). Creatine-Protein-Carbohydrate (10% CrM; 43% Whey Protein and 47% Maltodextrose); 2). Creatine-Carbohydrate (10% CrM; and 90% Maltodextrose); 3). Protein-Carbohydrate (50% Whey Protein and 50% Maltodextrose); 4). Protein-Carbohydrate (50% Casein Protein and 50% Maltodextrose); 5). Protein-Carbohydrate (50% Leucine and 50% Protein-Carbohydrate (50% Whey Protein and 50% Maltodextrose) or 6). Isocaloric Placebo (Crsytal Light®, a flavored non-energetic beverage). I will be asked to orally ingest my selected supplement at a daily dose of 1.5g/kg.bw/day in three equal servings throughout the day (described with measuring scoops provided). For example, one serving will be consumed midmorning, another soon after the workout in the afternoon (or similar time on non-training days), and the final serving in the evening before sleep. I will be instructed not to change my routine dietary intake. Compliance to the supplementation protocol will be monitored by having me return the empty supplement container at each testing session, at which point I will be given the required supplement dosage for the following weeks. I understand that if I do not take my supplements I will be removed from the study.

I understand that I will be required to participate in a periodized 3-day per week resistance-training program that will exercise both upper and lower extremities. Prior to the beginning the resistance training program workout, I will perform a standardized series of stretching exercises and then perform the resistance-training program consisting of eight exercises (bench press, lat pull-down, biceps curl, triceps press down, leg press, leg extension, leg curl, and leg press). I understand that I will perform 3 sets of 10 repetitions at an intensity of 80% of 1 RM. Upon successful completion of 3 sets of 10 repetitions, an increase of 5% of weight utilized will take place. A rest period of one

minute will separate each set. I will also keep a training log detailing amount of weight utilized, number of repetitions completed, and number of sets completed.

I understand that I will be required to report to the laboratory on Day 0 (prior to beginning the supplementation and resistance training protocol) to have my heart rate and blood pressure determined, to turn in my 4-day dietary records, have my body composition and muscle strength determined, and to have a muscle and blood sample obtained. I understand that I will report back to the laboratory at the end of week 4, 8 and 12 to undergo the same testing procedures as I went through prior to beginning the study; however, I will also 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 at the end of weeks 4 and 12. I understand that at each testing session in the laboratory, I will turn in my 4-day dietary records. I understand that if clinically significant side effects are reported, I will be referred to discuss the problem with Melyn Galbreath, RN who is the research nurse for the ESNL at Baylor University. Upon her 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.

I agree to do my best to: 1) follow the instructions outline by the investigators; 2) show up to all scheduled testing times; and 3) take supplements as instructed. I agree not to take any other nutritional supplements or performance enhancing aids during this study (i.e. vitamins/minerals, creatine, HMB, androstenedione, DHEA, etc). 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 I will 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: 1.) I have any known metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism; 2.) I have a history of pulmonary disease, hypertension, liver or kidney disease, musculoskeletal disorders, neuromuscular or neurological diseases, autoimmune disease, cancer, peptic ulcers, or anemia; 3.) I am taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (ie, thyroid, insulin, etc), psychotropic, neuromuscular/neurological, or androgenic medications; 4.) I have any bleeding disorders; 5.) I have any chronic infections (e.g., HIV).

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) or Matthew Cooke, Ph.D. (254-710-4025). I understand that if I experience any unexpected problems or adverse events from participating in this study I may be referred to discuss the problem with Dr. Lori Greenwood. Upon her 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 even though clinical data are available outlining the safety effects of many creatine supplements, the one used in this study is still relatively new to the market. Therefore, the potential medical benefits of the involved creatine and whey protein formulation is not yet well delineated. However, these compounds are currently available in over the counter nutritional supplements sold in United States and Europe. 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. However, as with the vast majority of nutritional supplements, I understand that the FDA may not have evaluated the safety or marketing claims of creatine. In addition, there are minor risks of muscular pain and soreness associated with the resistance training protocol required in this study which are not uncommon to any exercise program especially for individuals who do not resistance train on a regular basis.

