ABSTRACT

The Effects of Creatine Ethyl Ester Supplementation Combined with Resistance Training on Body Composition, Muscle Mass and Performance, and Intramuscular Creatine Uptake in Males

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Creatine monohydrate has become one of the most popular ingested nutritional supplements due to its potential enhancement of athletic performance. Creatine absorption from the serum into skeletal muscle occurs through the utilization of a membrane-spanning protein, CreaT1. Numerous creatine formulations have been developed primarily to maximize creatine absorption. Creatine ethyl ester (CEE) has been chemically modified by adding an ester group and is thought to increase creatine bio-availability by by-passing the CreaT1. This study examined how a seven week supplementation regimen with CEE affected body composition, muscle mass and performance, whole body creatine retention, as well physiological and molecular adaptations, associated with creatine uptake in nonresistance-trained males following a resistance-training program. Results demonstrated that CEE did not show any additional benefit to increases in muscle strength/performance or a significant increase in total muscle creatine when compared to creatine monohydrate or placebo. CEE supplementation did show a large increase in creatinine levels throughout the study.

The Effects of Creatine Ethyl Ester Supplementation Combined with Resistance Training on Body Composition, Muscle Mass and Performance, and Intramuscular Creatine Uptake in Males

by

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A Thesis

Approved by the Department of Health, Human Performance, and Recreation

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Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Master of Science in Education

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August 2008

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LIST OF ABBREVIATIONS

Cr – creatine
Crn – creatinine
Crea T1 – creatine transporter
CEE – creatine ethyl ester
ATP – adenosine triphosphate
ADP – adenosine diphosphate
PCr – phosphocreatine
Pi – inorganic phosphate
mRNA – message ribonucleic acid
PLA – placebo
1-RM – 1-repetition maximum

ACKNOWLEDGMENTS

I would like to thank Dr. Willoughby and Dr. Greenwood for giving me the opportunity to work on a research study of this magnitude. I am very appreciative of Dr. Willoughby's trust in my abilities perform all the biochemistry assays and willingness to allow me to learn muscle biopsy procedures. Special thanks to Dr. Talbert for working with my schedule during my defense. I would like to thank my parents John and Marie for all the support over the past couple of years. Thanks to my good friend Ryan Schoch who was instrumental in the completion of the study. I would also like to thank Dr. Matt Cook and Maureen Head who were vital in helping me complete the study. Thanks to my friends Brandon Maroney, Mike Joublanc, and Joe Zawack who gave me encouragement and support throughout the process, and Ginger who provided me with daily happiness.

CHAPTER ONE

Introduction

Creatine is found in small quantities within the brain, liver, kidneys, and testes, while approximately 95% of creatine stores are found in skeletal muscle (Greenhaff, 1997). Creatine or methyl guanidine acetic acid is supplied by exogenous sources such as fish and red meat and is endogenously synthesized from the amino acids arginine, glycine, and methionine (Bemben, 2005). Energy is provided to the body from the hydrolysis of ATP into adenosine diphosphate (ADP) and inorganic phosphate (Pi). The phosphagen system provides a rapid resynthesis of ATP from ADP with the use of phosphocreatine (PCr) through the reversible reaction of creatine kinase (Bemben & Lamont, 2005; Demant & Rhodes, 1999; Persky & Brazeau, 2001). Of the 95% of creatine stored within skeletal muscle, approximately 40% is free creatine and approximately 60% is PCr (Demant & Rhodes, 1999). The average 70 kg person has a total creatine pool of 120-140 g. Specifically, the range of creatine in skeletal muscle is 110-160 mmol/kg dry mass (Bemben & Lamont, 2005; Greenhaff, 1997; Mesa 2002). Creatine supplementation has the ability to increase skeletal muscle stores of creatine and PCr, which could therefore increase skeletal muscle's ability to increase ATP resynthesis from ADP. A study conducted by Yquel *et al.* (2002) employing 20 g of creatine for 6 days showed an increase in PCr concentrations after a maximal isometric contraction during 16 and 32 seconds of recovery. Resistance training along with creatine supplementation has typically been shown to be more beneficial at increasing body mass,

maximal strength, and weight lifting performance compared to placebo, but responses are variable (Rawson, 2003).

With the ergogenic benefits consistently being shown in both research settings and among the general population, creatine has become one of the most recognized ergogenic aids to date. Intramuscular stores of creatine are considered to be saturated at 160 mmol/kg dry mass; however, only 20% of users achieve this amount and another 20-30% do not respond to creatine supplementation at all (Greenhaff, 1997). Several hundred studies have examined creatine supplementation's effectiveness in improving muscle performance. Approximately 70% of these studies have shown statistically significant performance improvements, with the remaining studies generally producing non-significant trends (Kreider, 2003). Aside from differences such as experimental design, amount and duration of creatine dosage, training status of participants, etc., the variance in response to creatine supplementation may be due to regulatory mechanisms of a sodium-chloride dependent transport protein, the creatine transporter (CreaT1). CreaT1 is directly involved in the extracellular uptake of creatine to increase the pool of metabolically active creatine in muscle (Snow & Murphy, 2001). It appears that intramuscular creatine uptake is dependent on CreaT1 activity, which has resulted in numerous creatine formulations having been developed in an attempt to improve muscle creatine uptake and potentially increasing the efficacy of creatine supplementation (Loike *et al.*, 1988). Research directly examining how CreaT1 is expressed with creatine supplementation or resistance training in humans is virtually nonexistent, and it is imperative that future research examines CreaT1 activity in order to provide a better understanding of creatine metabolism (Schoch, Willoughby, & Greenwood, 2006).

Due to these variations in intramuscular creatine uptake in response to creatine supplementation, it has been suggested that creatine alone may have a limited ability to maximally activate the CreatT1. Numerous creatine formulations have been developed recently which combine creatine with carbohydrate, sodium, or esterified alcohol with the primary intent of improving cellular absorption and transport, which may maximize total intramuscular creatine concentration thereby improving muscular performance. These new products may prove beneficial for individuals portraying a down-regulation of CreaT1, whereby providing a mechanism to partially "bypass" normal creatine uptake. A comparison of creatine monohydrate, creatine with dextrose, and effervescent creatine showed added benefit when dextrose is combined with creatine, but no additional benefits of effervescent creatine compared to creatine monohydrate (Greenwood, 2003). Another study combined creatine with magnesium and showed no additional performance benefits compared to creatine monohydrate (Selsby, 2004). Additionally, creatine serum was ineffective at increasing creatine retention compared to creatine monohydrate (Kreider 2003).

The molecular structure of creatine consists of a negatively charged carboxyl group and a positively charged avelengt functional group (Persky, 2003). Creatine is a polar molecule and hydrophilic due to this composition, which limits creatine bioavailability. Esterification is a process widely used by pharmaceutical companies to increase bioavailability of certain prescription drugs with low bioavailability. In a continued attempt to more effectively increase intramuscular creatine levels, one of the latest variations of creatine is creatine ethyl ester (CEE), which has been chemically modified by adding an ester group to increase creatine bioavailability. Claims of

superiority over other forms of creatine have been made, but no scientific evidence has been published to substantiate these claims. Furthermore, the effectiveness of CEE has not yet been adequately researched and currently no peer-reviewed articles have been published on this formulation. As a result, concerns over CEE safety and effectiveness are still prevalent.

Problem Statement

How does a seven-week supplementation regimen with CEE affect skeletal muscle creatine concentration, muscle mass and performance, CreaT1 protein expression, as well as whole-blood and serum clinical safety markers in non-resistance-trained males while following a resistance-training program?

Purposes of Study

The primary purpose of the study is to determine the extent to which CEE affects muscle strength and mass, body composition, CreatT1 protein expression, and muscle creatine uptake, and whether any corresponding increases in intramuscular creatine levels are related to the CreaT1 expression in humans. The secondary purpose of the study is to determine the extent to which CEE affects whole-blood and serum clinical safety markers.

Null Hypotheses

H_1: Following CR and CEE supplementation, the serum levels of creatine and creatinine will not significantly increase after the loading and maintenance phase when compared to placebo.

H_2: Following CR and CEE supplementation, there will be no significant increase in relation to the expression of muscle protein after the loading and maintenance phase when compared to placebo.

H_3: Following CR and CEE supplementation, there will be no significant difference in relation to whole-blood and serum clinical chemistry markers after the loading and maintenance phase compared to placebo.

H_4: Following CEE supplementation, intramuscular total creatine levels will not significantly increase after the loading and maintenance phase when compared to placebo.

H_5: Following CEE supplementation, there will be no significant improvement in muscle strength after the loading and maintenance phase when compared to placebo.

H_6: Following CEE supplementation, there will be no significant improvement in body composition after the loading and maintenance phase when compared to placebo.

Directional Hypotheses

H_1: Following CR supplementation, intramuscular total creatine, levels will significantly increase after the loading and maintenance phase when compared to placebo.

H_2: Following CR supplementation, there will be a significant improvement in muscle strength after the loading and maintenance phase when compared to placebo.

H_3: Following CR supplementation, there will be a significant improvement in body composition after the loading and maintenance phase when compared to placebo.

Delimitations

- This study will incorporate thirty male participants between the ages of 18-35 who have not been involved in regular resistance-training (3x/week) over the past year, which includes at least one lower-body exercise session per week.

- Participants will be recruited from Baylor University and within surrounding Waco, Texas, if necessary via flyers and online advertisements.

- Participants will be excluded from the study if prior supplementation, with any potentially ergogenic dietary supplement with claims to enhance performance, has been ingested within the past 6 months.

Limitations

- Since this study is utilizing convenience sampling only within the city of Waco, as well as the small sample size (n=30), external validity to the greater population of nonresistance-trained males may be decreased; although, it is unlikely to be a significant concern.

- Participants are expected to adhere to the dosing protocol through the consumption of their randomly assigned supplement or placebo.

- Research participants will be responsible and expected to follow the prescribed resistance-training regimen throughout the course of the study.

Assumptions

- It is assumed that all participants will fully comply with the supplementation dosing and training protocol. However, to help control this issue, compliance forms for both supplement intake and training will be provided and collected at each testing session.

- The supplements are assumed to be pure in form and to contain the specified concentration of the creatine formulation as indicated by the manufacturer.

- It is assumed that all laboratory equipment is functioning properly in relation to validity and reliability measures. Proper calibration and the use of trained research staff will be utilized to reduce any potential errors.

- Subjects are assumed to have arrived to each testing state in a fasting state (>8 hours).

Definitions

- ATP – adenosine triphosphate

- BIA – bioelectrical impedance analysis

- Bioavailability – The fraction or percentage of an administered drug or other substance that becomes available in plasma or to the target tissue after administration.

- Creatine – An organic molecule derived from amino acids that plays an important role in energy utilization during muscular contraction. Also used as a potentially ergogenic nutritional supplement.
- Creatinine – End product of creatine metabolism, formed through nonenzymatic conversion of PCr and Cr.
- Creatine transporter – The molecular gateway protein that allows creatine to enter muscle tissue from the circulation.
- DEXA – dual energy x-ray absorptiometry
- Esterase – Enzyme who's activation cleavage a promoiety (ester) through hydrolysis leaving the active agent (creatine).
- Esterification – The process of chemically combining an alcohol and an acid resulting in the formation of an ester.
- mRNA – Messager ribonucleic acid. A coding molecule that provides genetic information for the cell to produce new proteins.
- MHC isoform – A large elongated protein consisting of a long tail extending to the carboxy terminal and a globular domain at the amino terminal
- RT-PCR – real-time polymerase chain reaction
- Myofibrillar Protein – Cylindrical structure within muscle that contains contractile proteins.
- Wingate – Cycle ergometer test measure anaerobic peak powers, mean power, total work, and fatigue index.

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CHAPTER TWO

Literature Review

Creatine Metabolism

Creatine is nitrogenous amino acid that has a net positive charge and molecular weight of 131 daltons (Mesa, 2002). Creatine, ά – methyl gunoidinoacetic acid, is provided through both endogenous and exogenous means. Endogenous synthesis of creatine takes place within the pancreas, kidney, and mainly the liver (Greenhaff, 1997; Wyss & Kaddurah-Daouk, 2000). Endogenous creatine synthesis involves three amino acids glycine, arginine, and methionine (Bloch, 1941). Within the kidney, L – arginine glycine amidinotranferase (AGAT) transfers an amide group of arginine to glycine, yielding L-ornithine and guanidinoacetate (Walker, 1979; Wyss & Kaddurah-Daouk, 2000). A second irreversible transfer of methyl group from s-adenosylmethinoine (SAM) to guanidinoacetate by guanidinoacetate methyltransferase (GAMT) within the hepatocyte forms creatine (Persky & Brazeau, 2001; Walker 1979; Wyss & Kaddurah-Daouk, 2000). Endogenous and exogenous sources contribute ~2 g/day to maintain the body's total creatine pool (Greenhaff, 1997; Persky, 2003). Creatine from endogenous and exogenous sources is transferred via the blood to skeletal muscle with normal plasma concentrations ranging from 50 to 100 μmol/L (Harris, 1992). A 70 kg person has a total body creatine pool of ~120g with 95% located within the skeletal muscle and the remainder in the brain, liver, kidneys, and testes (Greenhaff, 1997; Persky & Brazeau, 2001; Walker, 1979). The creatine concentration within skeletal muscle is ~ 40% free Cr and ~ 60% PCr (Bemben, 2005). Creatine is cleared by uptake in tissue such as skeletal

muscle or by renal elimination (Persky, 20003). Intramuscular total creatine levels, hormone levels, muscle mass, and kidney function are factors determining the rate of elimination (Persky & Brazeau, 2001). Creatine is nonenzymatically degraded into creatinine in an irreversible reaction at a rate constant (kCrn) of 0.017 day^{-1} (Fitch, 1968; Greenhaff, 1997; Persky, 2003). Skeletal muscle being the primary storage area for creatine is also the primary area for creatinine production (Persky, 2003). Approximately 1.1 %/day and 2.6 %/day of creatine and PCr, respectively, is nonenzymatically converted into creatinine, which totals ~1.7 %/day or ~2 g for a 70 kg individual (Wyss & Kaddurah-Daouk, 2000). Creatine synthesis (~1 g/day) and ingestion (~1 g/day) replaces the ~2 g/day loss of creatine due to degradation, which maintains the total creatine pool (Persky, 2003).

Creatine Function within Muscle Cells

In skeletal muscle ATP is hydrolyzed into ADP + Pi, thereby releasing energy for muscle contraction. ATP concentrations are exhausted within 1 -2 seconds during muscle contraction due to limited ~24 mmol/kg dry mass concentrations within skeletal muscle (Casey, 2000). Phosphocreatine is utilized through the reversible reaction of creatine kinase (CK) to maintain ATP levels within the muscle. PCr acts as an energy buffer through the rapid regeneration of ATP from ADP. Creatine produced during ATP hydrolysis diffuses from the utilization site to the mitochondrial membrane. Phosphocreatine is produced through the reaction of creatine, mitochondrial creatine kinase (M-CK) and ATP. PCr diffuses back through the cytosol to the myosin head for ATP resynthesis (Mesa, 2002; Demant, 1999). During exercise and ATP hydrolysis, H^+ and lactate are formed, which decreases pH within the muscle. During ATP resynthesis,

PCr utilizes H^+ during hydrolysis providing buffer for H^+, reducing acidosis and helping maintain a normal pH (Demant, 1999). Glucose and Glycogen metabolism are partially regulated by PCr. During exercise PCr levels are depleted, decreasing PFK inhibition (Bemben, 2005). PFK is the rate-limiting step for glycolysis, and with PFK inhibited glycolytic activity is decreased. During supplementation, total creatine levels are typical increased ~20 mmol/kg dry mass, with 20% being in the form of PCr (Greenhaff, 1997). PCr hydrolysis contributes to 50% of ATP production during a 6-second sprint at 250% VO2max, and 25% of ATP production during a 30-second sprint at 200% VO2max. If during creatine supplementation a 20% increase in PCr is seen, energy supply would be increased 5-10% for a 30 second sprint and 2.5 – 5% during a 6 second sprint (Mesa, 2002). This increase in energy availability is of obvious benefit during short duration high intensity exercises, which has been the primary rational for supplementation.

Body Composition and Muscle Hypertrophy

Various studies show that creatine supplementation (5-7 days) typically increases total body mass by 1 to 2 kg (Greenhaff, 1994; Persky & Brazeau, 2001; van Loon, 2003; Wyss & Kaddurah-Daouk, 2000). This increase in body mass may be a result of an increase in protein synthesis, a reduction in protein breakdown, or both. The increase in body mass has also been attributed to increases in muscle fiber size (Volek, 2004). After 12 weeks of creatine supplementation and resistance training, Volek *et al.* (1999) showed a 6.3% increase in fat free mass compared to a 3.1% increase in the placebo group. Volek also found an increase in Type I (35%), Type IIA (36%), and Type IIAB (35%) muscle fiber cross sectional area as compared to placebo group increase of 11%, 15%, and 6% respectively. Willoughby and Rosene (2001) showed that myosin heavy chain

isoform mRNA and protein expression were increased after 12 weeks of creatine supplementation combined with resistance training, and concluded that the increase in lean body mass may be due in part to increases in myosin heavy chain synthesis. Parise *et al.* (2001) showed acute creatine supplementation in men increases total body mass and fat free mass. However, the results were attributed to anti-catabolic actions within the muscle rather than an increase in whole body or mixed muscle protein synthesis. During supplementation, total body mass increases are caused by intracellular hydration due to the osmolarity of the creatine, which pulls water into the cell (Mesa, 2002). The increase in intracellular hydration may act as an anabolic signal, thus increasing protein synthesis within the cell (Mesa, 2002; Persky & Brazeau, 2001). Short term creatine supplementation (5-7 days), typically has lead to increases of up to 1.6 kg in total body mass (Kreider, 1998). In such a short duration, it is unlikely that changes in muscle protein synthesis or breakdown play a major factor with increases in total body mass. The increases in muscle hypertrophy are due to increases in intracellular water retention, which lead to the increase in cross sectional area seen within skeletal muscle (Bemben & Lament, 2005). During 3 days of supplementation 90% of the increase in body mass was shown to be due to increases in total body water without a significant effect on extra cellular volume (Ziegenfuss, 1998). Roughly two-thirds of the total body water is intracellular. Powers *et al.* (2003) examined changes in total body water and fluid shift after loading and maintenance phases during 28 days of supplementation, and after loading, there was a significant (4.6%) increase in body mass. When comparing increases in total body water to increases in total body mass there is no significant changes (Volek, 2004). This suggests that the initial increase in body mass is due to increases in intra

cellular water retention that correlates with the increase in body mass. It is unknown which of these factors play the primary role in increasing body mass, and what the exact mechanism that allows for this change; however, it is known that supplementation increases total body mass during both short and long term creatine usage.

Muscular Strength/Power

Creatine supplementation has shown a positive effect on muscle strength and power through the increased ATP availability and muscle hypertrophy (Mesa, 2002). The osmolarity of creatine increases intracellular water retention that allows for increased force production through increased leverage (Bemben & Lamont, 2005). Muscular strength is typically measured through a maximal effort test, such as bench press and leg press. A study by Volek *et al.* (1999) showed that 12 weeks of creatine ingestion led to increases in squat (4%) and bench press (5%) after one week. The placebo group showing a decline in the squat (15%) increased in the bench press (3%). At the completion of week 12, an increase in squat (32%) and bench press (24%) was seen compared to only squat (24%) and bench press (16%) in the placebo group (Volek, 1999). In another study Volek *et al.* (2004) measured strength during an over-reaching protocol of 5 days per week for 4 weeks. Squat and bench press measures showed a decline with the placebo group, while the creatine group did not (Volek, 2004). Not all studies have been positive. Stevenson and Dudley (2001) showed that a 20 g loading dose of creatine for 7 days had no effect on unilateral strength during multiple knee extensions exercise compared to placebo. Creatine supplementation has shown a positive ergogenic effect on muscle power output. Creatine use has shown to effect peak and mean power (W), time to peak power (seconds), total work (J), and fatigue index (%) when measured with a Wingate

ergometer (Bemben & Lamont, 2005). Casey *et al.* (2000) showed during short term (5-6 days) of creatine loading with 20 grams, there was an increase in maximal and total work performed with a mean increase of ~4%. This correlates with the increase in creatine muscle uptake (Greenhaff, 2000). In low dose of creatine (6g) and short duration (6 days) anaerobic working capacity was shown to increase by 9.4% when creatine was ingested with 35 g of carbohydrate (Stout, 1999). In another study, Hoffman *et al.* (2005) showed low dose and short duration had no effect on peak power, mean power, or total work, but the rate of fatigue was significantly reduced. Numerous studies have shown positive ergogenic effects, while others have shown no effect. The variance in results may be due to the form of creatine ingested, the duration of ingestion, and the total amount ingested.

