ABSTRACT

The Effects of a Creatine Supplement Formulation Containing Cinnamon Extract on Creatine Uptake, Creatine Transporter Expression, Insulin Signaling, and Muscle Performance in Males

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Creatine monohydrate has become one of the most popular ingested nutritional supplements used for its potential to enhance athletic performance. Numerous creatine formulations have been developed to maximize creatine absorption, and may also provide a means to either partially bypass or up-regulate the function of creatine transporter-1 (CreaT1). Cinnamon extract (Cinnulin$^{TM}$) has been observed to mimic the effects of insulin, thereby up-regulating glucose uptake and insulin signaling. This study examined how a seven-week supplementation regimen with creatine monohydrate combined with Cinnulin$^{TM}$ (CCI), creatine monohydrate (CR), or placebo (PLA) affected physiological and molecular adaptations in nonresistance-trained males following a prescribed resistance-training program. Results demonstrated that Cinnulin$^{TM}$ combined with creatine monohydrate elicited greater mean increases in relative 1-RM leg press, thigh lean mass, body water, and total Akt protein content when compared to creatine monohydrate alone, or placebo; however, intramuscular creatine increases between the CCI and CR groups demonstrated no significant differences.

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by

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A Thesis

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# CHAPTER ONE

## Introduction and Rationale

### *Background*

Creatine monohydrate, an endogenously synthesized nitrogenous amino acid compound which is also naturally present in food, has become one of the most widely ingested nutritional supplements. Creatine is primarily located in skeletal muscle and plays a pivotal role in cellular bioenergetics, specifically towards the reformation of a molecule essential for muscular contraction, adenosine triphosphate (ATP) (Bessman & Carpenter, 1985).

It has been well established in the past decade that creatine supplementation is effective towards increasing both intramuscular total creatine and phosphocreatine (PCr) concentrations and serving as an ergogenic aid (Earnest, Snell, Rodriguez, Almada & Mitchell, 1995; Kreider, Willoughby, Greenwood, Parise & Tarnopolsky, 2003; Soderlund, Balsom & Ekblom, 1994; Volek & Kraemer, 1996). PCr availability is vital towards the continuation of muscular force production through its ability to transphosphorylate adenosine diphosphate (ADP), thereby regenerating ATP (Greenhaff, Bodin, Soderlund & Hultman, 1994). However, although all energy systems, both aerobic and anaerobic, will at least partially utilize the phosphagen system, high-intensity, short duration, and repeated exercise bouts have been repeatedly observed in the literature to be the most effective mode of exercise that can be enhanced via creatine supplementation (Mesa, Ruiz, Ganzalez-Gross, Gutierrez Sainz & Castillo, 2002).

Of the several hundred studies examining creatine supplementation, approximately 70% proved to be statistically significant towards performance enhancement, with the remaining generally producing non-significant trends (Kreider, 2003). Aside from such issues as differences in 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 and, as a result, numerous creatine formulations have been developed in an attempt to improve muscle creatine uptake, potentially increasing the efficacy of creatine supplementation (Loike, Zalutsky, Kaback, Miranda & Silverstein, 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 provide a better understanding of creatine metabolism (Schoch, Willoughby, & Greenwood, 2006).

A number of unique creatine formulations have been developed (e.g. combining creatine with carbohydrate, sodium, or esterified alcohol), and possess the primary intent of improving cellular absorption and transport, thereby maximizing total intramuscular creatine concentration, ultimately improving muscular performance. These new products may prove beneficial for individuals portraying a down-regulation of CreaT1 by providing a mechanism to partially “bypass” normal physiological creatine uptake.

One of the latest developments includes combining creatine monohydrate with a cinnamon extract known as Cinnulin$^{TM}$. Creatine ingested with simple carbohydrates has been observed to increase muscle creatine uptake rate. Additionally, insulin has been reported to stimulate muscle blood flow, which may provide another means resulting in enhanced creatine availability (Steenge, Lambourne, Casey, Macdonald & Greenhaff, 1998). Cinnamon extract has been observed in the literature to potentiate an insulin effect and successive insulin signaling through the up-regulation of glucose transport (Qin, Nagasaki, Ren, Bajotto, Oshida & Sato, 2004). Therefore, combining creatine monohydrate with Cinnulin$^{TM}$ may increase muscle creatine uptake by stimulating skeletal muscle-mediated glucose uptake by way of glucose transporter-4 (GLUT-4) and paralleled up-regulation in CreaT1. Furthermore, various intermediates within the insulin signaling pathway responsible for glucose regulation may be up-regulated in response to cinnamon extract supplementation, such as insulin receptor substrate-1 (IRS-1), phosphatidylinosital 3-kinase (PI3K), and/or protein kinase B (Akt). Examining alterations in each of these insulin signaling compounds may assist in determining the efficacy of Cinnulin$^{TM}$. At this time, while there are several published studies on the effects of cinnamon extract, there is little research specifically examining insulin signaling within skeletal muscle, and there are no studies combining this extract with creatine monohydrate.