On four separate occasions during this study, I understand that I will have about 3-4 teaspoons (6-12 milliliters) of blood drawn from a vein in my forearm using a sterile needle and blood tubes by an experienced phlebotomist following an 8-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 faint 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/exercised leg. I understand that Darryn Willoughby, Ph.D., or Matthew Cooke, PhD., will perform all of the biopsies and that a local 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 I will have a small incision made in my skin and a biopsy needle introduced 1 cm into the incision. I also understand that the incision is so small that it will not require any stitches and will be simply closed with

a butterfly bandage and then covered with an adhesive bandage (band-aid). After the anesthetic wears off within 3-4 hours, I understand that the sensation at the biopsy site is comparable to that of a bruise and may persist for 24-36 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).

I understand that the main benefits that may be obtained from this study are the potential ergogenic benefits of creatine monohydrate plus whey protein supplementation, which may include increased muscular strength, power, and reduced recovery time from resistance training. 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. I understand that if I am an intercollegiate scholarship athlete I may not be eligible to receive payment to participate 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 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 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, that 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 and muscle 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, that 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 University, the investigator's institutions, and 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 researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

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 Matthew Cooke, Ph.D. (principal investigator, Department of Health, Human Performance & Recreation, 118 Marrs McLean Gymnasium, Baylor University, phone: 254-710-4025) or Darryn Willoughby, Ph.D. (co-investigator, Department of Health, Human Performance & Recreation, 120 Marrs McLean Gymnasium, Baylor University, phone: 254-710-3504) or one of the 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 _____ Subject'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 C

Baylor University: Exercise & Sport Nutrition Laboratory

"**The Effects of Combining a Whey-Protein-Carbohydrate or a Casein-Protein Supplement and Resistance Training on Body Composition, Muscle Strength, and Markers of Satellite Cell Activation in Older Males**"

Demographics ESNL Staff Initials: _____
Name:_____ Testing Session: _____ Group: _____
Date: _____D.O.B.: _____ Age: _____

Resting Measures ESNL Staff Initials:_____

Medical Clearance/Informed Consent
Informed Consent: _____ Food Log: _____
Reported Side-Effect Questionnaire: _____ Medical Clearance:_____

Physiological Parameters:

Height: _____in. Baseline Resting H.R.: _____bpm.
Weight: _____lb. Baseline Resting B.P.:_____/_____mmHg
Time: _____am Last Meal: _____am/pm
Max Test: _____ #1, #2, #3, #4 Hrs Fasted: _____hr

Blood Collection/Hemodynamic Measures:
T-Top x 2_____

Body Composition:
DEXA:_____
BIA: _____

Strength Measures:

Bench: _____ _____ _____ _____ _____ _____ _____ _____

Leg Press: _____ _____ _____ _____ _____ _____ _____ _____

Muscle Biopsy:_____

APPENDIX D

BAYLOR UNIVERSITY
ESNL

Medical History Inventory

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

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 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. Subject is ***cleared*** to participate in the study.

____ Exclusion criteria is/are present. Subject is ***not cleared*** to participate in the study.

Signed: _____ Date: _____

APPENDIX E

BAYLOR
UNIVERSITY

Dear Provider:

One of your patients would like to participate in a study entitled "***The Effects of Combining a Whey-Protein-Carbohydrate or Casein-Carbohydrate Supplement and Resistance Training on Body Composition, Muscle Strength, and Markers of Satellite Cell Activation in Older Males***" that is being conducted by the Exercise & Sport Nutrition Lab at Baylor University. In order to do so, he must meet the entrance criteria described below and have approval from his personal physician to participate in the study. The study will involve men between the ages of 55-75 who will be randomized to one of four supplementation groups. Over the duration of the study (12 weeks), the following tests will be administered on several occasions: fasting blood, muscle biopsies, body composition and bone density (DEXA), and maximal strength assessments. Also, the subject will be participating in a 12-week, 3 day/week resistance training regimen. The resistance training sessions will be supervised. Details about the study are described in the subject consent form that can be obtained from the subject or upon request to the study contact person given below. If you feel he meets the entrance criteria described below or that any existing medical condition that he may have is under control and would not be a limitation for him to participate in the study, please sign the medical clearance below.