Pharmacokinetics

Physiochemical properties of creatine along with splanchnic blood flow determine oral bioavailability (Mesa, 2002). Creatine contains a negatively charged carboxyl group and a positively charged guanidine group, which makes creatine a hydrophilic and polar molecule (Persky, 2003). The hydrophilic property limits passive diffusion across intestinal membranes. The exact process by which creatine is absorbed from the gastrointestinal tract has not been identified. Dash *et al.* (2001) used Caco-2 monolayers in an *in vitro* model to determine creatine transport across intestinal membranes. Creatine transport was limited in the movement across apical to basolateral membrane, which supports the theory of an active process for intestinal creatine uptake. An mRNA for a creatine transporter has been previously identified in gastrointestinal tract (Persky, 2003). Creatine transporter in the apical membrane layer in rat jejunal enterocyte has been identified, with features similar to other previously identified transporters throughout the

body (Tosco, 2004). The solubility of the creatine ingested also determines bioavailability. Peak plasma concentration were higher when creatine was ingested as a solution, versus a suspension, lozenge, or as meat (Harris, 2002). The increase in plasma creatine is also affected by dosage. With a 10 g dose, a 50-fold increase in resting plasma creatine is seen, compared to only a 2-fold increase with a 5 g dose (Schedel, 1999). Creatine absorbed within the gut or synthesized within the body travels to the target tissue via the blood. Once within the skeletal muscle, creatine is significantly limited in its diffusion capacity out of the cell. Therefore, a 70 kg person with 120 g within the creatine pool will lose 2g/day of creatine due to turnover into creatinine (Demant, 1999). The half-life of creatine degradation into creatinine with a pH of 1.4 is 55 days, a pH of 3.7 is 7.5 days, and a pH of 6.8 is 40.5 days, with the maximal rate occurring at a pH of between 3 and 4 (Persky, 2003). The amount of creatine ingested will determine serum creatine and creatinine concentrations. After an acute 20 g dose of creatine, plasma levels peaked 2 ½ hours after ingestion, which is a 50-fold increase along with a 13 % increase in creatinine after 3 hours (Schedel, 1999). Schedel *et al.* (1999) measured various acute dosages (2.5 g, 5 g, 10 g, 15g) with serum concentration and peak time values increasing linearly with the amount of creatine ingested. Maximum creatine absorption is typically occurs within 2 hours with up to a 10 g dose, and more than 3 hours with a does greater than 10 g (Persky, 2003). Schedel *et al.* (1999) suggested that creatine conversion to creatinine occurring within the stomach and gut for ~2 hours post ingestion is not a major factor affecting uptake. Creatine degradation is limited, which equates to 0.1 g of a 5 g dose that will be degraded each hour (Persky, 2003; Schedel, 1999). Creatine conversion to creatinine within the gut is minimal and not a major factor limiting creatine uptake;

however, once Cr is taken up by skeletal muscle, this tissue becomes the primary site of creatinine production (Fitch, 1966; Greenhaff, 1997; Persky, 2003).

Creatine Transporter

Increases in total creatine concentrations of 15-20% have been typically seen during supplementation (Mesa, 2002; Persky, 2003; Greenhaff, 1997). Creatine is transported against a concentration gradient into skeletal muscles. Blood plasma creatine levels range from 50-100 μmol/L; with an acute 5g or 20g dose plasma creatine level is increases 600-800 μmol/L and 2170 (+/-) 660 μmol/L respectively (Mesa, 2002). Variations have been seen with different subjects, due to skeletal muscle's creatine uptake ability. A specific creatine transport protein (CreaT1) regulates this uptake. CreaT1 is sodium-dependent with structural similarities to dopamine, y-amino butyric acid (GABA) and taurine (Snow, 2001). A second transporter CreaT2 has also been identified, but the activity has been primarily within the testes (Persky, 2003). Approximately 90% of creatine uptake is determined by CreatT1, though the exact means by which up- or down-regulation of the creatine transporter is not known (Snow, 2001). Exercise, hormones, and total creatine content within the muscle have shown to affect uptake. Catecholamines, thyroid hormone, insulin-like growth factor 1 (IGF-1), and insulin have shown a positive effect on creatine uptake (Persky, 2001). B_2-adrenergic receptors stimulated by catecholamines such as noradrenalin, isoproterenol, and clenbuterol activate cyclic AMP-dependent pathway, thereby increasing creatine uptake (Odoom, 1996). Creatine ingestion combined with carbohydrates has also been shown to increase uptake. Insulin actions are not direct but appear to stimulate CreaT activity (Steenge, 2000). Exercise also increases Cr uptake by possibly activating the CreaT

(Persky, 2001). Depletion of intracellular creatine levels in high oxidative muscle fibers has been shown to correlate with increases in Cr uptake (Brault, 2003). Skeletal muscle creatine content appears to be the largest factor regulating CreaT activity. During acute ingestion of creatine, an increase in extra cellular creatine allows for increased uptake, but once skeletal muscle is saturated an inhibition of uptake is seen (Snow, 2001). Long term Cr ingestion (6-10 weeks) has been shown to down-regulate the CreaT in rat skeletal muscles (Guerrero, 1998).

Creatine Esterification

The molecular structure of creatine consists of a negatively charge carboxyl group and a positively charged avelengt functional group (Persky, 2003). Creatine is a polar molecule and hydrophilic due to this makeup, which limits creatine bioavailability. Esterification is a process widely used by pharmaceutical companies to increase bioavailability of certain prescription drugs with low bioavailability. The most common process to increase bioavailability is to increase lipophilicity by masking carboxylic acid, phosphates, and other charged groups through the addition of an ester group (Taylor, 1996). The esterification process is used as a temporary means to increase bioavailability; once the substance is absorbed the esterified product is readily reversed back into the original state (Beaumont, 2003). Oral ingestion of a nutrient is the preferred delivery method for nutrients, but many hurdles need to be overcome before a said nutrient is delivered into the targeted area. Once ingested, a nutrients travels from the gastrointestinal track into the blood by various means depending on the nutrient. Passive transcellular, passive paracellular, carrier – mediated, and receptor-mediated endocytosis are the various means by which a nutrient can travel through the intestinal mucosal

membrane (Taylor, 1996). The pharmaceutical industry uses various classes of esters (alkyl, aryl, double cyclic, and amino acid) to achieve this delivery. Alkyl esters are the most common formulation, with an alkyl alcohol bound to the acid group of a substance (Beaumont, 2003). One of the most successful uses of esterification process in the pharmaceutical industry is with ACE inhibitors (Taylor, 1996). In 1922, Dox and Yoder conducted an experiment with creatine and various alcohols (methyl, ethyl, n-butyl) and dry hydrogen chloride, and discovered that the creatine chemical ring structure did not close but formed an esterification of the carboxyl group. In 2003, ProNutrient Technologies, Inc. filed with the FDA for NDI status for creatine ethyl ester. What makes the form of creatine unique is the chemical alteration of creatine's structure. While the FDA did grant GRAS status to this formulation, questions still remain. ProNutrient Technologies, Inc. provided three studies to prove the safety of CEE. An *in vitro* study conducted with mouse skeletal muscle, an *in vivo* study with adult male Sprague-Dawley rats, and an *in vivo* study with five human adult males. These studies were not published in peer review journals and toxicology reports were the only data provided to the Department of Health and Human Services. The *in vitro* study on mouse skeletal muscle cell showed no significant effect of the creatine ethyl ester on the cells viability, and the cell viability was shown to be similar to creatine monohydrate. The *in vivo* study in rats showed no significant changes in weight for the seven days of the study. Also, no significant differences in blood chemistries were seen and the researches concluded there is no evidence of toxicity with up to a 30 g equivalent dose in humans. The human *in vivo* study showed for the five subjects no abnormal blood or urine chemistries markers for the 238 to 414 days of ingestion. Claims of superiority over other forms of creatine have

been made, but no scientific evidence has been published to substantiate these claims. Highest bioavailability of pharmaceutical drugs using the esterification method is increased by 40-60% (Beaumont, 2003). Once esterified, a nutrient has to disassociate from the original nutrient to provide the active component. Enzymes within the systemic circulation or the target tissue release the active component (Majumdar, 2004). At what time and location within the body this active component is released will determine effectiveness of the esterification product. Beaumont *et al.* (2003) has provided two categories for which a nutrient may be cleared without full benefit of the esterification process. The first category describes the process in which the esterase enzyme does not liberate the active ingredient into systemic circulation. This could be due to enzyme activity in gut lumen, hydrolysis in intestinal cell with return to gut lumen, hydrolysis within the liver and excretion in bile, and additional metabolism of the active component after disassociation. The second category describes the process in which the active ingredient would be metabolized by non-esterase enzymes. Lipophilicity, solubility, molecular size, and other physiochemical properties determine absorption of a nutrient. If blood levels of a nutrient are increased it does not guarantee an increase delivery to the target tissue.

If these mechanisms allow for esterified creatine to disassociate in the blood, then creatine uptake into the muscle cell would still be dependent on the creatine transporter. If esterfied creatine does not disassociate with creatine in the blood but then penetrate the lipid by layer of the target tissue, by passing the creatine transporter, then there would be no down regulation of the transporter, which is one of the limiting factors in creatine uptake.

CHAPTER THREE

Methods

Participants

Thirty apparently healthy, recreationally active males between the ages of 18 and 30 were recruited on a voluntary basis to participate in this double-blind research study. The participants were not resistance trained [not following a consistent resistance training program (i.e. thrice weekly) for at least one year prior to the study]. Subjects were not able to participate if they had any contraindications, defined and outlined by the American College of Sports Medicine (ACSM) and if they had ingested any supplements claimed to have ergogenic properties within three months prior to the study. The potential participants were eligible only after an informed consent form was read and signed, in addition to completion of a successful medical screening examination.

Study Site

Within the Department of Health, Human Performance, and Recreation at Baylor University, all familiarization and testing sessions were performed within the Exercise and Sport Nutrition Laboratory (ESNL). Biochemical and sample analyses were executed in the Exercise and Biochemical Nutrition Laboratory (EBNL) at Baylor University.

Study Design

The study included baseline testing followed by double-blind supplementation with various creatine formulations for 47 days. Table 1 provides an outline of the study

design. The supplementation included both a loading phase of 0.3 g/kg of fat-free mass/day for five days, and a maintenance phase of 0.075 g/kg of fat-free mass/day for 42 days thereafter. Participants concomitantly followed a prescribed resistance-training program four times weekly and were required to report to the ESNL four times over the course of the study for testing sessions.

Table 1

Study Design

	Loading Phase	Maintenance Phase	
T1 Day 0	T2 Day 6	T3 Day 27	T4 Day 48
Food Log	Food Log	Food Log	Food Log
BIA	BIA	BIA	BIA
DEXA	DEXA	DEXA	DEXA
Blood Draw	Blood Draw	Blood Draw	Blood Draw
Muscle Biopsy	Muscle Biopsy	Muscle Biopsy	Muscle Biopsy
Leg Press 1-RM	Leg Press 1-RM	Leg Press 1-RM	Leg Press 1-RM
Bench Press 1-RM	Bench Press 1-RM	Bench Press 1-RM	Bench Press 1-RM
Wingate	Wingate	Wingate	Wingate

Independent and Dependent Variables

The independent variables were the creatine supplements and the placebo. Dependent variables included body composition, muscle strength and power, serum

creatine and creatinine, intramuscular, total creatine, CreaT1 protein expression, and whole-blood and serum clinical safety markers.

Entry and Familiarization Session

Participants interested in the study were initially interviewed in person or over the phone to determine if they appeared to qualify for participation in the study. If eligibility was believed to be met, an invitation to attend an entry/familiarization session was made. Once reporting to the ESNL, a medical history questionnaire and a general physical examination were completed to determine eligibility. When eligible, a familiarization session consisting of verbal and written explanation of the study protocol was given. Participants also performed a 1-RM leg and bench press test along with a Wingate anaerobic test. A time was set for initial testing and participants were instructed to refrain from exercise for 24 hours, fast for 8 hours, and record their dietary intake for 4 days prior to each of the four testing sessions

Strength Assessment

The leg press and bench press maximal strength test (Nebula, Versailles, OH) were performed by the participants to measure any changes in muscular strength during the course of the study. Four one repetition maximum (1-RM) strength tests were performed during the study. Initially, an estimated 50% (1-RM) measured from the previous testing 1-RM test, was utilized to complete 5 to 10 repetitions. After a two minute rest period, a load of 70% of estimated (1-RM) was utilized to perform 3 to 5 repetitions. Weight was gradually increased until a 1-RM was reached with each following lift, with a two-minute rest period in between each successful lift. Test-retest

reliability of performing these strength assessments on subjects within our laboratory has demonstrated low mean coefficients of variation and high reliability for the bench press (1.9%, intraclass $r = 0.94$) and leg press (0.7%, intraclass $r = 0.91$), respectively.

Anaerobic Wingate Test

Anaerobic capacity and power were determined during each of the four testing sessions. The determinations were made by performing a 30 second Wingate test on a computerized Lode cycle ergometer (Groningen, Netherlands). A warm up of 30 rpm for 120 seconds was followed by maximal sprint for 30 seconds against a workload of 0.075 kg/kg of body weight. Correlation coefficients of test-retest reliability of performing these assessments of absolute peak power and mean power on subjects within our laboratory has been found to be $r = 0.692$ and $r = 0.950$, respectively.

Anthropometric and Body Composition Testing Procedures

Total body mass (kg) were determined using a calibrated electronic scale with a precision of ± 0.02 kg (Detecto, Webb City, MO). Total body water (total, intracellular, and extracellular) was determined through use of bioelectrical impedance analysis (BIA) (Xitron 4200, San Diego, CA). The subjects laid in a supine position on a table and were swabbed with an alcohol pad on their right hand and foot. Four electrodes were placed on the body to allow a low energy high frequency of 500 micro amps @ 50 kHz to flow through the body; measuring resistance to the current within the body. The positive electrodes were placed on the hand. One electrode was placed on the posterior surface of the right wrist, between the radial and ulna styloid processes. The other was placed on the posterior surface of the right hand and the distal base of the second metacarpal. The

negative electrodes were placed on the foot. One was placed on the anterior surface of the right foot with the other placed at the distal end of the first metatarsal. Once connected, the subject's age, gender, weight, and height were entered into the unit and the analysis was started.

Percent body fat, fat mass, fat-free mass, and right thigh mass was determined using dual-energy x-ray absorptiometer [(DEXA) Hologic Discovery, Bedford, MA]. The subjects were asked to lay in a supine position in only shorts/t-shirt or a gown that was provided. The subjects were asked to lie motionless for approximately six minutes while the scan was being performed. The subjects were exposed to a low dosage of radiation at each scan. Approximately 1.5 mR of radiation was emitted during each scan. For the four testing sessions of the study, each subject was exposed to approximately 6 mR of radiation. The maximal amount of x-ray radiation exposure per year for non-occupation exposure is 500 mR, the radiation exposure was not significantly more than the background radiation in the local Waco area. Once the scans were completed they were analyzed following completion of the testing sessions. The DEXA scans were segmented into regions (right & left arm, right & left leg, and trunk). Each of these segments was analyzed for fat mass, lean mass, and bone mineral content. A sub region was utilized to determine right thigh mass. The isolated region extended medially to the pubic symphysis down to the head of the femur. During each of the four testing sessions total body mass, BIA, and DEXA was assessed. Supplement dosages were also modified based on fat-free mass determined by DEXA.

Dietary Analysis

Dietary intake was recorded for four days (three weekdays and one weekend) prior to each of the four testing sessions. The participant's diets were not standardized and the subjects were asked not to change their dietary habits during the course of the study. Diet logs were evaluated with the Food Processor dietary assessment software (EHSA Research, Salem, OR) to determine the average daily Kcal and macronutrient consumptions of fat, carbohydrate, and protein ingested.

Supplementation Protocol

Subjects were randomly assigned to ingest in a double blind manner capsules and powder which contained either dextrose placebo (Gaspari Nutrition, Lakewood, NJ) (AST Sport Science, Colorado Springs, CO), creatine (Integrity Nutraceuticals, Sarasota, FL), or creatine ethyl ester [(CEE) Labrada Nutrition, Houston, TX]. The creatine monohydrate capsules were 250 mg per capsule, with the creatine ethyl ester (CEE) containing 700 mg per capsule. After baseline testing procedures and lean body mass determination via DEXA, creatine or the placebo at a relative daily dose of 0.30 g/kg fat free body mass (approximately 20 g/day) for five days in the loading phase and, immediately following the loading phase, a relative daily dose of 0.075 g/kg fat free mass (approximately 5 g/day) during the 42-day maintenance phase were ingested by the subjects.

In order to standardize supplement intake throughout the study, participants were instructed to ingest the supplements in two equal intervals throughout the day during the loading phase (AM and PM), and at one constant interval during the maintenance phase (approximately 12:00 PM). Compliance to the supplementation protocol was monitored

by supplement logs and verbal confirmation. After completing the compliance procedures the subjects were given the required supplement dosage for the following supplementation period.

Resistance Training Protocol

Subjects participated in a 4-day per week resistance-training program split into two upper and two lower extremity workouts per week for a total of seven weeks. The upper body resistance-training program consisted of nine exercises (bench press, lat pull, shoulder press, seated rows, shoulder shrugs, chest flies, biceps curl, triceps press down, and abdominal curls) twice per week and a seven exercise lower extremity program (leg press or squat, back extension, step ups, leg curls, leg extension, heel raises, and abdominal crunches) performed twice per week. Subjects performed 3 sets of 10 repetitions with as much weight as they could lift per set (typically 70 – 80% of 1-RM). Rest periods between exercises lasted no longer than three minutes and rest between sets lasted no longer than two minutes. Training was conducted at the Student life Center (SLC) at Baylor University or an area gym, documented in training logs, and signed off to verify compliance and to monitor progress.

Muscle Biopsies and Venous Blood Sampling

Percutaneous muscle biopsies (50-70 mg) were obtained using a Bergstrom (5mm) needle, from the middle portion of the vastus lateralis muscle of the dominant leg at the midpoint between the patella and the greater trochanter of the femur, at a depth between one and two cm. An initial numbing agent (1.5 ml 1% Lidocaine) was given before incision. For the remaining three biopsies, attempts were 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 was made approximately 0.5 cm to the former from medial to lateral. After removal, the muscle specimens were immediately frozen in liquid nitrogen and then stored at -80^{o}C for later analysis. Muscle biopsies were taken at each of the four testing sessions.

Venous blood samples were obtained from the antecubital vein into 2, 7.5 ml and 1, 2.0ml collection tubes using a standard Vacutainer^{TM} apparatus. Once collected (2 7.5ml tubes) the samples were centrifuged for 15 minutes. The serum was removed and frozen at -80^{o}C for later analysis. The whole blood was analyzed the same day as collected. An 8-hour fast prior to blood donation was required for the participants before each of the four testing sessions.

Creatine Analysis

From the four muscle biopsies obtained, tissue samples were analyzed spectrophotometrically for total creatine, by the diacetyl/α-napthtol reaction. The samples were allowed to incubate at room temperature allowing for color formation, which was detected by a spectrophotometer at 520 nm. Then the samples were run against a standard curve of known creatine concentrations. In addition, serum creatine levels were determined from the four blood samples collected. The muscle tissue samples were cut (10-15 mg), then placed into a microfuge tube and placed into a vacuum centrifuge for 18-24 hours (Savant ISS110 SpeedVac^{TM} Concentrator, Thermo Scientific, Milford, MA). Once dried, the muscle samples were placed in an ultra-low freezer at -80^{o}C. A porcelain plate and pestle were used to grind the dried muscle samples into a powder. Any connective tissues were discarded and the powder was placed into pre

weighed microfuge tubes. The powdered muscle tissue was extracted in a 0.5 M perchloric acid/1 mM EDTA solution, relative ratio of 800 µL per 10 mg of powdered muscle. The samples were left on ice for 15 min, while vortexing periodically. Samples were centrifuged at 15,000 rpm at 4^{o}C for five minutes. The supernatant was transferred into microfuge tubes and neutralized with 2.1 M $KHCO_3$/ 0.3 M MOPS solution with a ratio of 1:5 followed by additional centrifuged at 15,000 rpm for 5 minutes. The supernatant was stored in an ultra-low freezer at -80^{o}C for later analysis. Muscle total creatine concentrations were determined by utilizing 40 µL of supernatant from the previous reaction and combining it with 140µL of ddH_20 and 20 µL of 0.4 N HCL, then heated at 65^{o}C for 10 minutes to hydrolyze phosphate groups. 40 µL of 2.0 N NaOH was used to neutralize the reaction. The samples were subsequently subjected to the diacetyl/α-napthtol reaction as previously described.

Creatine Transporter

Total RNA Isolation Method

Total cellular RNA was extracted from the homogenate of biopsy samples with a monophasic solution of phenol and guanidine isothiocyanate contained within TRI-reagent (Sigma Chemical Co., St. Louis, MO). The total RNA isolation methods were designed for smaller muscle samples to yield approximately 0.1 – 9.15 µg/mg muscle tissue. 500 µL of TRI-Reagent was added to each tube, and then the muscle sample was homogenized using a pestle. 100 µL of chloroform were added to each tube and shaken, then allowed to sit for 15 minutes at room temperature. The samples were centrifuged at 12,000 rpm for 15 minutes. The samples were separated into three distinct phases, a

lower (pink) organic phase which contained the protein, a middle (gray) interphase containing the DNA, and an upper (clear) aqueous phase containing the RNA. Using a sterile transfer pipette, the clear aqueous phase was transferred into a new microfuge tube. The remaining interphase and organic phase were stored in an ultra-low freezer at -80^{o}C. At this point, 250µL of 100% isopropanol were added to each tube and allowed to sit at room temperature for 10 minutes. Samples were then centrifuged at 12,000 rpm for 10 minutes, allowing for the formation of a RNA pellet. The supernatant was discarded, then 500 µL of 75% ethanol was added then vortexed to wash the pellet. The samples were centrifuged at 7500 rpm for five minutes then the supernatant was discarded. The washing process was repeated twice. The pellet was allowed to air dry for 10 minutes, then 50 µL of nuclease free water was added. The samples were stored in an ultra-low freezer at -80^{o}C for analysis; the RNA was not utilized in the present study.

Total Muscle Protein Isolation

Total protein remaining from the total RNA isolation procedure was isolated with isopropanol, ethanol, and 0.1 M guanidine hydrochloride. The DNA from the interphase was isolated by 150 µL of 100% ethanol added to each tube, which was inverted several times and allowed to stand for two minutes. The samples were centrifuged at 2000 rpm for five minutes, the supernatant was removed and transferred to microfuge tube by a sterile pipette. The DNA was discarded. The remaining organic phase containing protein was isolated. Specifically, 750 µL isopropanol was added and allowed to sit at room temperature for 10 minutes, followed by a 10-minute centrifugation period at 12,000 rpm. The resulting supernatant was discarded, and the outstanding pellet washed in 1 mL 0.1 M guanidine / 95% ethanol, allowed to stand for 20 minutes, and spun for five minutes at

7,500 rpm (process repeated three times). The supernatant was again discarded, followed by the addition of 1 mL 100% ethanol, vortexed, and allowed to stand at room temperature for 20 minutes. The mixture was then centrifuged for 5-minutes at 7,500 rpm, followed by the removal of the supernatant, and the pellet was air-dried for 10 minutes. At this point, 1 mL 0.1% SDS was then added to dissolve the pellet, but to aid in the process, plastic pestle grinding was needed. After one last centrifugation period at 10,000 rpm, the supernatant was transferred to new a microfuge tube (Willoughby *et al*., 2007).