### *Problem Statement*

How does a seven-week supplementation regimen with creatine monohydrate combined with Cinnulin$^{TM}$ (CCI), creatine monohydrate alone (CR), and placebo (PLA)

affect physiological and molecular adaptations in nonresistance-trained males following a prescribed resistance-training program?

### *Purpose of Study*

The purpose of this study was to determine the extent CCI, CR, and PLA groups affect the following: muscle performance and mass; body composition; resting energy expenditure; muscle creatine uptake; serum insulin and IGF-1; CreaT1 and GLUT-4 mRNA and protein expression; the activity of various components involved in the insulin signaling pathway; and clinical blood chemistry markers.

### *Hypotheses*

- $H_1$: Following CR and CCI supplementation, intramuscular total creatine, as well as serum creatine concentration, will significantly increase when compared to placebo.
- $H_2$: Following CR and CCI supplementation, there will be a significant improvement in muscle strength and power and body composition compared to placebo.
- $H_3$: Following CR and CCI supplementation, there will be no significant increase in relation to muscle expression of CreaT1 mRNA and protein compared to placebo.
- $H_4$: There will be no significant differences in GLUT-4 mRNA and protein expression between groups.
- $H_5$: There will be no significant differences in serum insulin and IGF-1, nor total content of the insulin signaling pathway (IRS-1, PI3K, Akt) between groups.
- $H_6$: There will be no significant differences in total content of the insulin signaling pathway (IRS-1, PI3K, Akt) between groups.
- $H_7$: There will be no significant differences in resting energy expenditure between groups.
- $H_8$: There will be no significant alterations in clinical chemistry outside of normal ranges following creatine supplementation.
- $H_9$: Following CR and CCI supplementation, there will be a significant increase in measures of body water retention.

*Delimitations*

- This study incorporated 21 male participants between the ages of 18-35 who have not been involved in regular resistance-training (i.e. three times per week), which includes at least one lower-body exercise session per week, over the past year.

- Participants were recruited from Baylor University and within surrounding Waco, Texas, through flyers.

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

*Limitations*

- Since this study utilized convenience sampling only within the city of Waco, as well as the small sample size (n=21), external validity to the greater population of nonresistance-trained males may be decreased.

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

- Research participants were responsible and expected to follow the prescribed resistance-training regimen throughout the course of the study; this was not a supervised training study.

- Dietary intake was not controlled, but participants were instructed not to alter their typical dietary habits over the course of the study.

*Assumptions*

- All participants fully complied with the supplementation dosing and training protocol. Compliance forms for both supplement intake and training were provided and collected at each testing session.

- The supplements were pure in form and contained the specified concentration of the creatine formulation and cinnamon extract (Cinnulin$^{TM}$) as indicated by the manufacturer.

- All laboratory equipment functioned properly in relation to validity and reliability measures. Proper calibration and the use of trained research staff were utilized to reduce any potential errors.

- Participants arrived to each testing session in a fasting state.
- Participants did not alter their dietary habits over the course of the study.

*Definition of Terms*

1. Adenosine Triphosphate (ATP) – an adenine nucleotide that is utilized as energy; a molecule essential for muscular contraction
2. Anaerobic – occurring or taking place without air or oxygen
3. Creatine– a nitrogenous compound derived from the amino acids arginine, glycine, and methionine; essential for the maintenance of ATP levels
4. Creatine "loading" phase – the period of time within a creatine supplementation protocol that is initiated to quickly saturate creatine muscle stores; typically specifies the daily consumption of 20 g of creatine for five days
5. Creatine "maintenance" phase – the period of time within a creatine supplementation protocol that is utilized to maintain creatine saturation; typically specifies the daily consumption of 5 g creatine following a loading phase
6. Creatine kinase – an enzyme that catalyzes the phosphorylation of creatine by ATP to form phosphocreatine
7. Creatine transporter-1 (CreaT1) – a sodium-chloride dependent membrane-spanning protein responsible for the uptake of creatine into skeletal muscle
8. Glucose transporter-4 (GLUT-4) – an insulin-regulated glucose transporter found in adipose tissues and striated muscle
9. Insulin – an anabolic hormone produced in the pancreas and plays a pivotal role in the controlling blood glucose levels in the body
10. Insulin receptor substrate-1 (IRS-1) – an insulin signaling intermediate, which becomes phosphorylated in response to the activation of the insulin receptor
11. Messenger RNA (mRNA) – the transcript of a gene encoding a protein
12. Myosin Heavy Chain (MHC) – a larger, thicker filament within the oligomeric protein, myosin, in skeletal muscle
13. Phosphatidylinosital 3-kinase (PI3K) – an enzyme that catalyzes the conversion of phosphatidylinositol to phosphatidylinositol 3-phosphate