> ***Exclusion Criteria*** *Approximately 60 apparently healthy untrained males will participate in the study. Subjects will not be allowed to participate in this study if have any metabolic disorder including heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism. Other exclusionary criteria include a history of pulmonary disease, hypertension, liver or kidney disease, musculoskeletal disorders, neuromuscular or neurological diseases, autoimmune disease, cancer, peptic ulcers, anemia. Subjects will also be excluded if they are taking any heart, pulmonary, thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, endocrinologic (i.e., thyroid, insulin, etc…), psychotropic, neuromuscular/neurological, or androgenic medications. Also, any bleeding disorders or chronic infections disqualify the subject from participating in the study.* ***The only exception will be if the prospective subject has a medical condition or history that the subject's personal physician feels is controlled and therefore would not be a limitation for them to participate in the study.*** *Subjects meeting eligibility criteria will be informed of the requirements of the study and sign informed consent statements in compliance with the Human Subjects Guidelines of Baylor University and the American College of Sports Medicine. Subjects will be required to obtain clearance to participate in the study from their personal physician before participating in baseline assessments.*

If you have any questions regarding this study, please contact Annie Allison at 254-710-4037 or annie_allison@baylor.edu

Medical Clearance

I medically clear _____to participate as a subject in this study.

Name _____ Date _____

Signature _____

Exercise & Sport Nutrition Lab
Center for Exercise, Nutrition & Preventive Health Research
Department Of Health, Human Performance & Recreation
One Bear Place 97313 · Baylor University · Waco, TX 76798-7313 · (254) 710-7277 · Fax (254) 710-3527 · www3.baylor.edu/HHPR/ESNL

APPENDIX F

Resistance Training Log

Name_____

Week 1 2 3 4 5 6 7 8 9 10 11 12

Workout	1	2	3
Bench Press	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____
Leg Press	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____
Bicep Curls	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____
Triceps Press	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____
Leg Curls	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____
Leg Extension	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____
Lat Pulls	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____	1x10 @ _____ 1x10@ _____ 1x10@ _____

REFERENCES

Akima, H., Takahashi, H., Kuno, S., Masuda, K., Masuda, T., Shimojo, H., Anno, I., Itai, Y. and Katsuta, S. (1999). Early phase adaptations of muscle use and strength to isokinetic training. *Medicine & Science in Sports & Exercise*, 31(4), 588-594.

Allen, R.E., Temm-Grove, C.J., Sheehan, S.M. and Rice, G. (1997). Skeletal muscle satellite cell cultures. *Methods Cell Biol.,* 52, 155–176.

Balagopal, P., Rooyackers, O., Adey, D., Ades, P. and Nair, K. (1997). Effects of aging on in vivo synthesis of skeletal muscle myosin heavy-chain and sarcoplasmic protein in humans. *Am J Physiol Endocrinol Metab, 273*, E790–E800.

Bemben, M.G., Witten, M.S., Carter, J.M., Eliot, K.A., Knehans, A.W. and Bemben, D.A. (2010). The effects of supplementation with creatine and protein on muscle strength following a traditional resistance training program in middle-aged and older men. *The Journal of Nutrition*, 14(2), 155-159.

Biolo, G., Tipton, K. D., Klein, S., & R, W. R. (1997). An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *APJ-Endiocrinology & Metabolism , 273*, E122-E129.

Bodine, S.C., Latres, E., Baumhueter, S., Lai, V.K., Nunez, L., Clarke, B.A., Poueymirou, W.T., Panaro, F.J., Na, E., Dharmarajan, K., Pan, Z,Q., Valenzuela, D.M., DeChiara, T.M., Stitt, T.N., Yancopoulos, G.D. and Glass, D.J. (2001). Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science,* 29, 1704–1708

Boirie, Y., Gachon, P., & Beaufrere, B. (1997). Splanchnic and whole-body leucine kinetics in young and elderly men. *Am. J. Clin. , 65*, 489-495.

Borer, K. (2003). Exercise Endocrinology. Champaing, IL, Human Kinetics.

Bounous, G., Gervais, F., Amer, V., & Batist, G. (1989). The influence of dietary whey protein on tissue glutathione and the diseases of aging. *Gold PClin Invest Med , 12* (6), 343-349.