Creatine Transporter Protein Expression

The muscle protein expression of CreaT1 was assessed through the use of ELISAs. A polyclonal antibodies specific for CreaT1 was purchased from Alpha Diagnostics (San Antonio, TX). Initially, The CreatT1 antibody was diluted to 1 μg/ml in coating buffer (Na2CO3, NaHCO3, and ddH2O, pH 9.6) and added to the microtiter plates. The plates were then covered and allowed to incubate overnight at room temperature. The next day, the wells were washed four times with approximately 300 μL wash buffer (1X phosphate buffered saline, Tween-20). Next, 200 μl of a blocking buffer (10X phosphate buffered saline, bovine serum albumin, ddH2O) was added to every well. The blocking buffer was added to prevent the non-specific binding of non-relevant proteins to the bottom of each well. Again, the plate was covered, allowed to incubate for one hour at room temperature and then washed four times. After, the wash step, 50 μL of the samples were added to each well, allowed to incubate at room temperature for one hour, and followed by another wash cycle. Next, a secondary antibody (IgG conjugated to HRP) was diluted to 5 μg/ml in a dilution buffer (10X phosphate buffered saline, Tween-

20, bovine serum albumin, ddH2O), added to each well, and then was incubated for one hour followed by another wash cycle. After plate washing was complete, 100 μL stabilized TMB chromogen was added. The plates were then covered and placed in the dark for the last 30-minute incubation. The final step required the addition of 100 μL of a stop solution (0.2 M sulphuric acid) to every well. The subsequent absorbances, which are directly proportional to the concentration of CreaT1 in the samples, were measured at a wavelength of 405 nm. There were no standards used in these ELISAs, thus no standard curve was created. Therefore, the absorbances relative to muscle weight were assessed and compared as delta changes.

Reported Side Effects from Supplements

Subjects reported (by questionnaires) at T2, T3, and T4, whether or not they tolerated the supplement, as well as reporting any medical problems/symptoms they may have encountered throughout the protocol of the study. (see Appendix E for questionnaire).

Statistical Analysis

Data was grouped into body composition variables, muscle performance variables, blood variables, and muscle variables and then each group was analyzed using separate 3 (group) x time (4) multivariate analysis of variance (MANOVA) with repeated measures on the time factor with SPSS for Windows Version 11.5 software (SPSS inc., Chicago, IL). Eleven sets of MANOVAs were utilized and grouped as follows: food log, anthropometric, creatine, muscle strength, muscle power, serum renal & hepatic, serum lipid, serum LDH & CK, serum clinical safety, whole blood red, and whole blood white.

Significant differences among groups were identified by a Tukey HSD post hoc test. Probability levels of ≤ 0.05 were adopted throughout. Approximate Cohen's d effect size = 1.35. 10 subjects per group x 3 groups = power of approximately 0.84 (http://stat.ubc.ca/~rollin/stats/ssize/n2.html).

CHAPTER FOUR

Results

Subject Demographics

Forty-two participants who were initially recruited for the study completed consent forms and participated in an initial familiarization session. Of the forty-two subjects recruited, thirty completed the seven week research study. Table 2 shows the sample size, along with the baseline means (±SD) for height, weight, and age for each of the three groups.

Table 2

Subject Baseline Demographics

PLA Group	
N size	10
Height (cm)	175.39 (7.8)
Weight (kg)	77.91 (18.4)
Age (years)	20.1 (1.4)
CR Group	
N size	10
Height (cm)	173.6 (9.1)
Weight (kg)	89.4 (22.1)
Age (years)	20.3 (1.5)
CEE Group	
N size	10
Height (cm)	177.55 (6.7)
Weight (kg)	73.7 (14.9)
Age (years)	20.8 (2.2)

Note: Values are reported as means (±SD)

Dietary Analysis and Supplement Compliance

Participants recorded their dietary intakes four days prior to each of the testing sessions. The diet logs were used to analyze the average caloric and macronutrient consumption and any possible changes in the participant's dietary habits during the course of the study. Total kcal, protein, carbohydrates, and fats were grouped together for statistical analysis. No significant multivariate Group x Time interactions ($p = 0.935$, effects size = 0.303) or multivariate Time main effect ($p = 0.781$, effect size = 0.338) were shown. However, a significant multivariate Group main effect ($p = 0.001$, effect size = 0.415) was observed. The significant Group main effects were seen for total kcal ($p = 0.001$, effect size = 0.415) and fat ($p = 0.006$, effect size = 0.324). Post Hoc testing revealed the significance for total kcal resided between the CEE group and the PLA group ($p = 0.012$) and CR group ($p = 0.001$). Post Hoc testing also showed significant differences in dietary fat intake between the CEE group and the PLA group ($p = 0.033$) and CR group ($p = 0.007$). Table 3 provides the average values (±SD) for total kcal and macronutrients during each of the testing sessions. Of note, significant error could have occurred with the reporting of the dietary intake of the subjects. All subjects were instructed not to make changes in their dietary habits during the study. The dietary information collected was self reported by each of the participants. The participants were college age students who had no previous experience recording dietary intake, except for the initial instructions during the familiarization session. Supplement compliance and side effects were based on self reporting on a provided supplement log during testing session. Based on these logs, all subjects followed supplementation protocols with no adverse effects being reported.

Table 3

Dietary Caloric and Macronutrient Intake

Group	Total Calories (kcal/day)	Protein (g/day)	Carbohydrate (g/day)	Fat (g/day)
PLA				
T1	1797 (889)	98 (45)	218 (74)	78 (49)
T2	1953 (537)	99 (26)	247 (72)	83 (23)
T3	2009 (433)	107 (37)	256 (60)	86 (22)
T4	2059 (726)	103 (23)	248 (84)	95 (39)
CR				
T1	1879 (929)	114 (53)	294 (84)	82 (27)
T2	1783 (884)	123 (73)	279 (106)	85 (30)
T3	1580 (517)	108 (30)	266 (91)	75 (21)
T4	1470 (300)	95 (21)	255 (98)	70 (19)
CEE				
T1	2744 (418)	114 (35)	318 (70)	110 (27)
T2	2858 (810)	119 (39)	332 (103)	116 (41)
T3	2589 (514)	109 (33)	288 (67)	112 (24)
T4	2765 (823)	124 (51)	307 (117)	115 (32)

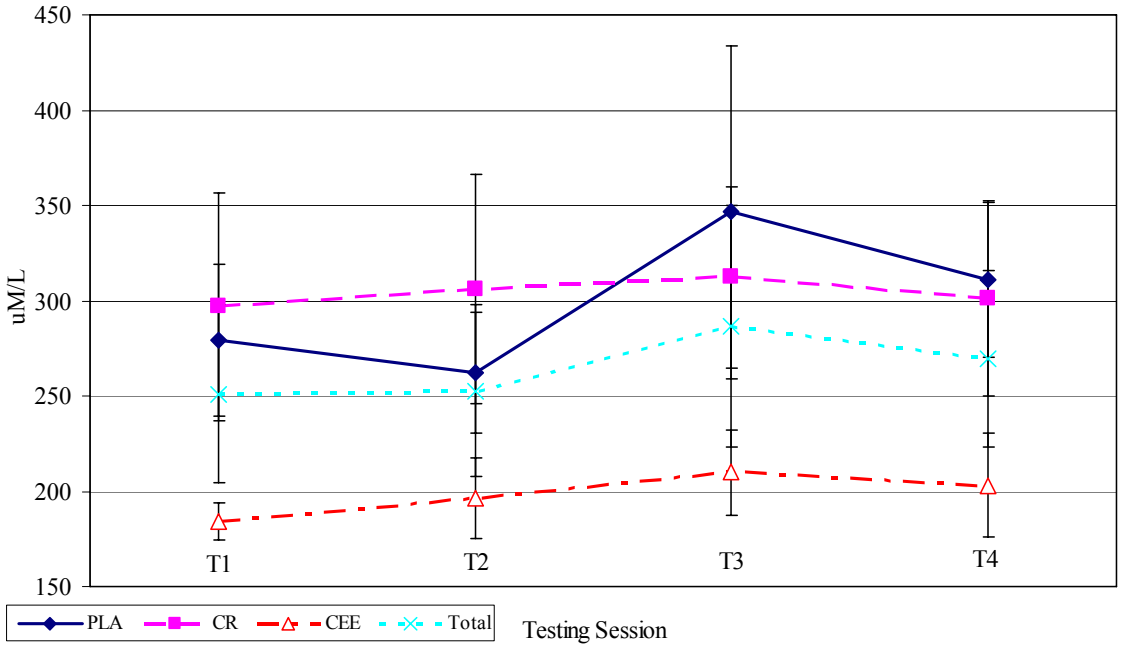
Note: Data are represented as means (±SD)

Creatine

The variables serum creatine, total muscle creatine, and creatine transporter protein content were grouped together for statistical analysis. No significant multivariate Group x Time interaction ($p = 0.986$, effects size = 0.163) or Time main effect ($p = 0.211$, effect size = 0.449) occurred. However, a significant multivariate Group main effect ($p = 0.001$, effect size = 0.368) was observed. Figures 1, 2, and 3 represent the mean values (±SD) and changes throughout the duration of the study for serum creatine, total muscle creatine, and creatine transporter protein content, respectively.

Serum Creatine

A significant Group main effect (p = 0.002, effect size = 0.391) was observed. Post hoc testing indicated significantly higher serum creatine concentrations in the PLA (p = 0.007) and CR (p = 0.005) groups compared to CEE. The mean correlation coefficient of variation between duplicates was 10.74%. The standard curve correlation coefficient between plates for serum creatine was 0.998.



	T1	T2	T3	T4
PLA†	279.47 (79.64)	262.10 (63.65)	346.39 (174.70)	311.31 (82.33)
CR^{+}	296.77 (118.81)	306.29 (120.13)	312.25 (95.56)	300.93 (102.00)
CEE	184.48 (19.39)	196.40 (42.10)	209.99 (45.00)	202.96 (54.44)
Total	251.11 (93.78)	252.84 (90.71)	286.70 (126.52)	269.28 (92.90)

Figure 1. Changes in serum creatine concentrations. Data expressed as mean (± SD).
Data table † and $^{+}$ indicates significant group main effect † (p = 0.007) and $^{+}$ (p = 0.005) compared to CEE.

Total Muscle Creatine

A significant Group main effect (p = 0.032, effect size = 0.240) for total muscle creatine content was observed (Figure 2). Post hoc testing revealed total muscle creatine content was significantly higher (p = 0.026) in the CR group compared to the PLA group.

The baseline total muscle creatine content for all groups was 106.3 mmol/kg (± 24.68). Intramuscular creatine levels can typically range from 110-160 mmol/kg with an average of approximately 120 mmol/kg for an average 70 kg male. The approximate average body weight for all of the participants was 80 kg; therefore, the baseline muscle creatine levels were within normal expected ranges. A separate ANOVA for muscle creatine criterion variable showed a strong trend towards a significant time effect ($p = 0.055$). Post hoc testing indicated that there was a weak trend ($p < 0.10$) for increased muscle creatine uptake after the loading phase at T2. The mean correlation coefficient of variation between duplicates was 1.53%. The standard curve correlation coefficient between plates for total muscle creatine was 0.998.

Creatine Transporter

A significant Group main effect ($p = 0.008$, effect size = 0.318) was observed for creatine transporter protein content (Figure 3). Post hoc testing revealed PLA creatine transporter protein content was significantly higher ($p = 0.008$), than the CEE group. A mild trend indicating the CR group to be significantly greater ($p = 0.073$), than the CEE group was also observed. There were no standard curves utilized for the creatine transporter assay. The mean correlation coefficient between duplicates was 10.80%.

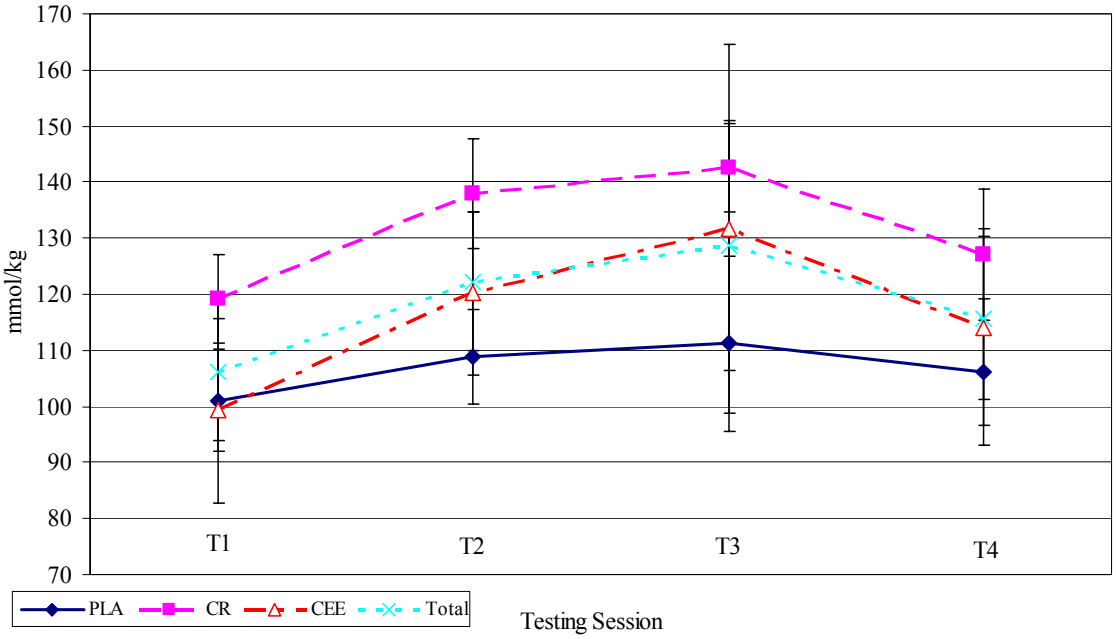
Creatine Hypothesis Conclusion

> H_o: Following CR and CEE supplementation, the serum levels of creatine will not significantly increase after the loading and maintenance phase when compared to placebo. Serum creatine did not significantly increases. The null hypothesis is accepted.

- H_o: Following Cr and CEE supplementation, there will be no significant increase in CreaT1 protein expression after the loading and maintenance phase when compared to placebo. Creat1 protein content did not significantly increase. The null hypothesis is accepted.

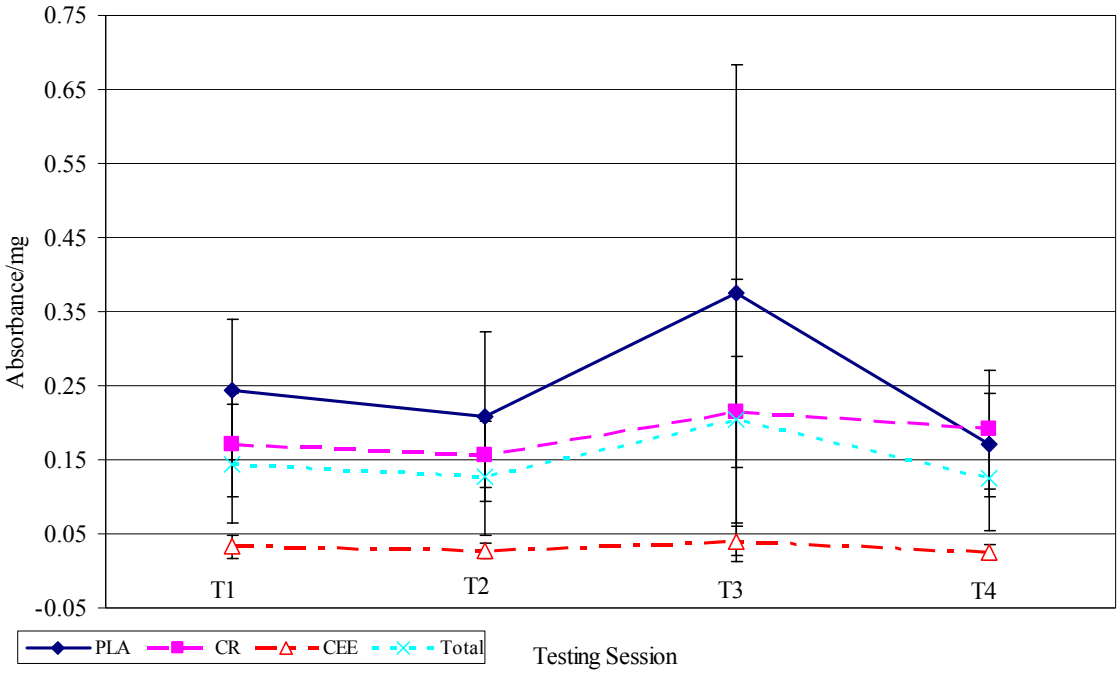
- H_o: Following CEE supplementation, intramuscular total creatine levels will not significantly increase after the loading and maintenance phase when compared to placebo. Total intramuscular creatine levels did not increase. The null hypothesis is accepted.

- H_1: Following CR supplementation, intramuscular total creatine, levels will significantly increase after the loading and maintenance phase when compared to placebo. While a trend was observed after the loading phase, no significant increase in intramuscular total creatine content for CR group was seen. The directional hypothesis is rejected.



	T1	T2	T3	T4
PLA	101.11 (18.14)	108.97 (16.80)	111.21 (31.21)	106.1 (26.09)
CR †	119.18 (15.65)	137.89 (19.56)	142.56 (15.80)	127.07 (23.27)
CEE	99.23 (32.69)	120.16 (28.92)	131.80 (65.73)	114.10 (35.01)
Total	106.25 (24.68)	122.26 (24.85)	128.64 (44.41)	115.70 (29.07)

Figure 2. Changes in total muscle creatine. Data are expressed as mean (± SD).
Data table † indicates significant group main effect ($p = 0.026$) compared to PLA.



	T1	T2	T3	T4
PLA †	0.2443 (.19)	0.2079 (.23)	0.3742 (.62)	0.1704 (.14)
CR +	0.1698 (.14)	0.1570 (.09)	0.2138 (.15)	0.1914 (.16)
CEE	0.0325 (.03)	0.0274 (.02)	0.0400 (.04)	0.0248 (.02)
Total	0.1447 (.16)	0.1271 (.16)	0.2033 (.38)	0.1251 (.14)

Figure 3. Changes in creatine transporter protein content. Data expressed as raw absorbance relative to muscle weight and presented as mean (± SD).
Data table † indicates significant group main effect (p = 0.008) for PLA compared to CEE. + indicates a mild trend for a group main effect (p = 0.073) for CR compared to CEE.

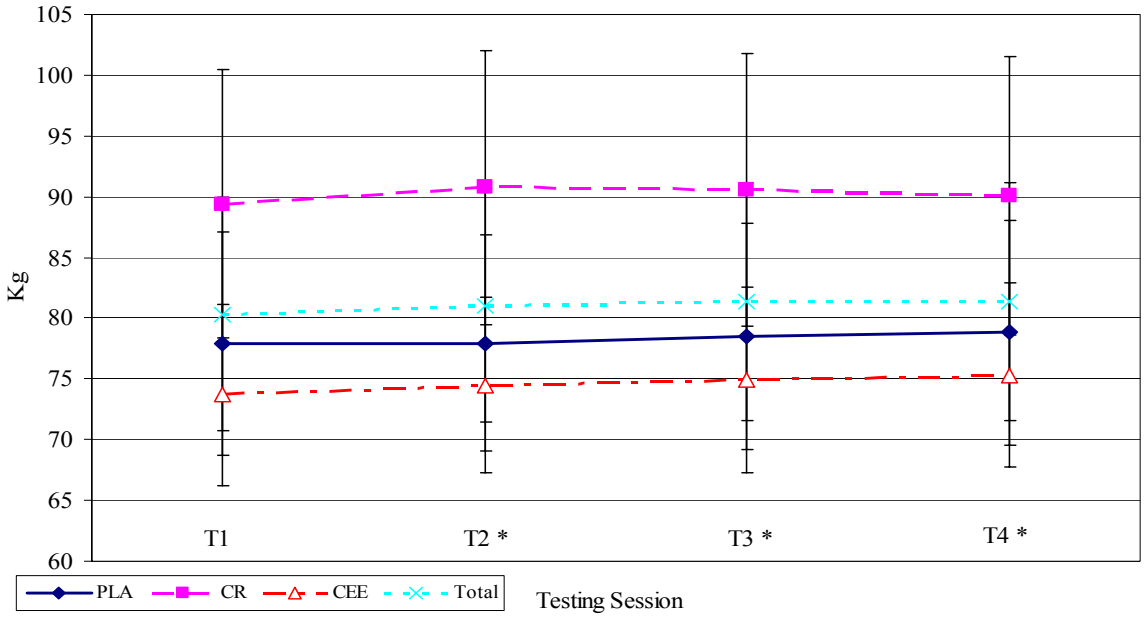
Anthropometric Variables

The variables bodyweight, fat-free mass, thigh mass, body fat, total, intracellular, and extracellular body water were grouped together for statistical analysis. There were significant multivariate main effects for Group (p = 0.015, effect size = 0.444) and Time (p = 0.004, effect size = 0.963). A multivariate trend for Group x Time interaction (p = 0.054, effect size = 0.869) was also observed. Figures 4, 5, 6, 7, 8, 9 and 10 represent the mean values (±SD) and changes throughout the duration of the study for bodyweight, fat-

free mass, thigh mass, body fat, total, intracellular, and extracellular body water, respectively.

Bodyweight

There was not a significant Group x Time interaction (p = 0.174, effect size = 0.107, or Group main effect (p = 0.173, effect size = 0.122). A significant Time main effect (p = 0.001, effect size = 0.206) was observed with bodyweight significantly increasing at T2 (p = 0.015), T3 (p = 0.006), and T4 (p = 0.027).



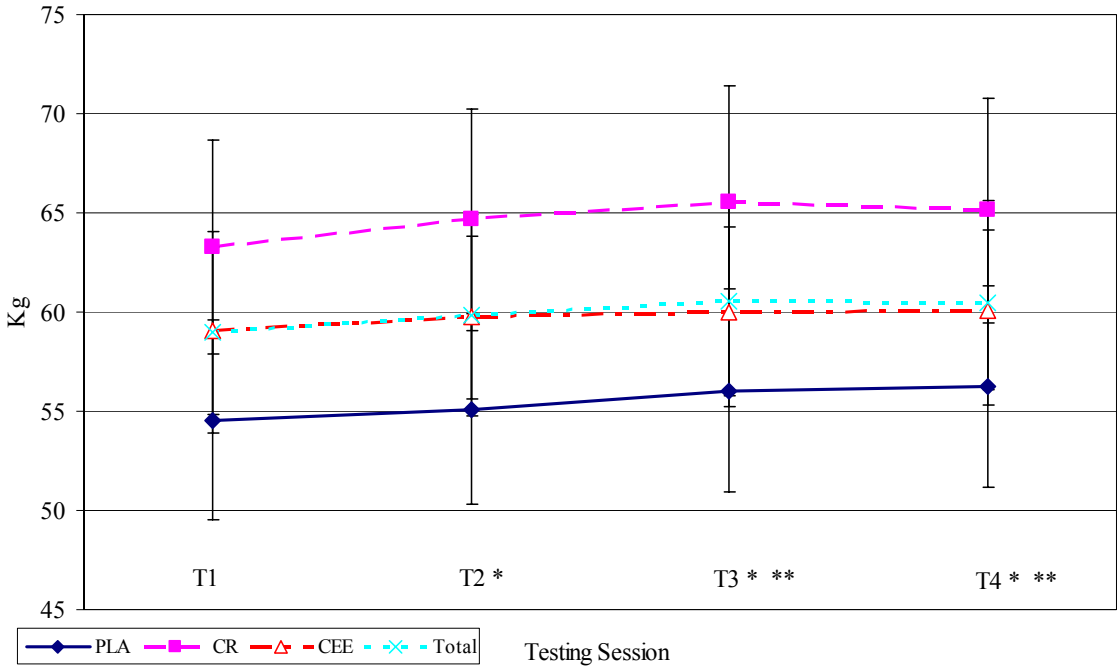
	T1	T2	T3	T4
PLA	77.91 (18.36)	77.94 (17.76)	78.52 (18.64)	78.80 (18.50)
CR	89.42 (22.08)	90.76 (22.60)	90.55 (22.54)	90.09 (22.86)
CEE	73.69 (14.94)	74.49 (14.48)	74.91 (15.19)	75.32 (15.21)
Total	80.34 (19.26)	81.06 (19.28)	81.33 (19.58)	81.40 (19.53)

Figure 4. Changes in bodyweight. Data are expressed as mean (± SD). * indicates a significant increase at T2 (p = 0.015), T3 (p = 0.006), and T4 (p = 0.027).

Fat-Free Mass

There was no significant Group x Time interaction (p = 0.126, effect size = 0.114) or Group main effect (p = 0.137, effect size = 0.137). A significant Time main effect

(p = 0.000, effect size = 0.497) was seen with fat-free mass. Post hoc testing indicated that fat-free significantly increased at T2 (p = 0.001), T3 (p = 0.000), and T4 (p = 0.000) compared to T1. There was also a significant increase from T2 to T3 (p = 0.012) and T2 to T4 (p = 0.022).



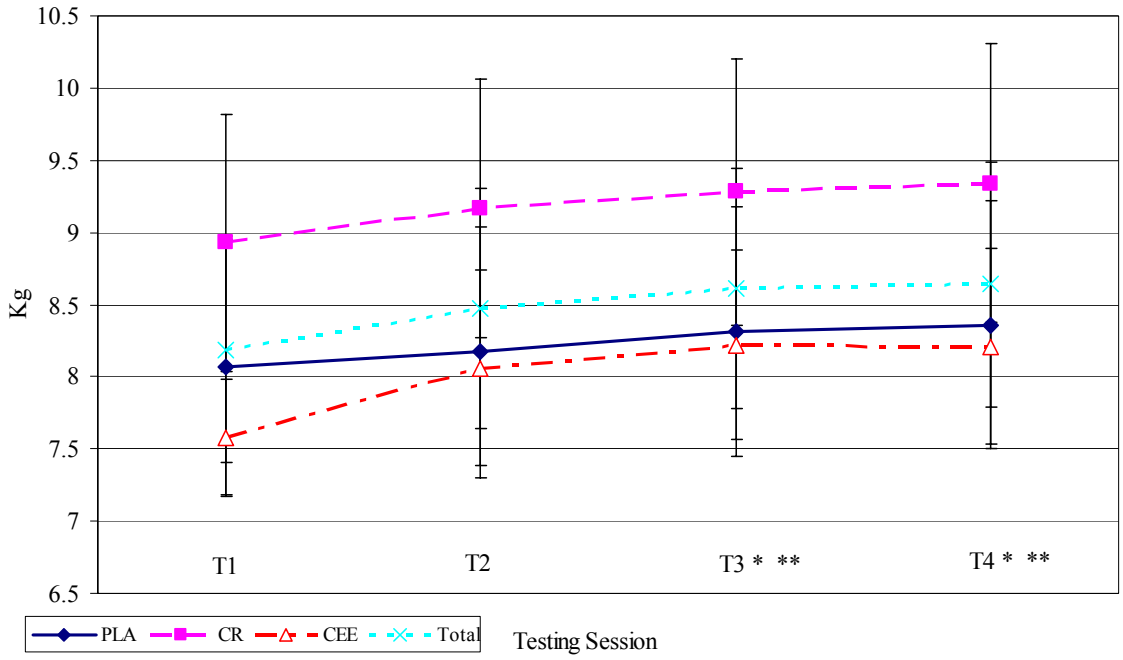
	T1	T2	T3	T4
PLA	54.55 (10.05)	55.10 (9.60)	56.05 (10.19)	56.25 (10.22)
CR	63.27 (10.79)	64.68 (11.18)	65.54 (11.68)	65.12 (11.39)
CEE	59.06 (8.46)	59.74 (8.16)	60.01 (8.52)	60.11 (8.11)
Total	58.96 (10.14)	59.84 (10.19)	60.53 (10.62)	60.49 (10.33)

Figure 5. Changes in fat-free mass. Data are expressed as mean (± SD). * indicates a significant increase at T2 (p = 0.001), T3 (p = 0.001), and T4 (p = 0.001) compared to T1. ** indicates a significant increase at T3 (p = 0.012) and T4 (p = 0.022).

Thigh Muscle Mass

There was no significant Group x Time interaction (p = 0.593, effect size = 0.046) or Group main effect (p = 0.236, effect size = 0.101) observed. However, a significant Time main effect (p = 0.002, effect size = 0.243) was observed. Post hoc testing revealed thigh muscle mass to be significantly increased at T3 (p = 0.017) and T4 (p = 0.016)

compared to T1. Increases were also seen at T3 ($p = 0.012$) and T4 ($p = 0.041$) compared to T2.

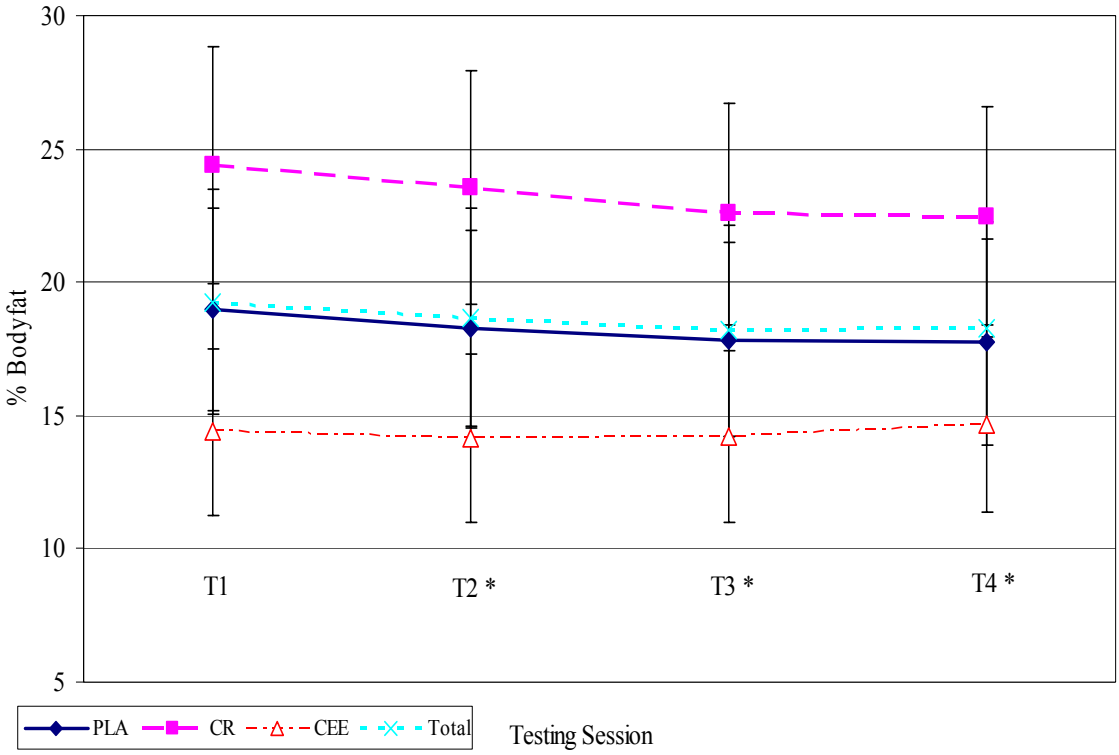


	T1	T2	T3	T4
PLA	8.07 (1.77)	8.17 (1.73)	8.31 (1.73)	8.36 (1.71)
CR	8.93 (1.78)	9.17 (1.79)	9.28 (1.84)	9.34 (1.93)
CEE	7.58 (.81)	8.06 (1.35)	8.22 (1.31)	8.21 (1.36)
Total	8.19 (1.57)	8.47 (1.66)	8.61 (1.66)	8.64 (1.70)

Figure 6. Changes in thigh muscle mass. Data are expressed as mean (± SD). * indicates a significant increase at T3 ($p = 0.017$), and T4 ($p = 0.016$) compared to T1. ** indicates a significant increase at T3 ($p = 0.012$) and T4 ($p = 0.041$) compared to T2

Body Fat Percentage

A significant Group x Time interaction ($p = 0.002$, effect size = 0.253) and significant Group main effect ($p = 0.043$, effect size = 0.208) was observed. Post hoc testing revealed the CR group had significantly ($p = 0.034$) more body fat than the CEE group. Additionally, significant Time main effects were observed ($p = 0.000$, effect size = 0.320) and post hoc testing indicated that significant decreases in body fat were measured at T2 ($p = 0.002$), T3 ($p = 0.000$), and T4 ($p = 0.003$) compared to T1.



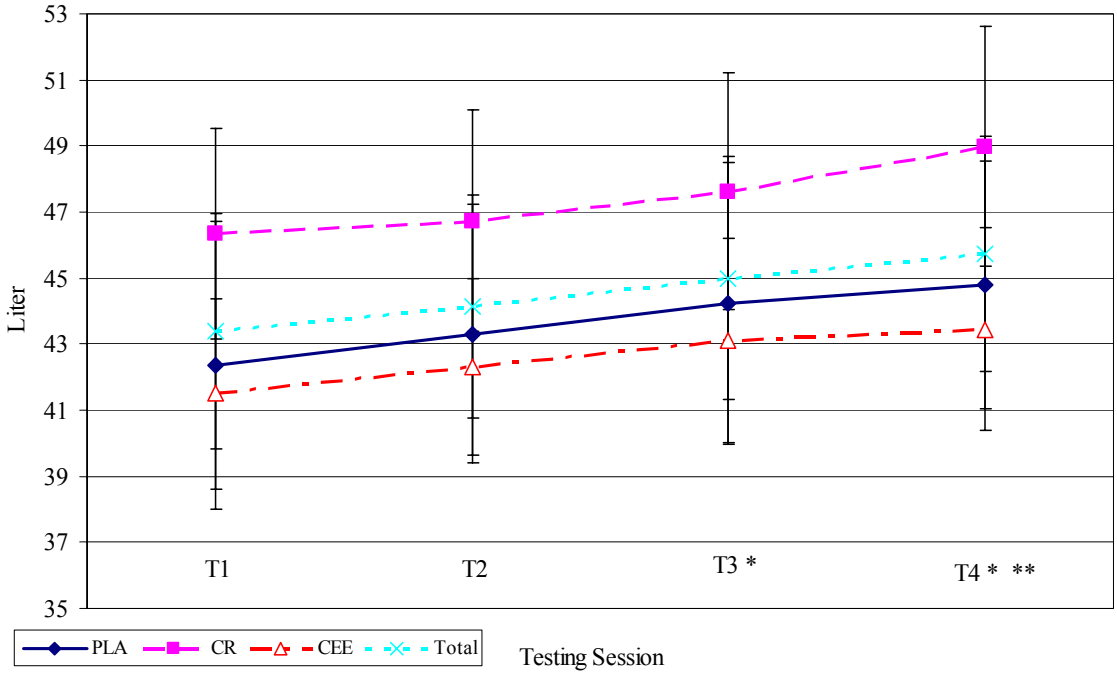
	T1	T2	T3	T4
PLA	19.00 (7.62)	18.30 (7.34)	17.84 (7.25)	17.76 (7.70)
CR	24.41 (8.90)	23.57 (8.74)	22.56 (8.36)	22.49 (8.22)
CEE^{+}	14.39 (6.23)	14.16 (6.35)	14.21 (6.47)	14.64 (6.58)
Total	19.27 (8.49)	18.68 (8.26)	18.20 (7.94)	18.30 (7.97)

Figure 7. Changes in body fat percentage. Data are expressed as mean (± SD). * indicates a significant increase at T2 ($p = 0.002$), T3 ($p = 0.000$), and T4 ($p = 0.003$).
Data table $^{+}$ indicates a significant difference ($p = 0.040$) between CR and CEE groups.

Total Body Water

There was not a significant Group x Time interaction ($p = 0.939$, effect size = 0.019) or a significant Group main effect ($p = 0.276$, effect size = 0.091) observed. However, a significant Time main effect ($p = 0.000$, effect size = 0.290) was observed. Post hoc testing indicated that total body water was significantly increased at T3 ($p =$

0.022) and T4 (p = 0.001) compared to T1. There was also a significant increase from T2 to T4 (p = 0.002).



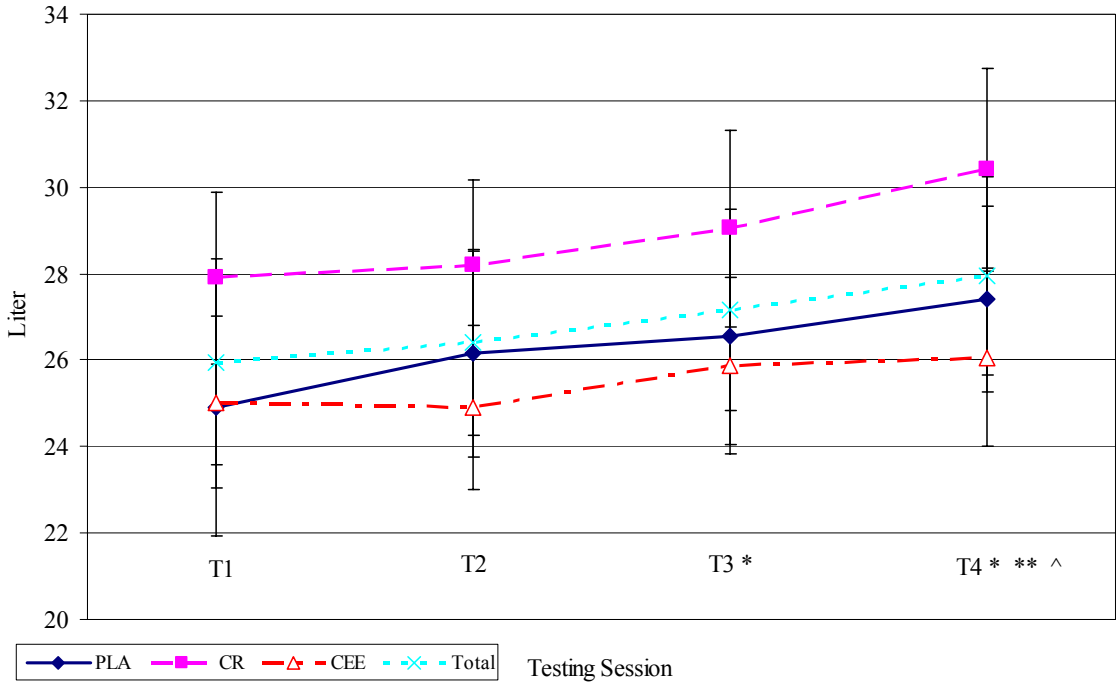
	T1	T2	T3	T4
PLA	42.36 (8.68)	43.32 (7.86)	44.23 (8.56)	44.79 (7.49)
CR	46.34 (6.38)	46.74 (6.72)	47.62 (7.16)	48.98 (7.28)
CEE	41.51 (5.77)	42.32 (5.36)	43.11 (6.20)	43.46 (6.10)
Total	43.40 (7.14)	44.13 (6.77)	44.99 (7.37)	45.74 (7.15)

Figure 8. Changes in total body water. Data are expressed as mean (± SD). * indicates a significant increase at T3 (p = 0.022) and T4 (p = 0.001) compared to T1. ** indicates a significant increase at T4 (p = 0.002) compared to T2.

Intracellular Body Water

No significant Group x Time interaction (p = 0.318, effect size = 0.082) or Group main effect (p = 0.198, effect size = 0.113) was observed for intracellular body water. However, a significant Time main effect (p = 0.000, effect size = 0.357) was observed and post hoc testing indicated there to be increases in intracellular body water at T3 (p =

0.023) and T4 (p = 0.000) compared to T1. There were also significant increases from T2 to T4 (p = 0.000) T3 to T4 (p = 0.002).



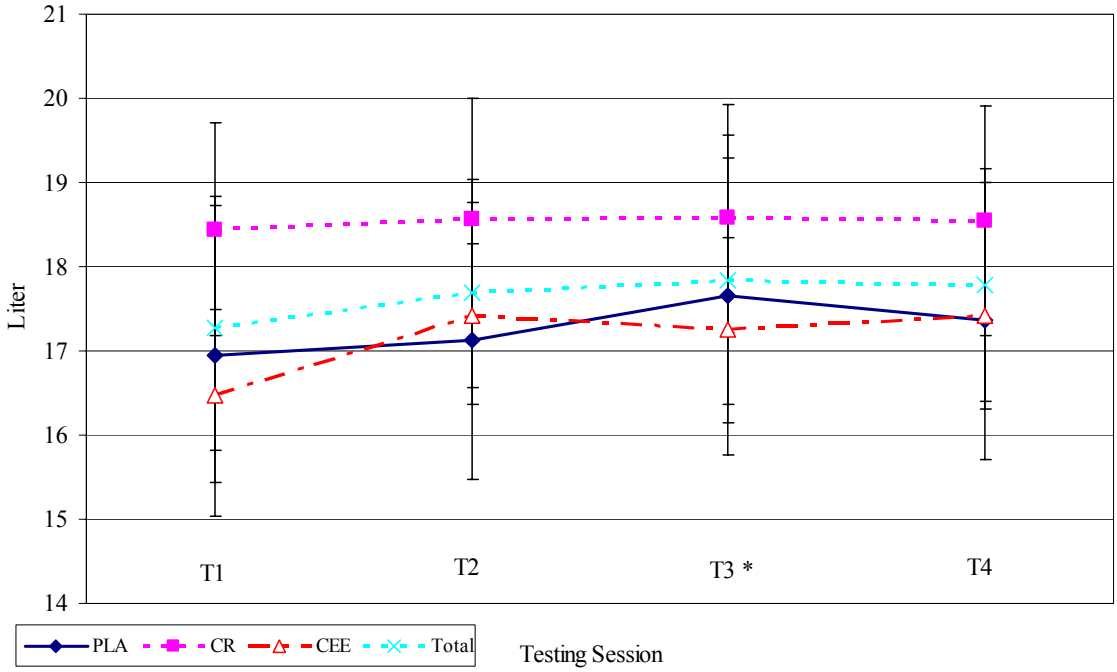
	T1	T2	T3	T4
PLA	24.90 (5.94)	26.15 (4.77)	26.57 (5.04)	27.42 (4.30)
CR	27.91 (3.97)	28.19 (3.96)	29.05 (4.53)	30.43 (4.62)
CEE	25.03 (3.98)	24.90 (3.78)	25.87 (4.11)	26.04 (4.03)
Total	25.95 (4.77)	26.41 (4.27)	27.16 (4.63)	27.96 (4.57)

Figure 9 . Changes in intracellular body water. Data are expressed as mean (± SD). * indicates a significant increase at T3 (p = 0.023) and T4 (p = 0.000) compared to T1. ** indicates a significant increase from T2 to T4 (p = 0.000). ^ indicates a significant increase from T3 to T4 (p = 0.002).