Braun, T. and Arnold, H. (1991). The four human muscle regulatory helix-loop-helix proteins Myf3-Myf6 exhibit similar hetero-dimerization and DNA binding properties. *Nucleic Acids Res , 19*, 5645-5651.

Cai, D., Lee, K., Li, M., Tang, M.K. and Chan, K.M. (2004). Ubiquitin expression is upregulated in human and rat skeletal muscles during aging. *Arch. Biochem. Biophys*., 425, 42-50.

Campbell, W. and Leidy, H.J. (2007). Dietary protein and resistance training effects on muscle and body composition in older persons. J Am Coll Nutr, 26(6), 696S-703S.

Chrusch, M., Chilibeck, P., and Al., E. (2001). Creatine Supplementation Combined with Resistance Training in Older Men. *Medicine & Science in Sports & Exercise* , 33(12), 2111-2117.

Ciechanover A. (1994). The ubiquitin-proteasome proteolytic pathway. *Cell,* 79(1), 13–21.

Clavel, S., Coldefy, A., Kurkdjian, E., Salles, J., Margaritis, I., & Derijard, B. (2006). Atrophy-related ubiquitin ligases, atrogin-1 and MuRF1 are up-regulated in aged rat tibialis anterior muscle. *Mechanisms of Ageing and Development*, 127(10), 794-801.

Cribb, P. (2006). Sarcopenia and whey proteins. Arlington, VA, USA: US Dairy Export Council.

Cuthbertson D, S. K., Atherton, P., Wackerhage, H., Taylor, P. and Rennie, M. (2005). Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle . *FASEB J* , 422–424.

Dangin, M., Boirie, Y., Garcia-Rodenas, C., Gachon, P., Fauquant, J. and Callier, P. (2001). The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am J Physiol Endocrinol* , E340-348.

Dangin, M., Guillet, C., Garcia-Rodenas, C., Gachon, P., Bouteloup-Demange, C. and Reiffers-Magnani, R. (2003). The rate of protein digestion affects protein gain differently during aging in humans. *The Journal of Physiology , 549*, 635-644.

Delbono, O., O'Rourke, K.S. and Ettinger, W.H. (1995). Excitation-calcium release uncoupling in aged single human skeletal muscle fibers. *Journal of Membrane Biology*, 148, 211-222.

Demling, R.H., DeSanti, L. (2000). Effect of a hypocaloric diet, increased protein intake and resistance training on lean mass gains and fat mass loss in overweight police officers. *Annals of Nutrition & Metabolism*, 44: 21-29.

Deschenes, M. R. (2004). Effects of aging on muscle fibre type and size. *Sports Medicine , 34*, 809-824.

Dhawan, J., & Rando, T. (2005). Stem cells in postnatal myogenesis: molecular mechanisms of satellite cell quiescence, activation and replenishment. *Trends Cell Biol* , 666–673.

Drummond, M., Dreyer, H. C., Pennings, B., Fry, C. S., Dhanani, S., Dillon, E. L.-M., et al. (2008). Skeletal muscle protein anabolic response to resistance exercise and essential amino acids is delayed with aging. *Journal of Applied Physiology , 104*, 1452-1461.

Esmarck, B., Andersen, J., Olsen, S., Richter, E., Mizuno, M., & Kjaer, M. (2001). Timing of postexercise protein intake is important for muscle hypertrophy with resistance training in elderly humans. *J Physiol , 535*, 301-311.

Evans, W. J. (2004). Protein Nutrition, Exercise and Aging. *Journal of the American College of Nutrition , 23*, 601S-609S.

Farnfield, M., Carey, K., & Cameron-Smith, D. (2005). Whey protein supplementation and resistance training to enhance muscle growth in young and older adults. *Asia Pac J Clin Nutr , 14*, S69.

Fiatarone, M. A., Marks, E. C., Ryan, N., Meredith, C. N., Lipsitz, L. A., & Evans, W. J. (1990). High-Intensity Strength Training in Nonagenarians. *Journal of the American Medical Association , 263* (22), 3029-3034.

Frontera, W., Meredith, C., O'Reilly, K., & Evans, W. (1990). Strength training and determinants of VO2 max in older men. *J Appl Physio , 68*, 329 –333.