Extracellular Body Water

There was not a significant Group x Time interaction (p = 0.215, effect size = 0.096) or Group main effect (p = 0.478, effect size = 0.053) for extracellular body water. However, a significant Time main effect was observed (p = 0.000, effect size = 0.140).

Post hoc testing indicated extracellular water to be significantly increased at T3 ($p = 0.042$) compared to T1.



	T1	T2	T3	T4
PLA	16.94 (3.80)	17.12 (3.30)	17.66 (3.79)	17.36 (3.29)
CR	18.44 (2.52)	15.56 (2.87)	18.58 (2.71)	18.55 (2.73)
CEE	16.47 (2.06)	17.42 (1.71)	17.25 (2.20)	17.42 (2.24)
Total	17.28 (2.91)	17.70 (2.69)	17.83 (2.92)	17.78 (2.75)

Figure 10 . Changes in extracellular body water. Data are expressed as mean (± SD). * indicates a significant increase at T3 ($p = 0.042$) compared to T1.

Anthropometric Hypothesis Conclusion

- H_o: Following CEE supplementation, there will be no significant improvement in body composition after the loading and maintenance phase when compared to placebo. The null hypothesis is accepted
-
- H_1: Following CR supplementation, there will be a significant improvement in body composition after the loading and maintenance phase when compared to placebo. The directional hypothesis is rejected, all body composition variables improved, but in similar scope to the placebo group.

Muscle Strength

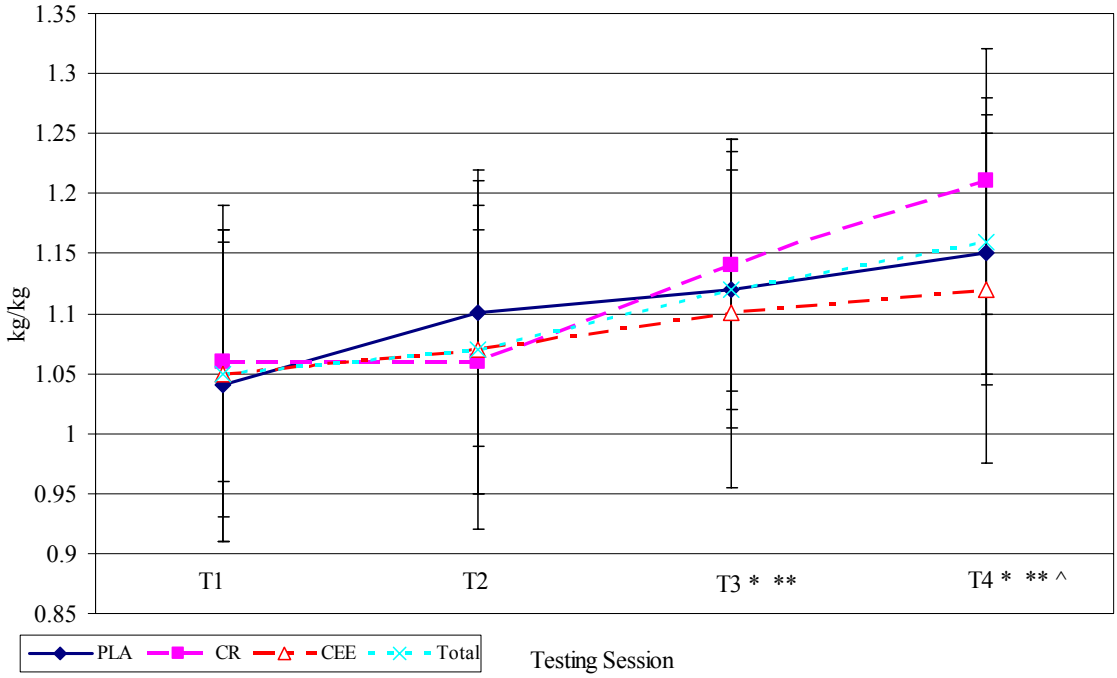
For bench and leg press strength, there was a mild trend for a Group x Time interaction ($p = 0.075$, effect size =0.331) and no significant multivariate Group main effect ($p = 0.893$, effect size = 0.021). However, a significant multivariate Time main effect ($p = 0.000$, effect size = 0.853) was observed. Figures 11 (bench press) and 12 (leg press) represent the mean values (±SD) for relative 1-RM strength throughout the duration of the study.

Relative 1-RM Bench Press

A significant Group x Time interaction ($p = 0.027$, effect size = 0.173) was observed (Figure 11); however, no significant Group main effect ($p = 0.946$, effect size = 0.004) was seen for relative 1-RM bench press. However, a significant Time main effect ($p = 0.000$, effect size = 0.515) was observed. Post hoc testing indicated that relative bench press was significantly increased at T3 ($p = 0.001$) and T4 ($p = 0.000$) compared to T1, significant increased at T3 ($p = 0.000$) and T4 ($p = 0.000$) compared to T2, and significantly increased at T4 ($p = 0.001$) compared to T3.

Relative 1-RM Leg Press

No significant Group x Time interaction ($p = 0.235$, effect size = 0.093) or Group main effect ($p = 0.894$, effect size = 0.008) were observed for relative 1-RM leg press (Figure 12). However, a significant Time main effect ($p = 0.000$, effect size = 0.513) was observed. Post hoc testing revealed that leg press 1-RM increased at T2 ($p = 0.021$), T3 ($p = 0.000$), and T4 ($p= 0.000$) compared to T1. Increases were also observed at T3 ($p = 0.000$) and T4 ($p = 0.000$) compared to T2.



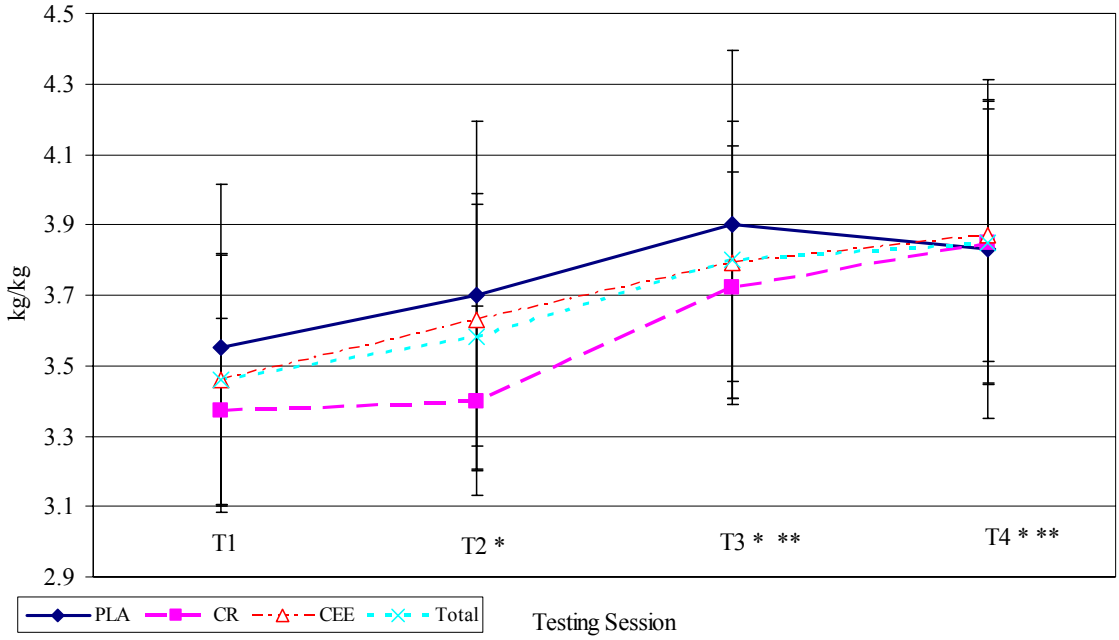
	T1	T2	T3	T4
PLA	1.04 (.26)	1.10 (.22)	1.12 (.20)	1.15 (.20)
CR	1.06 (.20)	1.06 (.22)	1.14 (.21)	1.21 (.22)
CEE	1.05 (.28)	1.07 (.30)	1.10 (.29)	1.12 (.29)
Total	1.05 (.24)	1.07 (.24)	1.12 (.23)	1.16 (.24)

Figure 11. Changes in relative 1-RM bench press. Data are expressed as mean (± SD). * indicates a significant increase at T3 ($p = 0.001$) and T4 ($p = 0.000$) compared to T1. ** indicates a significant increase at T3 ($p = 0.000$) and T4 ($p = 0.000$) compared to T2. ^ indicates a significant increase at T4 ($p = 0.001$) compared to T3

Muscle Strength Hypothesis Conclusion

- H_o: Following CEE supplementation, there will be no significant improvement in muscle strength after the loading and maintenance phase when compared to placebo. The null hypothesis is accepted.

- H_1: Following CR supplementation, there will be a significant improvement in muscle strength after the loading and maintenance phase when compared to placebo. The directional hypothesis is rejected.



	T1	T2	T3	T4
PLA	3.55 (.93)	3.70 (.99)	3.90 (.99)	3.83 (.96)
CR	3.37 (.53)	3.40 (.54)	3.72 (.66)	3.85 (.81)
CEE	3.46 (.71)	3.63 (.72)	3.79 (.67)	3.87 (.72)
Total	3.46 (.72)	3.58 (.76)	3.80 (.77)	3.85 (.80)

Figure 12. Changes in relative 1-RM leg press. Data are expressed as mean (± SD). * indicates a significant increase at T2 (p = 0.021), T3 (p = 0.000) and T4 (p = 0.000) compared to T1. ** indicates a significant increase at T3 (p = 0.000) and T4 (p = 0.000) compared to T2.

Muscle Power

For muscle power, mean, peak, and minimum power, time to peak power, rate of fatigue, peak and mean power/bodyweight were grouped for statistical analysis. There were no significant multivariate Group x Time interactions (p = 0.468, effect size 0.791), or multivariate main effects for Group (p = 0.705, effect size = 0.212) or Time (p =

0.179, effect size = 0.881) (Table 4). However, significant Time main effects were seen for mean power ($p = 0.001$, effect size = 0.218) and post hoc testing showed mean power to be increased at T3 ($p = 0.046$) and T4 ($p = 0.019$) compared to T1, along with increases seen at T4 ($p = 0.029$) compared to T2. A significant Time main effect ($p = 0.000$, effect size = 0.257) was observed for peak power with post hoc testing indicating peak power to be increased at T4 ($p = 0.000$) compared to T1. Additionally, peak power was increased at T4 compared to T2 ($p = 0.001$) and T3 ($p = 0.049$). No significant Time main effect ($p = 0.816$, effect size = 0.011) was observed for minimum power. A significant Time main effect was observed for time to peak power ($p = 0.001$, effect size = 0.211). Post hoc testing showed that time to peak power increased at T3 ($p = 0.041$) and T4 ($p = 0.015$) compared to T1 while T4 was increased ($p = 0.027$) compared to T2. A significant Time main effect was seen for rate of fatigue ($p = 0.000$, effect size = 0.258). Post hoc testing indicated that time to peak power significantly increased at T4 ($p = 0.000$) compared to T1, T2 ($p = 0.001$), and T3 ($p = 0.030$) compared to T4. A significant Time main effect ($p = 0.000$, effect size = 0.224) was observed for peak power/bodyweight. Post hoc testing indicated peak power/bodyweight increased at T4 compared to T1 ($p = 0.001$), T2 ($p = 0.004$) compared to T4, and T3 ($p = 0.047$) compared to T4. A significant Time main effect was observed for mean power/body weight ($p = 0.006$, effect size = 0.155). Post hoc testing showed only trends for mean power/bodyweight. The trends were observed at T4 ($p = 0.070$) compared to T1, also at T4 ($p = 0.092$) compared to T2.

Table 4

Wingate Results

Variable	Session	PLA Group (±SD)	CR Group (±SD)	CEE Group (±SD)	Time Effect p value	Group Effect p value	Group x Time p value
Wingate Mean Power	T1	623 (136)	679 (128)	615 (93)			
(W)	T2	633 (154)	695 (127)	623 (92)			
	T3	636 (166)	724 (128)	642 (111)			
	T4	657 (177)	713 (128)	648 (97)	* 0.001	0.414	0.638
Wingate Peak Power	T1	1171 (238)	1258 (243)	1107 (202)			
(W)	T2	1197 (313)	1208 (215)	1210 (181)			
	T3	1174 (229)	1322 (214)	1196 (193)			
	T4	1305 (256)	1326 (211)	1251 (174)	* 0.000	0.644	** 0.052
Wingate Minimum Power	T1	317 (70)	333 (56)	361 (37)			
(W)	T2	313 (93)	349 (73)	331 (34)			
	T3	309 (95)	363 (73)	345 (32)			
	T4	313 (102)	338 (86)	344 (54)	0.816	0.434	0.471
Wingate Time to Peak Power	T1	6.62 (.62)	3.38 (.82)	3.69 (.30)			
(Sec)	T2	3.66 (.49)	3.62 (.47)	3.80 (.39)			
	T3	4.20 (.56)	3.84 (.23)	3.87 (.10)			
	T4	4.10 (.33)	3.88 (.14)	3.91 (.18)	* 0.001	0.192	0.562

(table continues)

Variable	Session	PLA Group (±SD)	CR Group (±SD)	CEE Group (±SD)	Time Effect p value	Group Effect p value	Group x Time p value
Wingate Rate to Fatigue	T1	32.42 (8.25)	34.83 (8.29)	28.36 (7.81)			
(W/sec)	T2	33.63 (10.95)	32.55 (8.60)	33.54 (5.69)			
	T3	33.54 (6.22)	36.67 (7.96)	32.56 (7.02)			
	T4	38.34 (7.70)	37.84 (8.12)	34.77 (6.15)	* 0.000	0.622	** 0.092
Wingate Peak Power/Body Weight	T1	15.19 (2.30)	14.42 (2.62)	15.03 (1.03)			
(W/kg)	T2	15.47 (3.17)	13.61 (1.94)	16.47 (2.08)			
	T3	15.18 (2.24)	14.99 (2.27)	16.18 (2.24)			
	T4	16.82 (2.72)	15.21 (2.90)	16.91 (2.36)	* 0.000	0.268	** 0.069
Wingate Mean Power/Body Weight	T1	8.04 (1.04)	7.74 (1.04)	8.39 (.54)			
(W/kg)	T2	8.15 (1.10)	7.81 (1.00)	8.44 (.76)			
	T3	8.10 (1.13)	8.15 (.95)	8.62 (.64)			
	T4	8.32 (1.17)	8.08 (1.08)	8.69 (.70)	* 0.006	0.376	0.671

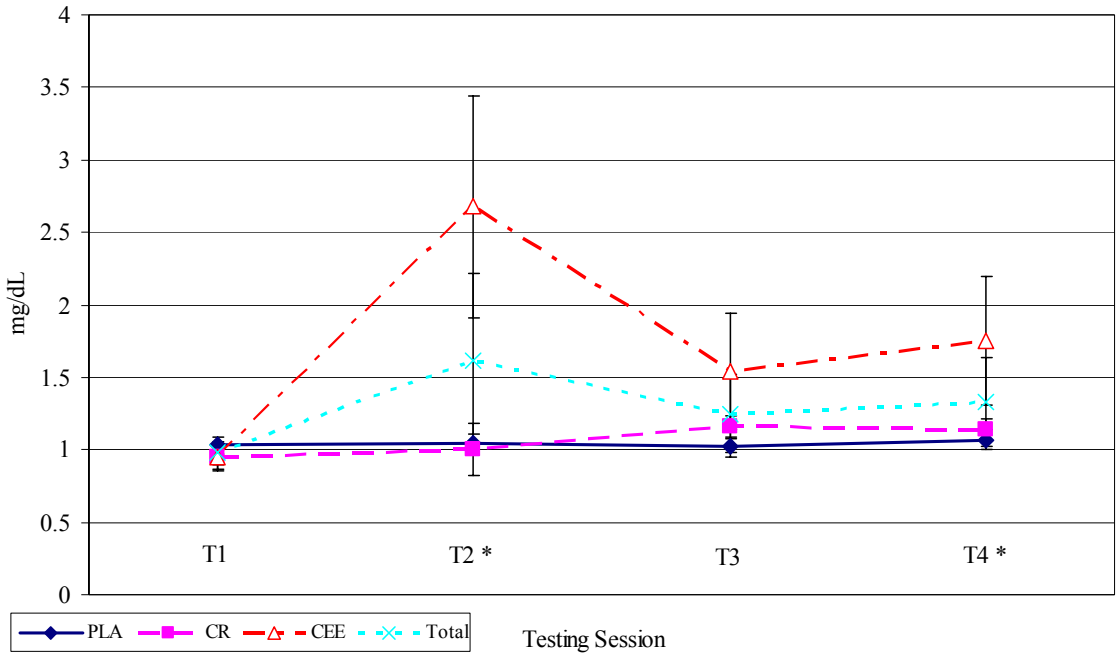
Note: This data represents the Wingate Muscle Power values during the course of the study. Values are represented as absolute means (± SD).
* indicates significant time main effects, ** indicates Group x Time interaction Trends

Serum Blood Markers

Serum Renal and Hepatic Variables

Serum GGT, URCA, BUN, CREA, BN/CR, ALP, AST, ALT were grouped together for statistical analysis. A significant multivariate Group x Time interaction ($p = 0.018$, effect size = 0.992) and significant multivariate Group main effect ($p = 0.000$, effect size = 0.629) were observed. However, only a trend for Time main effect ($p = 0.055$, effect size = 0.995) was observed. The group main effect was observed for creatinine ($p = 0.000$, effect size = 0.759). Post hoc testing indicated that serum creatinine was greater in the CEE group compared to the PLA group ($p = 0.000$) and CR group ($p = 0.000$). A Time main effect ($p = 0.030$, effect size = 0.134) was seen at T2 ($p = 0.028$) and T4 ($p = 0.013$). A significant Group x Time interaction ($p = 0.010$, effect size = 0.235) was also seen for Creatinine variable. CEE creatinine levels increased 3 fold after the loading phase, and continued to be elevated above normal values throughout the study. CR group of creatinine levels for the CR group did elevate, but stayed within the normal range of 0.8-1.3 mg/dL, while the PLA group stayed near baseline levels. Figure 13 shows mean (±SD) values and changes over time for serum creatinine. BUN/Creatinine (Table 5) ratio showed a Group x Time interaction ($p = 0.024$, effect size = 0.198). A trend for group main effect ($p < 0.061$) was observed. Post Hoc testing showed a trend ($p < 0.10$) between the PLA group and CEE group. Creatinine levels were elevated three fold, this may account for the interaction seen with in the BN/CR ratio. A Time main effect (Table 5) was seen for ALP ($p = 0.034$, effect size = 0.112). Post hoc testing indicated significant increased at T4 ($p = 0.045$) along with a trend at T4 ($p < 0.10$) compared to T3 for ALP. Table 5 gives the mean (±SD) for

the BN/CR and ALP variables. All other serum variables are grouped together in appendix D.



	T1	T2	T3	T4
PLA **	1.03 (.12)	1.04 (.13)	1.02 (.14)	1.07 (.13)
CR †	0.95 (.17)	1.00 (.36)	1.16 (.16)	1.14 (.14)
CEE	0.95 (.18)	2.68 (1.53)	1.54 (.80)	1.75 (.89)
Total	0.98 (.16)	1.61 (1.21)	1.25 (.53)	1.33 (.61)

Figure 13. Changes in serum creatinine. Data are expressed as mean (± SD). * indicates a significant increase at T2 (p = 0.028) and T4 (p = 0.013) compared to T1.
Data table. ** indicates CEE to be significantly greater than PLA (p = 0.001). † indicates CEE to be significantly greater than CR (p = 0.001).

Serum Lipid Variables

Serum TG, CHOL, HDL, and LDL were grouped together for statistical analysis . No significant multivariate Group x Time interaction (p = 0.175, effects size = 0.516) or Group main effect (p = 0.169, effect size = 0.204) occurred. A significant Time main effect was observed for the cholesterol (p = 0.021, effect size = 0.113) and LDL (p = 0.001, effect size = 0.173) variables (Table 6). Serum cholesterol had a trend at T4

(p<0.10) and T4 (p < 0.10) compared to T3. LDL cholesterol was significantly different at T4 (p = 0.003) along with a trend at T4 (p < 0.10) compared to T2. Table 6 gives the mean (±SD) for the CHOL and LDL variables. All other serum variables are grouped together in appendix D.

Table 5

Serum Renal & Hepatic Markers

Variable	Group	Session	Mean	± SD	Variable	Group	Session	Mean	± SD
BN/CR	PLA	T1	14.26	3.62	ALP	PLA	T1	69.2	22.36
		T2	15.78	4.29	U/L		T2	68.4	18.08
		T3	15.34	3.4			T3	70.6	18.79
		T4	15.63	3.5			T4	75.7	17.72
	CR	T1	13.76	2.95		CR	T1	60.13	17.63
		T2	19.73	16.85			T2	66.75	19.26
		T3	13.73	6.38			T3	65.88	13.85
		T4	13.36	3.25			T4	70.25	13.38
	CEE	T1	17.11	5.31		CEE	T1	76.4	16.77
		T2	7.18	4.21			T2	79.9	11.01
		T3	11.97	5.11			T3	78.9	16.08
		T4	11.34	6.22			T4	79.5	15.5

Serum kidney and liver variables mean and standard deviation for each testing session.

Serum LDH and CK Variables

Serum LDH and CK were grouped for statistical analysis. There was no significant Group x Time interaction (p = 0.260, effect size = 0.401). Additionally, there were no significant multivariate Group main effects (p = 0.142, effect size = 0.189) or Time main effects (p = 0.084, effect size = 0.555) observed. Appendix D contains the LDH and CK values with all the other serum variables.