Furuno, K., Goodman, M.N., Goldberg, A.L. (1990). Role of different proteolytic systems in the degradation of muscle proteins during denervation atrophy. *J Biol Chem*, 265(15):8550–8557.

Grounds, M.D. 2002. Reasons for the degeneration of ageing skeletal muscle: a central role for IGF-1 signalling. *Biogerontology*, 3: 19-24.

Ha, E., & Zemel, M. B. (2003). Functional properties of whey, whey components, and essential amino acids: mechanisms underlying health benefits for active people. *Journal of Nutritional Biochemistry , 14*, 251-258.

Hakkinen, K. and A. Pakarinen (1993). Acute Hormonal Responses to Two Different Fatiguing Heavy Resistance Protocols in Male Athletes. *Journal of Applied Physiology 74(2): 882-887.*

Hasten, D., Pak-Loduca, J., Obert, K., & Yarasheski, K. (2000). Resistance exercise acutely increases MHC and mixed muscle protein synthesis rates in 78-84 and 23-32 yr olds. *Am J Physiol Endocrinol Metab , 278*, E620-E626.

Hawke, T.J., Garry, D.J. (2001). Myogenic satellite cells: physiology to molecular biology. *Journal of Applied Physiology*, 91: 534-551.

Holloszy, J. O. (2000). The biology of aging. *Mayo Clinic Proceedings , 75*, S8-9.

Holterman, C., & Rudnick, M. (2005). Molecular regulation of satellite cell function. *Seminar of Cell Developmental Biology , 16*, 575-584.

Izquierdo, M., K. Hakkinen, et al. (2001). Effects of Strength Training on Muscle Power and Serum Hormones in Middle-Aged and Older Men. *J Appl Physiol 90(4): 1497-1507.*

Jagoe, R.T., Goldberg, A.L. (2001). What do we really know about the ubiquitin-proteasome pathway in muscle atrophy? *Curr. Opin. Clin. Nutr. Metab. Care,* 4: 183–190

Janssen, I., Shepard, D., Katzmarzyk, P., & Roubenoff, R. (2004). The healthcare costs of sarcopenia in the United States. *Journal of the American Geriatric Society , 52*, 80-85.

Jefferson, L.S., Kimball, S.R. (2001). Amino acid regulation of gene expression. *J Nutr,* 131:2460S–6S.

Jejurikar, S.S., Kuzon Jr., W.M.. (2003). Satellite cell depletion in degenerative skeletal muscle. *Apoptosis*, 8(6): 573-578.

Katsanos, C., Kobayashi, H., Sheffield-Moore, M., Aarsland, A., & Wolfe, R. (2006). A high proportion of leucine is required for optimal stimulation of the rate of muscle protein synthesis by essential amino acids in the elderly. *Am J Physiol Endocrinol Metab , 291*, E381-E387.

Katsanos, C., Kobayashi, H., Sheffield-Moore, M., Aarsland, A., & Wolfe, R. (2005). Aging is associated with diminished accretion of muscle proteins following ingestion of a small bolus of amino acids. *Am. J. Clin. Nutr , 82*, (in press).

Katsanos, C., Chinkes, D., Paddon-Jones, D., Zhang, X-j., Aarsland, A., Wolfe, R. (2008). Whey protein ingestion in elderly persons results in greater muscle protein accrual than ingestion of its constituent essential amino acid content. *Nutrition Research*, 28:651-658.

Kim, J., Kosek, D., Petrella, J., Cross, J., & Bamman, M. (2005). Resting and load-induced levels of myogenic gene transcripts differ between older adults with demonstrable sarcopenia and young men and women. *Journal of Applied Physiology , 99*, 2149-2158.

Kleijn, M., Scheper, G.C., Voorma, H.O., Thomas, A.A. (1998) Regulation of translation initiation factors by signal transduction. *Eur J Biochem,* 253:531–544.

Koopman, R., & van Loon, L. (2009). Aging, exercise and muscle protein metabolism. *J Appl Physiol , 106*, 2040-2048.

Koopman, R., Verdijk, L., Beelen, M., Gorselink, M., Kruseman, A., Wagenmakers, A., et al. (2008). Co-ingestion of leucine with protein does not further augment post-exercise muscle protein synthesis rates in elderly men. *British Journal of Nutrition , 99*, 571-580.

Koopman, R., Walrand, S., Beelen, M., Gijsen, A., Kies, A. (2009). Dietary protein digestion and absorption rates and the subsequent postprandial muscle protein synthetic response do not differ between young and elderly men. *The Journal of Nutrition,* 139 (9): 1707-1713.

Kosek, D. J., Kim, J.-s., Petrella, J. K., Cross, J. M., & Bamman, M. M. (2005). Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. *Journal of Applied Physiology , 101*, 531-544.

Kosek, D., Kim, J., Petrella, J., Cross, J., & Bamman, M. (2006). Efficacy of 3 days/wk resistance training on myofiber hypertrophy and myogenic mechanisms in young vs. older adults. *J Appl Physiol , 101*, 531–544.

Kraemer, W. and N. Ratamess (2005). Hormonal Responses and Adaptations to Resistance Exercise and Training. *Sports Medicine 35(4): 339-361.*

Kumar, V., Selby, A., Rankin, D., Patel, R., Atherton, P., Hildebrandt, W., et al. (2009). Age-related differences in the dose–response relationship of muscle protein synthesis to resistance exercise in young and old men. *Journal of Physiology , 587*, 211-217.

Lecker, S.H. , Jagoe, R.T., Gilbert, A., Gomes, M., Baracos, V., Bailey, J., Price, S.R., Mitch W.E., Goldberg, A. L. (2004). Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. *FASEB J.,* 18**:** 39–51

Leveille, S. G., Guralnik, J. M., Ferrucci, L., & Langlois, J. A. (1999). Aging Successfully until Death in Old Age: Opportunities for Increasing Active Life Expectancy . *American Journal of Epidemiology , 149* (7), 654-664.

Lexell, J., Downham, D., & Sjostrom, M. (1986). Distribution of different fibre types in human skeletal muscle: fibre type arrangement in m. vastus lateralis from three groups of healthy men between 15 and 83 years. *Journal of Neurological Science , 72*, 211-222.

Lexell, J., Downham, D.Y., Larsson, Y., Bruhn, E., Morsing, B. (1995). Heavy-resistance training in older Scandinavian men and women: short- and long-term effects on arm and leg muscles. *Scandinavian Journal Of Medicine & Science In Sports*, 5 (6), 329-41.

Lissner, L., Heitmann, B.L. (1995). Dietary fat and obesity: evidence from epidemiology. *Eur J Clin Nutr,* 49 (2): 79-90.

Manton, K., & X, G. (2001). Changes in the prevalence of chronic disability in the United States black and non-black population above age 65 from 1982 to 1999. *Proc Natl Acad Sci USA* , 6354-6359.

Mitchell, S., M. Harman, et al. (2001). Longitudinal Effects of Aging on Serum Total and Free Testosterone Levels in Healthy Men. *The Journal of Clinical Endrocrinology & Metabolism 86(2): 724-731.*

Morais, J., Chevalier, S., & Gougeon, R. (2006). Protein turnover and requirements in the healthy and frail elderly. *J Nutr Health Aging , 10*, 272–83.

Mozdziak, PE, Greaser, M., & Schultz, E. (1998). Myogenin, MyoD, and myosin expression after pharmacologically and surgically induced hypertrophy. *Journal of Applied Physiology , 84*, 1359-1364.

Musaro, A., McCullagh, K., Paul, A., Houghton, L., Dobrowolny, G., Molinaro, M., Barton, E.R., Sweeney, H.L., Rosenthal, N. (2001). Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle, *Nat. Genet.* 27: 195–200.

Nakshabendi, I.M., McKee, R., Downie, S., Russell, R.I., Rennie, M.J. (1999). Rates of small intestinal mucosal protein synthesis in human jejunum an ileum. *Am J Physiol Endocrinol Metab, 277: E1028-E1031.*

Nicklas, B., A. Ryan, et al. (1995). Testosterone, Growth Hormone and IGF-1 Responses to Acute and Chronic Resistive Exercise in Men Aged 55-70. *Int J Sports Med 16(7): 445-450.*

Paddon-Jones, D., Sheffield-Moorea, M., Katsanosa, C. S., Zhanga, X.-J., & Wolfea, R. R. (2006). Differential stimulation of muscle protein synthesis in elderly humans following isocaloric ingestion of amino acids or whey protein. *Experimental Gerentology , 41* (2), 215-219.