Table 6

Serum Lipid Markers

Variable	Group	Session	Mean	± SD	Variable	Group	Session	Mean	± SD
CHOL	PLA	T1	143.2	27.94	LDL	PLA	T1	85.1	26.96
mg/dL		T2	137.1	22.57	mg/dL		T2	79.7	25.85
		T3	137.2	26.55			T3	83.5	27.59
		T4	150	27.47			T4	93.3	29.59
	CR	T1	162.1	37.43		CR	T1	101.8	30.58
		T2	168.3	35.26			T2	106.8	30.62
		T3	170.1	29.83			T3	109.1	29.87
		T4	173.4	36.07			T4	110.7	31.21
	CEE	T1	136.5	36.98		CEE	T1	79.3	35.54
		T2	139.8	30.52			T2	82.4	32.45
		T3	136.5	29.65			T3	81.7	29.98
		T4	149.2	23.32			T4	92.9	28.33

Serum lipid variables mean and standard deviation for each testing session.

Serum Clinical Safety

Serum GLU, CA, TP, ALB, and TBIL were grouped together for statistical analysis. No significant Group x Time interaction ($p = 0.380$, effect size = 0.565) was observed. Additionally, there was no significant multivariate Group main effect ($p = 0.108$, effect size = 0.271) or Time main effect ($p = 0.476$, effect size = 0.545). Appendix D contains all the serum clinical safety values with all the other serum variables.

Serum Hypothesis Conclusion

- H_o: Following CR and CEE supplementation, the serum levels of creatinine will not significantly increase after the loading and maintenance phase when compared to placebo. CEE did significantly increase compared to CR and PLA groups. The null hypothesis for creatinine is rejected

- H_o: Following CR and CEE supplementation, there will be no significant difference in relation to whole-blood and serum clinical chemistry markers after the loading and maintenance phase compared to placebo. For serum clinical chemistry markers, there were no significant differences between the groups. The null hypothesis is accepted.

Whole Blood Markers

Whole Red Blood

Whole Red Blood markers (RBC, HGB, HCT, MCV, MCH, MCHC) were grouped for statistical analysis (Table 7). No significant multivariate Group x Time interaction ($p = 0.832$, effect size = 0.556) or Group main effect ($p = 0.399$, effect size = 0.228) were observed. However, a significant multivariate Time main effect ($p = 0.043$, effect size = 0.541) was observed. Post hoc testing indicated that RBC was significantly increased at T2 ($p = 0.009$) and T3 ($p = 0.001$) compared to T1. In addition, RBC was also increased at T4 compared to T2 ($p = 0.004$) and T3 ($p = 0.001$). Post hoc testing showed that HGB was significantly increased at T2 ($p = 0.025$) and T3 ($p = 0.004$) compared to T1, while significant increases in HGB were also seen at T4 ($p = 0.017$) compared to T3. Post hoc testing also showed that HCT significantly increased at T2 ($p = 0.004$) and T3 ($p = 0.003$) compared to T1. In addition, HCT was also increased T4 compared to T2 ($p = 0.000$) and T3 ($p = 0.001$). Table 7 shows all mean (±SD) for whole red blood variables.

Table 7

Whole Red Blood Variables

Variable	Group	Session	Mean	± SD	Variable	Group	Session	Mean	± SD
RBC	PLA	T1	5.42	0.31	HGB	PLA	T1	15.5	1.3
M/uL		T2	5.27	0.29	g/dL		T2	15.21	1.18
		T3	5.15	0.22			T3	14.91	1.18
		T4	5.43	0.39			T4	15.66	1.58
	CR	T1	5.2	0.41		CR	T1	15.15	0.95
		T2	5.07	0.33			T2	14.8	0.75
		T3	5.04	0.37			T3	14.74	0.88
		T4	5.18	0.39			T4	15.12	1.09
	CEE	T1	5.22	0.21		CEE	T1	15.4	0.87
		T2	5.04	0.26			T2	14.97	0.9
		T3	5.07	0.25			T3	14.96	0.67
		T4	5.14	0.24			T4	15.04	0.66
HCT	PLA	T1	49.02	3.82	MCV	PLA	T1	90.47	4.17
%		T2	47.56	3.3	fL		T2	90.24	3.78
		T3	46.55	2.32			T3	90.46	3.73
		T4	49.1	4.07			T4	90.36	3.35
	CR	T1	47.96	3.45		CR	T1	92.41	3.74
		T2	46.7	2.39			T2	92.28	3.72
		T3	46.68	2.68			T3	92.8	3.85
		T4	48.57	3.61			T4	94.02	6.39
	CEE	T1	48.12	2.45		CEE	T1	92.28	4.79
		T2	46.47	3.42			T2	92.19	4.57
		T3	47.06	2.75			T3	92.95	4.79
		T4	47.63	3.07			T4	92.63	5.12
MCH	PLA	T1	28.65	2.01	MCHC	PLA	T1	31.64	1.26
pg		T2	28.87	1.93	g/dL		T2	31.97	1.15
		T3	28.99	2.24			T3	32.01	1.42
		T4	28.84	1.99			T4	31.91	1.29
	CR	T1	29.54	2.2		CR	T1	31.62	1.1
		T2	29.27	1.69			T2	31.7	0.99
		T3	29.34	1.38			T3	31.63	0.79
		T4	29.25	1.58			T4	31.18	1.29
	CEE	T1	29.58	1.58		CEE	T1	32.05	0.92
		T2	29.69	1.43			T2	32.23	0.87
		T3	29.59	1.48			T3	31.83	1.03
		T4	29.25	1.47			T4	31.62	1.05

Red blood variables mean and standard deviation for each testing session.

Table 8

Whole White Blood Variables

Variable	Group	Session	Mean	± SD	Variable	Group	Session	Mean	± SD
WBC	PLA	T1	5.93	1.15	NEU	PLA	T1	3.48	0.92
K/uL		T2	5.96	1.25	%N		T2	3.29	1.01
		T3	4.99	0.97			T3	2.48	0.74
		T4	5.86	1.5			T4	3.17	1.15
	CR	T1	7.09	1.45		CR	T1	4.08	1.24
		T2	7.15	1.87			T2	3.89	1.09
		T3	6.08	0.76			T3	3.05	0.54
		T4	6.91	2.09			T4	3.91	1.78
	CEE	T1	6.9	2.15		CEE	T1	4.02	2.01
		T2	6.36	1.37			T2	3.58	1.19
		T3	6.05	1.36			T3	3.3	0.81
		T4	6.07	1.74			T4	3.3	1.2
LYM	PLA	T1	1.65	0.32	MONO	PLA	T1	0.58	0.21
%L		T2	1.79	0.35	%M		T2	0.58	0.2
		T3	1.77	0.44			T3	0.48	0.16
		T4	1.89	0.45			T4	0.54	0.19
	CR	T1	2.07	0.77		CR	T1	0.68	0.31
		T2	2.37	0.86			T2	0.63	0.19
		T3	2.17	0.58			T3	0.59	0.13
		T4	2.22	0.59			T4	0.57	0.16
	CEE	T1	2.07	0.7		CEE	T1	0.56	0.08
		T2	2.01	0.73			T2	0.55	0.17
		T3	1.87	0.58			T3	1.11	1.86
		T4	1.97	0.76			T4	0.51	0.14
EOS	PLA	T1	0.18	0.1	BASO	PLA	T1	0.06	0.03
%E		T2	0.24	0.17	%B		T2	0.07	0.03
		T3	0.2	0.14			T3	0.06	0.02
		T4	0.18	0.1			T4	0.07	0.04
	CR	T1	0.18	0.1		CR	T1	0.07	0.03
		T2	0.18	0.12			T2	0.08	0.03
		T3	0.18	0.08			T3	0.08	0.03
		T4	0.14	0.07			T4	0.07	0.03
	CEE	T1	0.2	0.15		CEE	T1	0.06	0.02
		T2	0.17	0.12			T2	0.05	0.02
		T3	0.26	0.28			T3	0.08	0.06
		T4	0.21	0.14			T4	0.07	0.03

White blood variables mean and standard deviation for each testing session.

Whole White Blood

Whole White Blood markers (WBC, NEU, LYM, MONO, EOS, and BASO) were grouped together for statistical analysis (Table 8). No significant multivariate Group x Time interaction ($p = 0.883$, effects size = 0.534) or Group main effect ($p = 0.693$, effect size = 0.170) occurred. Significant Time main effects were seen for WBC ($p = 0.011$, effect size = 0.131), which were shown to be trends at T3 ($p < 0.10$) and T3 ($p < 0.10$)) compared to T2. NEU had a Time main effect ($p = 0.002$, effect size = 0.170) at T3 ($p = 0.012$), and at T3 ($p = 0.007$) compared to T2. Table 8 shows all mean (±SD) for whole white blood variables.

Whole Blood Hypothesis Conclusion

- H_o: Following CR and CEE supplementation, there will be no significant difference in relation to whole-blood and serum clinical chemistry markers after the loading and maintenance phase compared to placebo. For whole blood clinical chemistry markers, there was no significant difference between the groups. The null hypothesis is accepted.

CHAPTER FIVE

Discussion

The purpose of this study was to examine the effects of creatine ethyl ester supplementation during a seven week training study compared to supplementation with creatine monohydrate and a placebo. Following a 5-day loading phase and a 42-day maintenance phase, creatine ethyl ester was examined for changes in muscle strength and mass, body composition changes, intramuscular total creatine uptake and creatine transporter protein content. Since creatine ethyl ester is a novel creatine formulation with no published safety data to date, safety of the formulation was a secondary purpose of this study. Therefore, the effects of creatine ethyl ester on whole-blood and serum clinical safety markers were also examined.

Serum and Total Muscle Creatine Concentrations

Studies have shown acute ingestion of 5g or 20g of creatine to increase serum levels of creatine (Mesa, 2002). Contrary to other studies, serum creatine concentrations (Figure 1) did not increase for the supplement groups during the course of the study. The mean serum creatine levels of the PLA and CR groups were significantly higher compared to the CEE group. Total muscle creatine (Figure 2) was shown to be significantly greater in the CR group compared to the PLA group. Total muscle creatine concentration for the CEE group was not significantly different than either the PLA or CR groups. The CR group had a higher total creatine concentration than the PLA group. There was not a significant increase in total muscle creatine levels for any of the groups

during the study, but a weak trend was seen for increases in intramuscular creatine concentrations after the loading phase. This is contrary to most other studies with creatine monohydrate showing significant intramuscular increases (Casey & Greenhaff, 2000; Demant & Rhodes, 1999; Greenhaff *et al*. 1994; Harris *et al*., 1992); however, it has been estimated that 20-30% of people do not respond to creatine supplementation (Greenhaff, 1997).

Creatine Transporter Protein Content

Creatine transporter protein content was shown to be greater in the PLA group ($p = 0.008$) compared to CEE group (Figure 3). The CR group showed a mild trend ($p = 0.073$) in creatine transporter protein content compared to the CEE group. While the CEE group had a lower creatine transporter protein content than the other two groups, there was no significant increase in protein content for during the duration of the study for any of the groups. Intramuscular creatine uptake has shown to be dependent on creatine transporter activity (Loike *et al*., 1988). However, in the present study supplementation with CEE or CR was shown not to change the creatine transporter protein content within the muscle. The possible rationale for this occurrence could be based on previous data illustrating that during chronic creatine ingestion (6-10 weeks) down regulation of the creatine transporter in rat muscle has been shown to occur (Guerrero, 1998).

Muscle Mass and Body Composition

Untrained participants were selected to perform a seven week (4 day per week) training program and were expected to have changes in muscle mass and body

composition independent of supplementation. Compared to T1 (Figure 4), all groups showed significant increases in body weight at each of the three testing sessions. While all groups increased in body weight, there was no significant difference between the three groups. Various studies have shown an average of 1-2 kg of total body mass increases with initial supplementation (5-7 days) 20g/day of creatine (Greenhaff, 1994; Persky & Brazeau, 2001; van Loon, 2003; Wyss & Kaddurah-Daouk, 2000). Total body weight increases after the 5-day loading phase were 0.03 kg, 1.39 kg, and 0.80 kg for PLA, CR, and CEE, respectively. Kreider (1998) indicated short duration (5-7 days) of creatine supplementation (20 – 25 g/day) typically leads to increases of up to 1.6 kg in total body mass. The total body mass increase observed with the CR group was within typical ranges previously seen (Balsom *et al*.,1993; Snow *et al*.,1998), even though there were no significant differences between the groups. For the body composition variables body fat percentage, fat-free mass, and thigh mass, there were no significant differences in fat-free mass between any of the three groups. However, collectively fat-free mass (Figure 5) was shown to increase at each testing session T2 ($p = 0.001$), T3 ($p = 0.001$), and T4 ($p = 0.001$) compared to T1. Fat-free mass was also significantly increased at T3 and T4 compared toT2. Fat-free mass increases after the 5-day loading phase were 0.55 kg, 1.41 kg, and 0.68 kg for PLA, CR, and CEE, respectively. Previous studies have shown 12 weeks of creatine supplementation with resistance exercise (Volek *et al*., 1999) and short (5 days loading and 4 days of maintenance) supplementation with creatine to increase fat-free mass (Parise *et al.* 2001). As anticipated with an untrained population, increases in bodyweight and fat-free mass were expected due to a training effect. In line with fat-free mass increases, thigh mass increases (Figure 6) were also observed throughout the

duration of the study. Thigh mass increases after the 5-day loading phase were 0.10 kg, 0.24 kg, and 0.48 kg for PLA, CR, and CEE, respectively. In contrast to total body mass and fat free mass, the CEE group showed the largest increase in thigh muscle mass. Body fat (Figure 7) was shown to significantly decrease at T2, T3, and T4. Both PLA and CR groups had reductions in body fat throughout the study, and the reduction in the CR group was significantly different that the CEE group. Specifically, body fat percent was shown to decrease 1.24 and 1.92 for the PLA and CR groups, respectively, whereas the CEE group underwent a slight 0.25 increase. It should be noted, that the CR group a baseline body fat percent that was higher than the CEE group. Even though bodyweight and fat-free mass were not statistically different, the CR group may have had a greater potential for fat loss than the CEE group. As such, the reduction of body fat observed with the PLA and CR groups was mostly likely due to the resistance training rather than supplementation.

Body Water

Total, intracellular, and extracellular body water are of particular interest for the CEE group. Claims by the manufactures of creatine ethyl ester have stated a difference in the retention of body water compared to other forms of creatine, specifically creatine monohydrate. Through the use of the esterfication process, creatine is proposed to bypass the creatine transporter which would allow for more creatine to enter the target cell and minimize the amount of extracellular water retained during supplementation. A potential benefit of creatine supplementation is through the action of an anabolic signal for skeletal muscle hypertrophy, with increases in total and intracellular water (Mesa,

2002; Persky & Brazeau, 2001). Roughly two-thirds of the increases in total body water seen during supplementation are intracellular, with no fluid shift occurring (Powers e*t al*., 2003; Ziegenfuss, 1998). Mean increases in total body water (Figure 8) from T1 to T4 were 2.43L, 2.64L, and 1.95L for PLA, CR, and CEE groups, respectively. For all groups, total body water was shown to significantly increase at T3 and T4 compared to T1. Mean increases in intracellular body water (Figure 9) from T1 to T4 were 2.52L, 2.52L and 1.01L for PLA, CR, and CEE groups, respectively, and intracellular body water was significantly increased at T3 and T4. For extracellular water (Figure 10), mean increases from T1 to T4 were .42L .11L and .50L for PLA, CR, and CEE groups, respectively, extracellular body water was only significantly increased at T3. Collectively, changes in total, intracellular, and extracellular body water were not significantly different between the supplement and placebo groups. However, the mean increases for total and intracellular body water from T1 to T4 were greatest for the CR group. Extracellular water increases from baseline were actually largest for the CEE groups. Claims by the manufactures of CEE stating extra cellular water retention is minimized were shown to be unfounded. Previous research has shown creatine supplementation to increase total body water, yet no fluid shift occurs (Powers, Arnold, Weltman, Perrin, Mistry, Kahler, *et al*., 2003). In trained population, Kutz and Gunter (2003) observed increases in total body water with creatine supplementation, but not a placebo during resistance training. In contrast, in the present study the population was untrained, with increases in body water observed in the PLA group. The observed changes were mostly likely due to the resistance training program itself rather than the supplementation.

Muscle Strength/Power

Various studies have shown improvement in muscle strength and power through the use of creatine supplementation (Bemben & Lamont, 2005; Casey *et al*., 2000; Volek, 1999). Relative muscle strength was measured through the use of 1-RM test for both bench press and leg press. Bench press (Figure 11) was shown to increase at T3 and T4 compared to T1. Leg press (Figure 12) showed an increase during T2, T3, and T4 compared to T1. However, there were no differences between the three groups.

With the exception of minimum power, mean and peak power, time to peak power, rate of fatigue, and peak and mean power/bodyweight all showed a significant improvement over the course of the study (Table 4). However, the muscle power measures had no significant differences between the three groups. Other studies have shown no benefits for increases in muscle power with supplementation (Hoffman *et al*., 2005). An increase in muscle performance typically correlates with an increase in creatine muscle uptake (Greenhaff, 2000). There was no significant increase in total muscle creatine content with the supplement groups. The PLA group did not consume creatine and showed similar improvements in muscle strength and performance, which indicates the improvements that were seen were most likely from the strength training program, not due to the creatine supplements.

Clinical Safety Data

Minimal data is available on creatine ethyl ester. Serum and whole blood were analyzed to evaluate any potential safety concerns. Some serum and whole blood variables (ALP, CHOL, LDL, RBC, HGB, HCT, WBC, and NEU) changed during the

course of the study (Tables 5, 6, 7, and 8). This was likely due to a training effect or normal variation in the participants' diets, rather than the supplementation, due to all (except creatinine and BN/CR) values remaining within normal clinical range. For serum creatinine (Figure 13), the CEE group underwent significant increases compared to the PLA and CR groups at T2 and T4. Serum creatinine is of significance importance because creatinine is the by-product of creatine degradation. Creatine is nonenzymatically converted into creatinine at roughly 1.7% daily for a typical 70 kg individual (Wyss & Kaddurah-Daouk, 2000). Creatine is also degraded by the gut into creatinine at an estimated a rate of 0.1g of a 5g dose per hour. This indicates that the GI track is not a major source of creatinine production; therefore, skeletal muscle is the primary site of creatinine production. (Persky, 2003; Schedel, 1999). With increases in muscle saturation of creatine, creatinine levels will increase due to reduction in the skeletal muscle uptake (Greenhaff, 1997). Skeletal muscle total creatine content underwent non-significant increases after the loading phase. However, creatinine levels were significantly increased at T2 and T4 compared to T1.

Supplementation was based on fat-free mass for all groups but was comparable to a 20g loading phase and a 5g maintenance phase typically seen with creatine supplementation. When CEE is bound with an ester compound and not pure creatine, the structure still yields approximately 17.4g of creatine for a 20 g dose and 4.37 g for a 5 g dosage (Dox & Yoder, 1922). The supplement loading phase in the present study consisted of two 10 g dosages based on the premise that for a 10 g dose, maximal absorption usually occurs within two hours (Persky, 2003). Blood draws were not taken specifically after supplementation, yet creatinine levels were approximately tripled during

T2 (2.68 mg/dL ±SD 1.53) compared to baseline (0.95 mg/dL ±SD .18) for the CEE group. The creatinine level for the CEE group during the duration of the study remained elevated above the normal range of (0.8-1.3 mg/dL). However, even though serum creatinine was elevated with the CR group, serum levels of creatinine stayed within normal clinical ranges throughout the study.

Conclusions

Creatine ethyl ester did not show any additional benefit to increase muscle strength or performance. Additionally, body weight, fat-free mass, thigh mass, and body fat were not significantly enhanced with creatine ethyl ester supplementation compared to placebo or creatine monohydrate groups. Increases in body water were similar to the placebo and creatine monohydrate groups. The vast majority of the improvement seen can be attributed to the training protocol itself, not the supplementation. Creatine ethyl ester supplementation did show a large increase in creatinine levels throughout the study. With no significant increase in total muscle creatine content, it can be concluded that a large portion of the creatine ethyl ester was being degraded within the GI track during ingestion. Furthermore, the skeletal muscle uptake of creatine ethyl ester uptake was not significant enough to increase skeletal muscle creatine levels without significant degradation occurring.

APPENDICES

APPENDIX A

Informed Consent

BAYLOR UNIVERSITY

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

Title of Investigation: The Effects of Different Creatine Supplement Formulations Containing Cinnamon Extract (Cinnulin^{TM}) or Ethyl Ester on Creatine Transporter Expression, Muscle Creatine Uptake, and Whole Body Creatine Retention in Males

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Sponsors: Integrity Nutraceuticals International (Sarasota, FL)

Rationale:

Creatine is a naturally occurring amino acid derivative that is essential in the regulation of muscular energy stores, and it contributes to the generation of ATP (adenosine triphosphate), which is essential for muscular contraction. In the past decade, creatine has been used as a potentially ergogenic supplement, and it has been shown to improve performance in muscular strength and power activities, enhance short bursts of muscular endurance, and allow for greater muscular overload in order to improve training effectiveness. Creatine content of muscle fibers is dependent primarily upon rates of

creatine uptake, and to a lesser extent, creatine retention and the slow degradation of creatine into creatinine. Creatine uptake into the muscle is dependent on the creatine transporter, a membrane-spanning protein that transfers creatine from the blood into the muscle fibers. It is likely that content and activity of the creatine transporter protein is important in controlling intramuscular creatine levels. Intramuscular creatine content may regulate the amount of creatine transporter present in muscle. Creatine transport activity has also been demonstrated to be affected by the sodium concentration across the cell membrane in culture. The regulation of total creatine metabolism within the muscle is still poorly understood; therefore, different formulations of creatine have been established in attempt to improve muscle creatine uptake and biochemically improve the ergogenic effects of creatine.