Paddon-Jones, et al. (2004). Essential amino acid and carbohydrate supplementation ameliorates muscle protein loss in humans during 28 days bedrest. *J. Clin. Endocrinol. Metab. , 89*, 4351-4358.

Pearson, R.B., Thomas, G. (1995). Regulation of p70S6K/p85S6K and its role in the cell cycle. *Prog Cell Cycle Res*, 1:21–32.

Petrella, J., Kim, J., Cross, J., Kosek, D., & Bamman, M. (2006). Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. *Am J Physiol Endocrinol Metab , 291*, E937–E946.

Petrella, J., Kim, J., Mayhew, D., Cross, J., & Bamman, M. (2008). Petrella JK, Kim JS, Mayhew DL, Cross JM, Bamman MM. Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. *J Appl Physio , 104*, 1736–1742.

Phillips, S., Hartman, J., Wilkinson, S. (2005). Dietary protein to support anabolism with resistance exercise in young men. *Journal of the American College of Nutrtion*, 24 (2): 134S-139S.

Price, S.R. (2003). Increased transcription of ubiquitin-proteasome system components: molecular responses associated with muscle atrophy. *Int. J. Biochem. Cell. Biol*., 35: 617–628.

Proud, C.G., Denton, R.M.(1997). Molecular mechanisms for the control of translation by insulin. *Biochem J*, 328:329–341.

Renganathan, M., Messi, M.L., Delbono, O. (1998). Overexpression of IGF-1exclusively in skeletal muscle prevents age-related decline in the number of dihypropyridine receptors. *The Journal of Biochemistry and Molecular Biology*, 273(44): 28845-28851.

Rieu, I., Balage, M., Sornet, C., Giraudet, C., Pujos, E., Grizard, J., et al. (2006). Leucine supplementation improves muscle protein synthesis in elderly men independently of hyperaminoacidaemia. *Journal of Physiology , 575* (1), 305-315.

Sabourin, L., & Rudnicki, M. (2000). The molecular regulation of myogenesis. *Clinical Genetics , 57*, 16-25.

Sandri, M., Sandri, C., Gilbert, A., Skurk, C., Calabria, E., Picard, A., Walsh, K., Schiaffino, S., Lecker S.H., Goldberg, A., Foxo, A.L. (2004). Transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy, *Cell* 117: 399–412.

Sayer, A. A., Syddall, H. E., Martin, H. J., Dennison, E. M., Anderson, F. H., & Cooper, C. (2006). Falls, sarcopenia, and growth in early life: findings from the Hertfordshire Cohort Study. *American Journal of Epidemiology , 164* (7), 665-671.

Shel, M., & Yates, F. (2001). Kinetics of human aging: I. Rates of senescence between ages 30 and 70 years in healthy people. *J Gerontol Biol Sci , 56*, B198-B208.

Short, K., Vittone, J., Bigelow, M., Proctor, D., & Nair, K. (2004). Age and aerobic exercise training effects on whole body and muscle protein metabolism. *AJP-Endocrinology & Metabolism , 286*, E92-E101.

Sinha-Hikim, I., Cornford, M., Gaytan, H., Lee, M., & Bhasin, S. (2006). Effects of testosterone supplementation on skeletal muscle fiber hypertrophy and satellite cells in community-dwelling older men. *J Clin Endocrinol Metab , 91*, 3024–3033.

Snow, M.H. (1977). The effects of aging on satellite cells in skeletal muscles of mice and rats. *Cell Tissue Res*, 185: 399–408.

Stitt, T.N., Drujan,D., Clarke, B.A., Panaro, F., Timofeyva, Y., Kline, W.O., Gonzalez, M.,Yancopoulos, G.D., Glass, D.J. (2004). The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors, *Mol. Cell* 14: 395–403.

Symons, T., Sheffield-Moore, M., Wolfe, R., Paddon-Jones, D. (2009). A Moderate Serving of High-Quality Protein Maximally Stimulates Skeletal Muscle Proten Synthesis in Young and Elderly Subjects. *American Dietetic Association*, 109: 1582-1586.