Creatine ethyl ester is a new formulation and is supposedly a membrane permeable form of creatine that theoretically can enter the cells without having to use the creatine transporter molecules. However, the effectiveness of creatine ethyl ester is unknown, as there are presently no published studies available on creatine ethyl ester. Creatine combined with carbohydrate has been suggested to increase muscle creatine uptake because the creatine transporter is thought to be activated by insulin. Cinnulin^{TM} is a cinnamon extract that has been shown to improve the insulin effect through increases in glucose transport. Therefore, the combination of creatine with cinnulin may increase muscle creatine uptake. However, at this time while there are several published studies on the effects of cinnulin extract, there are no studies combining cinnulin with creatine.

The primary goal of this study is to gain a preliminary understanding of the effects of creatine ethyl ester and creatine+cinnulin supplementation on muscle creatine uptake and whether any corresponding increases in intramuscular creatine levels are related to the activity of the creatine transporter in humans.

Description of the Study:

I will be one of 40 apparently healthy recreationally active males between the ages 18 to 30 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. A trained individual will examine me to determine if I am qualified to participate in this study. If I am cleared to participate in the study, I will be familiarized to the testing procedures. 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 24-hour dietary analysis form that I am to complete the 24 hours 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 five 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 body weight and then randomly assigned to ingest in a double blind manner a supplement containing either a: 1) dextrose placebo; 2) creatine monohydrate; 3) creatine ethyl; or 4) creatine + cinnulin. I will be asked to orally ingest my selected supplement at a relative daily dose of 0.3 g/kg lean body mass (≈ 17-20 g/day) for 5 days in the loading phase and, immediately following the loading phase, a relative daily dose of 0.075 g/kg lean body mass (≈ 5-7 g/day) during the 42-day maintenance phase. I will be instructed not to change my routine dietary intake and to ingest the supplements in 2 equal intervals in the AM and PM of each day during the

loading phase, and at 12:00 pm during the maintenance phase. Compliance to the supplementation protocol will be monitored by having me return the empty supplement container at the end of each week, at which point I will be given the required supplement dosage for the following week. I understand that if I do not take my supplements I will be removed from the study, and I also understand that I will cease supplementation on Day 48 following the 42-day maintenance phase.

I understand that I will be required to participate in a periodized 4-day per week resistance-training program split into two upper and two lower extremity workouts per week for a total of 8-weeks. Prior to the workout, I will perform a standardized series of stretching exercises and then perform an upper body resistance-training program consisting of nine exercises (bench press, lat pull, shoulder press, seated rows, shoulder shrugs, chest flies, biceps curl, triceps press down, and abdominal curls) twice per week and a seven exercise lower extremity program (leg press, back extension, step ups, leg curls, leg extension, heel raises, and abdominal crunches) performed twice per week. I understand that I will perform 3 sets of 10 repetitions with as much weight as I can lift per set (typically 70 – 80% of 1RM), and that I my training may be conducted at the Student Life Center (SLC) at Baylor University or an area gym. However, I understand that I must document my training session in training logs, and the logs must be signed by study personnel to verify compliance and monitor progress.

I understand that I will be required to report to the laboratory on Day 6 (at the end of the 5-day loading phase) to have my heart rate and blood pressure determined, to turn in my 24-day dietary records, to complete a reports of side effects from supplementation questionnaire to determine if I have experienced any unexpected problems or adverse events from participating in this study, have my body composition and muscle strength determined, and to have a muscle and blood sample obtained. 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 understand that following 5-day loading phase, I will immediately begin the 42-day maintenance phase of supplementation. 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 may be removed from the study.

Exclusionary Criteria

I understand that in order to participate in the study, a trained individual will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if: 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.

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 Dr. Darryn Willoughby (254-710-3504). 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 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 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 two used in this study are still relatively new to the market. Therefore, the potential medical benefits of these two creatine supplement formulations are 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. 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, creatine ethyl ester, or creatine + cinnulin 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 $200 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 this study was partially funded by Integrity Nutraceuticals International. (Sarasota, FL). 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 Darryn Willoughby, Ph.D. (principal investigator) or Ryan Schoch, B.S. (Masters Level Research Assistant, Department of Health, Human Performance & Recreation, 117 Marrs McLean Gymnasium, Baylor University, phone: 254-710-4011) 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 B

IRB Proposal

Part 1: Signature Page

1. Name Darryn S. Willoughby, Ph.D., FACSM, FISSN, CSCS, CISSN
2. Email Address (optional) Darryn_Willoughby@baylor.edu
3. Complete Mailing Address P.O. Box 97313
4. Position Associate Professor
5. Faculty Advisor (if researcher is Graduate Student) _____
6. Department/School HHPR/SOE & Biomedical Science Institute
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 Different Creatine Supplement Formulations Containing Cinnamon Extract (Cinnulin^{TM}) or Ethyl Ester on Creatine Transporter Expression, Muscle Creatine Uptake, and Whole Body Creatine Retention in 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 Subjects 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 subjects in research, contact Dr. Stanford at extension 2961.

Signature of Principal Investigator

06/27/06
Date

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

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

Part 2: Introduction & Rationale

Creatine is a naturally occurring amino acid derivative that is essential in the regulation of muscular energy stores, and it contributes to the generation of ATP (adenosine triphosphate), which is essential for muscular contraction (1). In the past decade, creatine has been used as a potentially ergogenic supplement, and it has been shown to improve performance in muscular strength and power activities, enhance short bursts of muscular endurance, and allow for greater muscular overload in order to improve training effectiveness (2-5). Generally, a creatine supplementation protocol includes a 3 to 7 day loading phase of 4 to 5 grams each day, which has been demonstrated to significantly increase intramuscular creatine and phosphocreatine stores (4,6). Immediately following the loading phase is a maintenance phase with a daily dose of 3-5 grams for approximately 4-6 weeks. In humans, the increase of intramuscular creatine content following supplementation shows a considerable amount of variability, if an increase is induced at all (7).

Creatine content of muscle fibers is dependent primarily upon rates of creatine uptake, and to a lesser extent, creatine retention and the slow degradation of creatine into creatinine. Creatine uptake into the muscle is dependent on the creatine transporter, a membrane-spanning protein that transfers creatine from the blood into the muscle fibers. It is likely that content and activity of the creatine transporter protein is important in controlling intramuscular creatine levels (8). It is evident that control of the creatine transporter is important in the regulation of intramuscular creatine content.

Intramuscular creatine content may regulate the amount of creatine transporter present in muscle. Creatine transport activity has also been demonstrated to be affected by the sodium concentration across the cell membrane in culture (9). The regulation of total creatine metabolism within the muscle is still poorly understood; therefore, different formulations of creatine have been established in attempt to improve muscle creatine uptake and biochemically improve the ergogenic effects of creatine.

Creatine ethyl ester (CEE) is a new formulation and is creatine monohydrate with an ester attached. Esters are organic compounds that are formed by esterification, the reaction of carboxylic acid and alcohols. CEE is a supposedly a membrane permeable form of creatine that theoretically can enter the cells without having to use the creatine transporter molecules. There seems to be a limit for muscle creatine uptake when the transport system is down-regulated below a concentration of 150 mmol/l. So in this regard, whether the muscle creatine uptake resulting from CEE is any higher than those achievable with creatine monohydrate based on a 30-day period of supplementation (at five grams per day) is unknown, as there are presently no published studies available on CEE.

Creatine combined with carbohydrate has been suggested to increase muscle creatine uptake because the creatine transporter is thought to be activated by a similar tyrosine kinase- related mechanism that activates the insulin receptor. Since carbohydrate increases the levels of insulin in the blood with subsequent binding of the insulin receptor, as the insulin receptor is activated the creatine transporter may be concomitantly activated. Cinnulin^{TM} is a cinnamon extract that has been shown to potentiate the insulin effect and subsequent insulin signaling through up-regulation of glucose transport (10). Therefore, the combination of creatine with cinnulin may exacerbate muscle creatine uptake by stimulating skeletal muscle mediated glucose uptake and concomitant up-regulation in the creatine transporter. However, at this time while there are several published studies on the effects of cinnulin extract, there are no studies combining cinnulin with creatine.

The primary goal of this study is to gain a preliminary understanding of the effects of creatine ethyl ester (CEE) and creatine+cinnulin (CCI) supplementation, compared to creatine monohydrate (CM) and placebo, on muscle creatine uptake and whole body creatine retention, and whether any corresponding increases in intramuscular creatine levels are related to the activity of the creatine transporter in humans.

Part 3: Methodology

Methods

Subjects

Forty 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 18-30 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 by the laboratory nurse. All eligible subjects will sign university-approved informed consent documents and approval will be granted by the Institutional Review Board for Human Subjects. 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 creatine supplements and the placebo that will be used for the control groups. Dependent variables will include urinary creatine and creatinine, serum creatine and creatine, intramuscular free creatine, total creatine, and phosphocreatine, intramuscular creatine transporter mRNA and protein expression, and whole body creatine retention.

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. Once reporting to the lab, participants will complete a medical history questionnaire and undergo a general physical examination to determine whether they meet eligibility criteria. Participants 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 24 hours prior to each of the five testing sessions occurring: 1) prior to the first dose of supplement (Day 0); 2) on

Day 6, after the 5-day loading phase; 3) on Day 8, after the first 7 days of the maintenance phase; 4) on Day 29, after the 28-day maintenance phase; and 5) on Day 29, after a 28-day washout period following the 4-week maintenance phase.

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 five muscle samples will be obtained: 1) prior to the first dose of supplement (Day 0); 2) on day 6, after the 5-day loading (which will also serve as the pre-maintenance phase biopsy); 3) on Day 15, after the first 14 days of the maintenance phase; 4) on day 29, after the 28-day maintenance phase; and 5) on day 29, after a 28-day washout period following the 4-week maintenance phase.

Venous blood samples will be obtained from the antecubital vein into a 10 ml collection tubes 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 five blood samples will be obtained: 1) prior to the first dose of supplement (Day 0); 2) on day 6, after the 5-day loading (which will also serve as the pre-maintenance phase blood sample); 3) on Day 15, after the first 14 days of the maintenance phase; 4) on day 29, after the 28-day maintenance phase; and 5) on day 29, after a 28-day washout period following the 4-week maintenance phase.

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 proposed creatine supplementation regimen on general markers of tissue degradation, immune function, and clinical safety.

Using a colorimetric, spectrophotometeric assay, the serum and urinary levels of creatine will be assessed. Using the same assay, the skeletal muscle levels of creatine, total creatine, and phosphocreatine will also be assessed. Using immuno (Western) blotting, skeletal muscle creatine transporter protein expression will be assessed. Creatine transporter gene expression will be determined by way of the real-time quantitative polymerase chain reaction procedure. These assays will help evaluate the potential effectiveness of different creatine supplement formulations containing either CM, CEE, or CCI on muscle creatine uptake, whole body creatine retention, and creatine transporter activity.

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 of the five testing sessions when blood and muscle samples are obtained, and these time points are: 1) prior to the first dose of supplement (Day 0); 2) on day 6, after the 5-day loading (which will also serve as the pre-maintenance phase blood sample); 3) on Day 15, after the first 14 days of the maintenance phase; 4) on day 29, after the 28-day maintenance phase; and 5) on day 29, after a 28-day washout period following the 4-week maintenance phase.

Dietary Analysis

Subjects will be required to record their dietary intake for 24 hours prior to each of the five testing sessions where blood and muscle samples are obtained. The participants' diets will not be standardized and subjects will be asked not to change their dietary habits during the course of the study. The 24-hour 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

Subjects will be randomly assigned to ingest in a double blind manner capsules containing: 1) dextrose placebo; 2) creatine monohydrate (CM); 3) creatine+cinnulin (CCI); or 4) creatine ethyl ester (CEE). After baseline testing procedures and lean body mass determination via DEXA, subjects will ingest creatine or the placebo at a relative daily dose of 0.30 g/kg fat free body mass (≈ 20 g/day) for 5 days in the loading phase and, immediately following the loading phase, a relative daily dose of 0.075 g/kg fat free mass (≈ 5 g/day) during the 28-day maintenance phase. The CCI supplement will include 250 mg of cinnulin per each 5 gm creatine dose. Therefore, for the loading phase the CCI group will ingest 20 g of creatine and 1 g of cinnulin/day and 5 g of creatine and 250 mg of cinnulin/day during the maintenance phase. All groups will cease supplementation on Day 29 following the 28-day maintenance phase.

In order to standardize supplement intake throughout the study, participants will be instructed to ingest the supplements in equal intervals at 8:00 am, 12:00 pm, 4:00 pm, and 8:00 pm each day during the loading phase, and at 12:00 pm during the maintenance phase. Dextrose and all creatine formulation powders will be comprised of similar mesh size, texture, taste, and appearance and will be prepared and blinded by an objective third-party. Compliance to the supplementation protocol will be monitored by having the participants return empty supplement containers at the end of each week. In addition, participant's compliance will be verified by weekly verbal communication. After completing the compliance procedures the subjects will be given the required supplement dosage for the following week.

Training Protocol

Subjects will participate in a periodized 4-day per week resistance-training program split into two upper and two lower extremity workouts per week for a total of 8-weeks. Prior to the workout, subjects will perform a standardized series of stretching exercises. The subjects will then perform an upper body resistance-training program consisting of nine exercises (bench press, lat pull, shoulder press, seated rows, shoulder shrugs, chest flies, biceps curl, triceps press down, and abdominal curls) twice per week and a seven exercise lower extremity program (leg press or squat, back extension, step ups, leg curls, leg extension, heel raises, and abdominal crunches)

performed twice per week. Subjects will perform 3 sets of 10 repetitions with as much weight as they can lift per set (typically 70 – 80% of 1RM). Rest periods between exercises will last no longer than 3 minutes and rest between sets will last no longer than 2 minutes. Training will be conducted at the Student Life Center (SLC) at Baylor University or an area gym, documented in training logs, and signed off to verify compliance and monitor progress.

Urine

In order to determine the effects of the supplements on urinary creatine and creatinine levels and whole body creatine retention, participants will be asked to collect a total of nine 24-hour urine samples throughout the course of the study. Each participant will be provided with 3 L urine collection containers in order to collect 24-hour urine samples on Day 0 prior to the 5-day loading phase, and then also on Days 3 and 5 (samples 1-3) of the loading phase. Participants will then also collect 24-hour urine samples on Days 7, 14, 21, 28 during the 28-day maintenance phase (samples 4-7), and also on Days 14 and 28 (samples 8, 9) of the 28-day washout period. The 24-hour urine sample time will be initiated at 8 am the day before testing is scheduled. Participants will be asked to record the number of times they urinated each day as well as total fluid intake. Participants will be asked to refrigerate their urine samples during the 24-hour time period, after which they will submit the urine samples when reporting to the ESNL for testing.

Strength Assessment

In order to determine possible effects of the supplements on muscular strength, participants will perform four one-repetition maximum (1-RM) tests on the leg press sled: 1) at baseline (Day 0); 2) on Day 6 after the 5-day loading phase; and 3) on Day 15, after the first 14 days of the maintenance phase; and 4) on Day 29 after the 28-day maintenance phase. Subjects will warm up by completing 5 to 10 repetitions at approximately 50% of the estimated 1-RM. The subject 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 subject will attempt to lift the weight for one repetition. If the lift is successful, the subject will rest for 2 minutes before attempting the next weight increment. This procedure will be continued until the subject fails to complete the lift. The 1-RM will be recorded as the maximum weight that the subject is able to lift for one repetition.

Creatine Analysis

Urine, serum, and muscle tissue samples will be analyzed spectrophotometrically for total creatine, free creatine, and phosphocreatine by the diacetyl/α-napthtol reaction. Urine creatine levels will be determined from each of the nine urine samples provided while serum and muscle creatine levels will be determined from the five blood and muscle samples provided. Additionally, from each muscle sample obtained, free creatine and phosphocreatine will also be determined.

Creatine Transporter mRNA Expression

Muscle tissue samples will be analyzed for creatine transporter mRNA transcription levels by quantitative real-time polymerase chain reaction (RT-PCR). Samples will then be run on an iCycler iQ RT-PCR system (Bio-Rad, Hercules, CA) along with a standard curve of known concentrations of human skeletal muscle cDNA to determine transcription levels of the creatine transporter gene. Creatine transporter mRNA expression will be determined from each of the five muscle samples obtained.

Creatine Transporter and Insulin Signaling Pathway Protein Expression
From the five muscle tissue samples obtained, expression of creatine transporter protein and proteins of the insulin signaling pathway (IGF-R, IRS-1, Akt/protein kinase B, mTOR, p70S6kinse, and GSK-3) using enzyme linked immunoabsorbent assay (ELISA). The expression of eIF4E and 4EBP-1 will be determined by immuno (Western) blot analysis.

Assessment of Hemodynamic Safety Markers (Heart Rate & Blood Pressure). At each of the five testing sessions where blood and muscle samples are obtained: 1) prior to the first dose of supplement (Day 0); 2) on day 6, after the 5-day loading (which will also serve as the pre-maintenance phase blood sample); 3) on Day 15, after the first 14 days of the maintenance phase; 4) on day 29, after the 28-day maintenance phase; and 5) on day 29, after a 28-day washout period following the 4-week maintenance phase, participants will undergo assessment of heart rate and blood pressure. Heart rate will be determined by palpitation of the radial artery using standard procedures. Blood pressure will be assessed in the supine position after resting for 5-min using a mercurial sphygmomanometer using standard procedures.

Reported Side Effects from Supplements
At the first four testing sessions where blood and muscle samples are obtained and participants are ingesting supplement, 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. Participants will not be required to complete the questionnaire at the end of the 28-day wash-out period.

Research Team

Darryn S. Willoughby, PhD, FACSM, FISSN, CSCS, CISSN. 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. He has conducted a vast amount of research focusing on the biochemical and molecular regulatory mechanisms regarding exercise performance and nutrition. Dr. Willoughby will be the principal supervisor of the project. He will perform all of the muscle biopsies, and oversee all aspects of the study and perform the majority 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 serve as a co-investigator for the study and will assist in the collection and analysis of data.

Richard B. Kreider, PhD, EPC, FACSM. Dr. Kreider is Professor and Chair of the Department of Health, Human Performance, & Recreation at Baylor University. Dr. Kreider is an internationally recognized exercise scientist and has conducted a vast amount of research primarily focusing on the role of exercise and nutrition and health and performance. Dr. Kreider will serve as a consultant and assist in providing administrative oversight for the study.

Ryan Schoch, B.S. Mr. Schoch is a graduate student in the Exercise Physiology Masters Degree program in the Department of Health, Human Performance, & Recreation at Baylor University. Mr. Schoch will be using the data generated during this study for his Master's thesis. He will recruit and familiarize subjects, administer the supplements, collect urine, perform the blood draws, assist with the muscle biopsies, and assist in the majority of the biochemical and clinical chemistry assays involved in the project.

Lisa Champ, B.S. Ms. Champ is a graduate student in the Exercise Physiology Masters Degree program in the Department of Health, Human Performance, & Recreation at Baylor University. She will assist in all areas involved in the project.

Melyn Galbreath, MSN, RN. Ms. Galbreath is a nurse practitioner who serves as the laboratory nurse and is also pursuing her Ph.D. in Exercise, Nutrition, and Preventative Health. She will assess weekly hemodynamic safety measurements, review the reported side effects from supplement questionnaire, and be involved in 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).

Procedures

Medical Monitoring. Interested participants will be invited to familiarization sessions. During this time, participants will sign consent forms and complete medical history information. Participants will then undergo a general exam by the research nurse to determine whether the subject meets entry criteria to participate in the study. This exam will include evaluating the medical and training history questionnaires and performing a general physical examination according to ACSM exercise testing guidelines. Based on this examination, participants will be assessed for their risk of cardiovascular disease and contraindications to exercise and then a recommendation will be made on whether the participant meets entry criteria and may therefore participate in the study. Trained, non-physician exercise specialists certified in CPR will supervise participants undergoing testing and assessments. A telephone is in the laboratory in case of any emergencies, and there will be no less than two researchers working with each participant during testing sessions. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance. Participants will be informed to report any unexpected problems or adverse events they may encounter during the course of the study to Darryn S. Willoughby, Ph.D. or Melyn Galbreath, RN. If clinically significant side effects are reported, the participants will be referred to discuss the problem with the laboratory nurse, and if deemed necessary Ms. Galbreath 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 each of the five testing sessions where blood and muscle samples are obtained, participants will undergo assessment of heart rate and blood pressure. Heart rate will be determined by palpitation of the radial artery using standard procedures. Blood pressure will be assessed in the supine position after resting for 5-min using a mercurial sphygmomanometer using standard procedures.

Reported Side Effects from Supplement Questionnaires. At the first four testing sessions where blood and muscle samples are obtained and participants are ingesting supplement, 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. Participants will not be required to complete the questionnaire at the end of the 28-day wash-out period.

Estimated Energy Intake/Dietary Inventories. For 24 hours prior to each of the five testing sessions where blood and muscle samples are obtained, 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 five testing sessions. Dietary intake will be assessed using the Food Processor IV Nutrition Software.

Body Composition Assessments. 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 subject 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. 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 subjects performing one repetition maximum (1 RM) on the isotonic Nebula hip/leg sled. Subjects will warm-up (2 sets of 8 – 10 repetitions at approximately 50% of anticipated maximum) on the hip/leg sled. Subjects will then perform successive 1 RM lifts starting at about 70% of anticipated 1RM and increasing by 10 – 20 lbs until the subject reaches their 1RM.