Tatsumi R., Anderson J.E., Nevoret C.J., Halevy O., Allen R.E. (1998). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Developmental Biology*. 194 (1): 114-128.

Tang, J.E., Moore, D.R., Kujbida, G.W., Tarnopolsky, M.A., Phillips, S.M. (2009). Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. *J Appl Physiol 107: 987-992.*

Timmerman, K.L., Volpi, E. (2008). Amino acid metabolism and regulatory effects in aging. *Curr Opin Clin Nutri Metab Care*, 11 (1): 49-55.

Tipton, K., Elliot, T., Cree, M., Wolf, S., Sanford, A., & Wolfe, R. (2004). Ingestion of Casein and Whey Proteins Result in Muscle Anabolism after Resistance Exercise. *Medicine & Science in Sports & Exercise* , 36(12):2073-2081.

Trumbo, P., Schlicker, S., Yates, A., & Poos, M. (2002). Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein and amino acids. *J Am Diet Assoc , 202*, 1621–1630.

Vellas, B., Hunt, W., Romero, L., Koehler, K., Baumgartne, R., & Garry, P. (1997). Changes in nutritional status and patterns of morbidity among free-living elderly persons: a 10-year longitudinal study. *Nutrition , 13*, 515-519.

Verdijk LB, K. R. (2007). Verdijk LB, Koopman R, Schaart G, Meijer K, Savelberg HH, van Loon LJ. Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. *Am J Physiol Endocrinol Metab , 292*, E151–E157.

Verdijk, L. B., Gleeson, B. G., Jonkers, R. A., Meijer, K., Savelberg, H. H., Dendale, P., et al. (2009). Skeletal Muscle Hypertrophy Following Resistance Training Is Accompanied by a Fiber Type–Specific Increase in Satellite Cell Content in Elderly Men. *The Journals of Gerontology Series A , 64A* (3), 332-339.

Volpi, E., Mittendorfer, B., Rasmussen, B., & Wolfe, R. (2000). The response of muscle protein anabolism to combined hyperaminoacidemia and gluscose-induced hyperinsulinemia. *J Clin Endocrinol Metab , 85*, 4481-4490.

Volpi, E., Mittendorfer, B., Wolfe, S. E., & Wolfe, R. R. (1999). Oral amino acids stimulate muscle protein anabolism in the elderly despite higher first-pass splanchnic extraction. *APJ-Endocrinology & Metabolism , 277*, E513-520.

Wang, X., Campbell, L.E., Miller, C.M., Proud, C.G. (1998). Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem J* 334:261–267.

Wing, S.S., Goldberg, A.L. (1993) Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. *Am J Physiol*. 264:E668–E676.

Welle, S., & Thornton, C. (1998). High-protein meals do not enhance myofibrillar synthesis after resistance exercise in 62-75 yr-old men and women. *Am J Physiol , 274*, E677-E683.

Welle, S., C, T., & Statt, M. (1995). Myofibrillar protein synthesis in young and old human subjects after three months of resistance training. *APJ-Endocrinology and Metabolism , 268*, E422-E427.

Welle, S., Brooks, A.I., Delehanty, J.M., Needler, N.,Thornton, C.A. (2003). Gene expression profile of aging in human muscle. *Physiol. Genomics* 14: 149-159

Yarasheski, K. E., Pak-Loduca, J., Hasten, D. L., Obert, K. A., Brown, M. B., & Sinacore, D. R. (1999). Resistance exercise training increases mixed muscle protein synthesis rate in frail women and men >76 yr old. *APJ-Endocrinology and Metabolism , 277* (1), E118-E125.

Yarasheski, K. E., Zachwieja, J. E., & Bier, D. M. (1993). Acute effects of resistance exercise on muscle protein synthesis rate in young and elderly men and women. *APJ-Endocrinology and Metabolism , 265* (2), E210-E214.

Yarasheski, K., Zachwieja, J., & Bier, D. (1993). Acute effects of resistance exercise on muscle protein synthesis rate in young and elderly men and women. *Am J Physiol Endocrinol Metab , 265* , E210-E214.