Creatine Analysis. Urine, blood serum, and muscle tissue samples will be analyzed for total creatine, free creatine, and phosphocreatine by the diacetyl/α-napthtol reaction. Unmodified urine and serum samples are ready for analysis, while muscle tissue must be prepared. Muscle tissue will be homogenized in a 0.5 M perchloric acid/1 mM EDTA solution, spun in a centrifuge at 10,000 rpm for 2 minutes, and the supernatant will neutralized with 2.1 M potassium bicarbonate/0.3 M MOPS solution, at which point the supernatant is ready for analysis. Total creatine of samples will be determined by reaction with 6.94 mM α-napthtol and a 1:2500 dilution of diacetyl. The reaction will incubate for 40 minutes at room temperature in the dark, and color formation will be detected by spectrophotometer at 520 nm. The samples will be run against a standard curve of known creatine concentrations. Free creatine will be determined by combining homogenate with 0.4 N hydrochloric acid and heating at 65˚C for 10 minutes. The homogenate will then be neutralized with 2.0 N sodium hydroxide, and subsequently subjected to the diacetyl/α-napthtol reaction as previously described. Phosphocreatine concentration may be calculated as free creatine concentration subtracted from total creatine concentration.

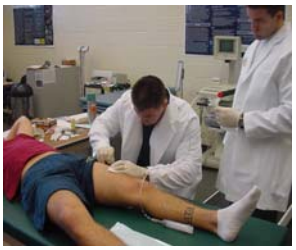
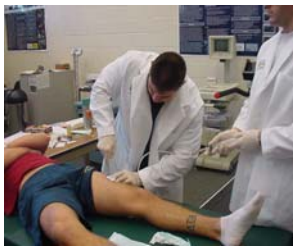
Creatine Transporter mRNA Expression. Muscle tissue samples will be analyzed for creatine transporter mRNA transcription levels by quantitative real-time polymerase chain reaction (RT-PCR). The tissue will be homogenized with Tri Reagent (Sigma, St. Louis, MO) and total RNA will be isolated by isopropanol/ethanol extraction. Total RNA will then be used to generate cDNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), which will be used as a template in the RT-PCR reaction. Primers will be designed according to the human creatine transporter gene sequence as published by the National Center for Biotechnology Information. Samples will then be run on an iCycler iQ RT-PCR system (Bio-Rad, Hercules, CA) along with a standard curve of known concentrations of human skeletal muscle cDNA to determine transcription levels of the creatine transporter gene.

Creatine Transporter Protein Expression. Muscle tissue samples will be analyzed for creatine transporter expression by Western blot analysis. 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) and then transferred to a nitrocellulose membrane. The membrane will be incubated with a commercially available primary antibody designed to bind to human creatine transporter. The membrane 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. Blotted membranes will be digitized by densitometry using a Chemi-Doc XRS imaging system (Bio-Rad, Hercules, CA).

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., Melyn Galbreath, Ph.D., or laboratory technician's trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. Up to this point in his professional career, Dr. Willoughby has successfully performed several thousand blood Draws without any complications. The phlebotomists and lab technicians will wear personal protective clothing gloves, lab coats, etc.) when handling blood samples. Subjects will be seated in a phlebotomy chair. Their arm will be cleaned with a sterile alcohol wipe and sterile gauze. A standard rubber tourniquet will then be placed on the brachium. An antecubital vein will be palpated and then a 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 subject 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 subjects. 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 subject 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.

Equipment

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

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 leg extension (Body Masters, Inc., Rayne, LA). Equipment and testing will be contained within the EBNL.

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 Protein Analyses. Blood samples will also be used to assess hormone profiles photometrically using either enzyme-linked immunoabsorbent assays (ELISA) 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 □avelength against a known standard curve.

Muscle Protein Analyses. Muscle protein samples will be separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Criterion Blotter (Bio-Rad, Hercules, CA) and then transferred to a nitrocellulose membrane where they will be incubated with the appropriate antibodies to carry out the immunoblotting procedure. Blotted membranes will be digitized by way of densitometry using a Chemi-Doc XRS imaging system (Bio-Rad, Hercules, CA). Muscle tissue samples will also be analyzed for creatine transporter mRNA transcription levels by quantitative real-time polymerase chain reaction (RT-PCR). Samples will then be run on an iCycler iQ RT-PCR system (Bio-Rad, Hercules, CA) along with a standard curve of known concentrations of human skeletal muscle cDNA to determine transcription levels of the creatine transporter gene. Creatine transporter mRNA expression will be determined from each of the five muscle samples obtained.

Participants

Recruitment

Forty non-resistance-trained (no regular, consistent resistance training for at least one year) male participants between the ages 18 to 30 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/subjects/) and sent via campus mail is attached.

Selection Criteria

Participants will not be allowed to participate in the second 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, or anemia;
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., creatine, 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 and unused supplements) in the study will be paid $200. Subjects may receive information regarding results of these tests if they desire. If subjects are Baylor students, they will not receive any academic credit for participating in this study.

Potential Risks

Participants who meet eligibility criteria will be exposed to a very low level of electrical current that will be passed through each subject'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.

Creatine monohydrate 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 CEE and CCI creatine 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 subjected 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. 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 five times during the study using standard phlebotomy procedures. This procedure may cause a small amount of pain when the needle is inserted into the vein as well as some bleeding and bruising. The subject may also experience some dizziness, nausea, and/or faint if they are unaccustomed to having blood drawn.

Complications resulting from the muscle biopsy are rare, especially in this case where the biopsy is similar to receiving a routine intramuscular injection. As with the blood draw, however, there is a risk of infection if the subject does not adequately cleanse the area for approximately 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 subjects will be asked if they have known allergies to local anesthetics (e.g. Lidocaine, Xylocaine, etc.) that they may have been previously given during dental or hospital visits. Participants with known allergies to anesthesia medications will not be allowed to participate in the study. Darryn Willoughby, 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 subjects 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 subject during testing. In the event of any unlikely emergency one researcher will check for vital signs and begin any necessary interventions while the other researcher contacts Baylor's campus police at extension 2222. Instructions for emergencies are posted above the phone in the event that any other research investigators are available for assistance.

Potential Benefits

The main benefit that participants may obtain from this study is that if these creatine nutritional supplements are effective there is a possibility that they may gain insight into how to possibly enhance muscle creatine uptake with supplementation that typically occurs in conjunction with resistance training as well as improved health profiles. 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. This information will be helpful to athletes and non-athletes alike who use nutritional creatine supplements during training with the intent of improving creatine uptake and subsequent muscular performance to know whether they are effective or not.

Assessment of Risk

Even though clinical data are available outlining the safety effects of many creatine supplements, because they are still relatively new to the market the potential medical benefits of the different creatine supplement formulations are not yet well delineated. Although, creatine is available in a number of over the counter nutritional supplements, initial results suggest that these supplements may provide benefit at increasing muscle creatine 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 CCI or CEE may effectively increase muscle creatine levels for active 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 subjects participating in this study outweigh the potential risks.

Compensation for Illness or Injury

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

Confidentiality

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

Data Presentation & Publication

Data will be presented at an appropriate scientific conference (e.g., American 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 Integrity Nutraceuticals International. (Sarasota, FL). Researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

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APPENDIX C

Muscle Biopsy Wound Care

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

For approximately 24 hours post biopsy

- leave the bandage(s) on for 24 hours (unless unexpected bleeding or pain occurs, which should be immediately reported to the lab)
- lightly clean around the bandage(s) if necessary
- report back to the lab within 24 hours to have the old bandage(s) removed, the incision inspected and new bandages applied
- refrain from vigorous physical activity with the leg during the first 24 hours post-biopsy

After the 24-hour followup, (for approximately 72 hours post biopsy)

- leave the butterfly bandage in place
- adequately cleanse the area surrounding the bandage with soap and water every 4-6 hours, and pat the area dry
- reapply a fresh adhesive bandage

At approximately 72 hours post biopsy

- return to the lab
- allow the incision sight to be inspected, butterfly removed, and new bandages applied
- leave these bandages on for 24 hours (unless unexpected bleeding or pain occurs)
- return to normal hygiene practices unless complications arise

Possible pain & side effects

Soreness of the area comparable to that of a bruise will likely persist for 24 hours, and possibly even 36 hours, after the biopsy procedure. Following the procedures outlined above should significantly minimize pain and possible bleeding of the area. However, some subjects experience no significant pain post biopsy.

☎🗎🗄🗐✆ If needed, the subject may take non-prescription analgesic medication such as Ibuprofen to relieve pain if needed.

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

If any questions or complications arise please contact:

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

or

Melyn Galbreath, R.N.
Room # 122 Marrs McLean Gym
(254) 710-7277 or 710-7199

APPEDIX D

TABLE D.1

Serum Clinical Chemistry

Variable	1 = PLA 2 = CR 3 = CEE	Testing Session	Mean	Standard Deviation
Triglycerides (mg/dL)	1	1	68.900	32.185
		2	77.400	40.656
		3	63.700	28.930
		4	84.800	50.820
		Total	73.700	38.148
	2	1	119.400	86.845
		2	95.800	40.830
		3	116.700	105.016
		4	87.100	24.433
		Total	104.750	64.281
	3	1	82.300	36.676
		2	76.900	23.402
		3	94.000	24.381
		4	68.700	22.978
		Total	80.475	26.859
	Total	1	90.200	64.200
		2	83.367	35.781
		3	91.467	65.988
		4	80.200	34.928
		Total	86.309	50.224
Cholesterol (mg/dL)	1	1	143.200	27.944
		2	137.100	22.566
		3	137.200	26.549
		4	150.000	27.471
		Total	141.875	26.133
	2	1	162.100	37.433
		2	168.300	35.260
		3	170.100	29.835
		4	173.400	36.075
		Total	168.475	34.651
	3	1	136.500	36.978
		2	139.800	30.517
		3	136.500	29.647
		4	149.200	23.323
		Total	140.500	30.116

(table continues)

Variable	1 = PLA 2 = CR 3 = CEE	Testing Session	Mean	Standard Deviation
	Total	1	147.267	34.973
		2	148.400	32.233
		3	147.933	31.969
		4	157.533	30.614
		Total	150.283	32.447
HDL (mg/dL)	1	1	48.900	10.376
		2	47.700	7.304
		3	46.900	9.267
		4	48.000	8.380
		Total	47.875	8.832
	2	1	47.300	12.500
		2	46.700	5.945
		3	45.500	5.911
		4	50.500	8.423
		Total	47.500	8.195
	3	1	44.500	7.091
		2	45.600	5.562
		3	46.400	5.190
		4	46.200	7.569
		Total	45.675	6.353
	Total	1	46.900	10.046
		2	46.667	6.155
		3	46.267	6.797
		4	48.233	8.050
		Total	47.017	7.762
LDL (mg/dL)	1	1	85.100	26.963
		2	79.700	25.855
		3	83.500	27.593
		4	93.300	29.594
		Total	85.400	27.501
	2	1	101.800	30.579
		2	116.800	30.622
		3	109.100	29.872
		4	110.700	31.209
		Total	109.600	30.571
	3	1	79.300	35.537
		2	82.400	32.449
		3	81.700	29.982
		4	92.900	28.333
		Total	84.075	31.575

(table continues)

Variable	1 = PLA 2 = CR 3 = CEE	Testing Session	Mean	Standard Deviation
	Total	1	88.733	31.652
		2	89.633	31.288
		3	91.433	30.890
		4	98.967	29.907
		Total	92.192	30.934
Gamma-Glutamyl Transferase (U/L)	1	1	32.700	7.790
		2	31.900	6.385
		3	33.900	11.836
		4	37.200	11.526
		Total	33.925	9.384
	2	1	38.625	18.353
		2	38.500	15.492
		3	37.750	14.008
		4	37.875	15.995
		Total	38.188	15.962
	3	1	31.000	11.165
		2	30.300	5.756
		3	29.200	6.408
		4	28.200	4.211
		Total	29.675	6.885
	Total	1	33.786	12.624
		2	33.214	9.946
		3	33.321	11.112
		4	34.179	11.713
		Total	33.625	11.349
Lactic Dehydrogenase (U/L)	1	1	110.200	17.866
		2	110.600	17.009
		3	116.800	28.030
		4	118.000	8.031
		Total	113.900	17.734
	2	1	155.429	48.425
		2	135.714	30.429
		3	129.857	21.965
		4	130.571	22.861
		Total	137.893	30.920
	3	1	112.500	20.206
		2	115.875	18.826
		3	116.000	18.315
		4	128.375	22.532
		Total	118.188	19.970

(table continues)

Variable	1 = PLA 2 = CR 3 = CEE	Testing Session	Mean	Standard Deviation
	Total	1	126.950	37.665
		2	121.500	24.554
		3	121.050	22.032
		4	126.550	19.806
		Total	124.013	26.014
Uric Acid (mg/dL)	1	1	5.660	1.111
		2	5.860	0.873
		3	5.390	0.796
		4	5.830	1.124
		Total	5.685	0.976
	2	1	6.163	2.313
		2	5.963	1.633
		3	6.250	1.672
		4	6.200	1.585
		Total	6.144	1.801
	3	1	5.250	1.074
		2	5.600	0.982
		3	5.330	0.663
		4	6.250	1.448
		Total	5.608	1.042
	Total	1	5.657	1.523
		2	5.796	1.136
		3	5.614	1.118
		4	6.086	1.345
		Total	5.788	1.281
Glucose (mg/dL)	1	1	88.200	6.828
		2	83.300	9.866
		3	87.900	5.705
		4	91.000	3.742
		Total	87.600	6.535
	2	1	88.200	10.444
		2	87.700	4.668
		3	89.100	6.402
		4	88.900	6.691
		Total	88.475	7.051
	3	1	82.800	14.078
		2	84.200	6.070
		3	83.900	5.507
		4	86.200	5.391
		Total	84.275	7.761

(table continues)

Variable	1 = PLA 2 = CR 3 = CEE	Testing Session	Mean	Standard Deviation
	Total	1	86.400	10.795
		2	85.067	7.220
		3	86.967	6.111
		4	88.700	5.590
		Total	86.783	7.429
Blood Urea Nitrogen (mg/dL)	1	1	14.700	4.029
		2	16.200	3.736
		3	15.600	3.777
		4	16.500	2.953
		Total	15.750	3.624
	2	1	12.875	2.800
		2	14.875	4.612
		3	15.500	6.071
		4	14.875	2.295
		Total	14.531	3.945
	3	1	15.600	3.658
		2	15.000	4.497
		3	16.100	4.433
		4	15.900	3.957
		Total	15.650	4.136
	Total	1	14.500	3.626
		2	15.393	4.157
		3	15.750	4.576
		4	15.821	3.151
		Total	15.366	3.878
Creatinine (mg/dL)	1	1	1.030	0.116
		2	1.040	0.126
		3	1.020	0.140
		4	1.070	0.134
		Total	1.040	0.129
	2	1	0.950	0.169
		2	1.000	0.359
		3	1.163	0.160
		4	1.138	0.141
		Total	1.063	0.207
	3	1	0.950	0.184
		2	2.680	1.529
		3	1.540	0.802
		4	1.750	0.890
		Total	1.730	0.851

(table continues)

Variable	1 = PLA 2 = CR 3 = CEE	Testing Session	Mean	Standard Deviation
	Total	1	0.979	0.157
		2	1.614	1.213
		3	1.246	0.529
		4	1.332	0.613
		Total	1.293	0.628
BUN/Creatinine Ratio	1	1	14.260	3.623
		2	15.780	4.293
		3	15.340	3.395
		4	15.630	3.496
		Total	15.253	3.702
	2	1	13.763	2.951
		2	19.725	16.848
		3	13.725	6.375
		4	13.363	3.251
		Total	15.144	7.356
	3	1	17.110	5.308
		2	7.180	4.205
		3	11.970	5.107
		4	11.340	6.217
		Total	11.900	5.209
	Total	1	15.136	4.279
		2	13.836	10.664
		3	13.675	5.018
		4	13.450	4.807
		Total	14.024	6.192
Calcium (mg/dL)	1	1	9.360	0.470
		2	9.320	0.225
		3	9.230	0.510
		4	9.500	0.400
		Total	9.353	0.401
	2	1	8.730	1.022
		2	9.160	0.337
		3	9.030	0.474
		4	9.100	0.457
		Total	9.005	0.573
	3	1	8.830	1.189
		2	9.010	0.407
		3	8.890	0.420
		4	8.980	0.253
		Total	8.928	0.567

(table continues)

Variable	1 = PLA 2 = CR 3 = CEE	Testing Session	Mean	Standard Deviation
	Total	1	8.973	0.954
		2	9.163	0.345
		3	9.050	0.475
		4	9.193	0.431
		Total	9.095	0.551
Total Protein (g/dL)	1	1	7.550	0.462
		2	7.460	0.398
		3	7.340	0.479
		4	7.630	0.254
		Total	7.495	0.398
	2	1	7.070	0.938
		2	7.230	0.455
		3	7.110	0.463
		4	7.330	0.377
		Total	7.185	0.558
	3	1	6.900	1.031
		2	7.010	0.425
		3	6.800	0.302
		4	6.900	0.320
		Total	6.903	0.519
	Total	1	7.173	0.865
		2	7.233	0.452
		3	7.083	0.465
		4	7.287	0.434
		Total	7.194	0.554
Albumin (g/dL)	1	1	4.720	0.301
		2	4.610	0.110
		3	4.550	0.295
		4	4.740	0.212
		Total	4.655	0.230
	2	1	4.300	0.600
		2	4.410	0.213
		3	4.310	0.260
		4	4.520	0.437
		Total	4.385	0.377
	3	1	4.290	0.642
		2	4.380	0.257
		3	4.270	0.323
		4	4.320	0.297
		Total	4.315	0.380

(table continues)

Variable	1 = PLA 2 = CR 3 = CEE	Testing Session	Mean	Standard Deviation
	Total	1	4.437	0.556
		2	4.467	0.222
		3	4.377	0.310
		4	4.527	0.362
		Total	4.452	0.363
Total Bilirubin (mg/dL)	1	1	0.710	0.644
		2	0.670	0.663
		3	0.590	0.409
		4	0.630	0.533
		Total	0.650	0.563
	2	1	0.350	0.158
		2	0.470	0.206
		3	0.420	0.193
		4	0.510	0.197
		Total	0.438	0.189
	3	1	0.450	0.217
		2	0.480	0.210
		3	0.460	0.196
		4	0.500	0.226
		Total	0.473	0.212
	Total	1	0.503	0.418
		2	0.540	0.415
		3	0.490	0.284
		4	0.547	0.346
		Total	0.520	0.366
Alkaline Phosphatase (U/L)	1	1	69.200	22.360
		2	68.400	19.256
		3	70.600	18.787
		4	75.700	17.720
		Total	70.975	19.531
	2	1	60.125	17.635
		2	66.750	19.256
		3	65.875	13.851
		4	70.250	13.382
		Total	65.750	16.031
	3	1	76.400	16.768
		2	79.900	11.010
		3	78.900	16.079
		4	79.500	15.501
		Total	78.675	14.840

(table continues)

Variable	1 = PLA 2 = CR 3 = CEE	Testing Session	Mean	Standard Deviation
	Total	1	69.179	19.611
		2	72.036	16.781
		3	72.214	16.822
		4	75.500	15.662
		Total	72.232	17.219
Aspartate Aminotransferase (U/L)	1	1	35.100	45.140
		2	62.600	74.998
		3	22.500	7.230
		4	24.600	9.766
		Total	36.200	34.284
	2	1	25.250	10.498
		2	150.625	293.196
		3	37.125	16.856
		4	27.500	8.281
		Total	60.125	82.208
	3	1	34.400	39.475
		2	32.800	25.914
		3	28.000	16.207
		4	24.900	11.160
		Total	30.025	23.189
	Total	1	32.036	35.304
		2	77.107	163.683
		3	28.643	14.632
		4	25.536	9.628
		Total	40.831	55.812
Alanine Aminotransferase (U/L)	1	1	27.800	4.590
		2	29.000	7.972
		3	27.700	6.816
		4	31.100	14.059
		Total	28.900	8.359
	2	1	28.125	4.422
		2	37.000	19.435
		3	30.500	4.660
		4	28.250	4.097
		Total	30.969	8.154
	3	1	36.400	26.252
		2	35.700	26.895
		3	31.800	10.779
		4	29.700	4.057
		Total	33.400	16.996

(table continues)

Variable	1 = PLA 2 = CR 3 = CEE	Testing Session	Mean	Standard Deviation
	Total	1	30.964	16.089
		2	33.679	19.316
		3	29.964	7.942
		4	29.786	8.779
		Total	31.098	13.032
Total CK (kat/L)	1	1	92.000	80.579
		2	127.200	99.417
		3	117.200	59.964
		4	118.600	74.225
		Total	113.750	78.546
	2	1	150.429	122.767
		2	439.714	286.630
		3	306.571	155.774
		4	305.000	197.342
		Total	300.429	190.628
	3	1	133.750	82.441
		2	247.625	169.627
		3	183.375	143.933
		4	223.375	227.361
		Total	197.031	155.841
	Total	1	129.150	95.756
		2	284.750	233.635
		3	209.950	148.503
		4	225.750	194.526
		Total	212.400	168.105

APPENDIX E

Adverse Reactions to Supplementation Form

Reported Side Effects From Supplement Questionnaire Follow-up Assessment

Subject Name: _____ Subject #: _____ Date: _____

Day	6 (after loading)	27 (3 weeks of maintenance)	48 (6 weeks of maintenance)
Are you adhering to the supplementation protocol?			
Rate the ***frequency*** of the following symptoms according to the scale where: 0 = none 1 = minimal (1-2 per/wk) 2 = slight (3-4 per/wk) 3 = occasional (5-6 per/wk) 4 = frequent (7-8 per/wk) 5 = severe (9 or more per/wk)			
Dizziness?			
Nausea and/or upset stomach?			
Headache?			
Fast or racing heart rate?			
Heart skipping or palpitations?			
Shortness of breath?			
Nervousness?			
Blurred Vision?			
Any other unusual or adverse effects?			
Rate the ***severity*** of the following symptoms according to the scale where: 0 = none 1 = minimal 2 = slight 3 = moderate 4 = severe 5 = very severe			
Dizziness?			
Nausea and/or upset stomach?			
Headache?			
Fast or racing heart rate?			
Heart skipping or palpitations?			
Shortness of breath?			
Nervousness?			
Blurred Vision?			
Any other unusual or adverse effects?			

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