14. Phosphocreatine (PCr) – a phosphagen that is capable of transferring its phosphate to adenosine diphosphate, ultimately regenerating ATP

15. Reverse transcription polymerase chain reaction (RT-PCR) – PCR in which the starting material is RNA; requires the enzyme reverse transcriptase

16. Total adenine nucleotide (TAN) – the sum of the concentration of ATP, ADP, and AMP

17. Translation - the synthesis of a polypeptide from a mRNA template

# CHAPTER TWO

## Review of Literature

### *Creatine Metabolism*

Creatine, α-methyl guanidinoacetic acid, is a compound that is endogenously synthesized by the liver, kidney, and pancreas. In addition, creatine is also naturally present in food, particularly meat products and fish. Based upon a 70 kg individual, approximately 1 g/day is endogenously synthesized, with another 1 g/day obtained via typical carnivorous diets. In sum, endogenous and exogenous creatine totals about 2 g/day, but it should be noted that larger individuals likely portray a greater turnover rate. Creatine synthesis and dietary ingestion is matched by the spontaneous degradation into the waste product, creatinine, which diffuses into circulation and is eliminated within the urine (Persky & Brazeau, 2001).

Creatine synthesis necessitates three amino acids, arginine, methionine, and glycine, which participate in a two-step reaction to produce creatine (Walker, 1979; Wyss & Kaddurah-Daouk, 2000). The first reaction in this pathway, occurring in the kidney, is catalyzed by the rate-limiting enzyme, arginine:glycine amidinotransferase (AGAT), in which arginine transfers its amidino group to glycine, resulting in the formation of guanidinoacetate (Walker, 1979; Wyss & Kaddurah-Daouk, 2000). Guanidinoacetate has been postulated to then be transported to the liver cells by the blood (Wyss & Kaddurah-Daouk, 2000). Next, in the hepatocyte, the methyl group of S-adenosyl-L-methionine is transferred to guanidinoacetate at the amidino group via S-adenosyl-L-methionine:N-

guanidinoacetate methyltransferase (GAMT), resulting in creatine formation (Walker, 1979; Wyss & Kaddurah-Daouk, 2000). Various factors have been observed to regulate creatine synthesis such as thyroid hormone, growth hormone, testosterone, ornithine, dietary deficiencies, and feedback inhibition of AGAT prior to AGAT mRNA translation (Walker, 1979; Wyss & Kaddurah-Daouk, 2000).

In humans, creatine is principally located within skeletal muscle (~95%), with the remaining (~5%) present in brain, eyes, kidneys, and testes (Walker, 1979). PCr is the major storage form of creatine in muscle, which is produced through the transfer of a phosphate group to creatine. In skeletal muscle, approximately 60% is PCr, with the remaining 40% existing as free creatine, which together constitutes total creatine concentration (Persky & Brazeau, 2001). Following the discovery of PCr (Eggleton & Eggleton, 1927; Fiske & Subbarow, 1927) and the creatine kinase (CK) reaction pathway (Lohmann, 1934), much research began to center upon the physiological roles of the CK system.

### *Physiological Roles of PCr during Anaerobic and Aerobic Exercise*

The intracellular concentration of PCr plays a significant role during the immediate bioenergetic system, which is most active at high intensity, short duration, and repeated exercise bouts. Through the depletion of intracellular PCr stores, intracellular ATP concentration is maintained. A freely reversible reaction occurs, in which PCr phosphorylates ADP to replenish ATP stores, catalyzed via the enzyme, CK. PCr levels within the muscle are almost three to four times more abundant than intramuscular ATP concentration. Although PCr is more copious than ATP, the rate in which ATP is utilized is likely to exceed the overall energy substrate regeneration necessary at activities of high

intensity. However, the PCr supply is sufficient in providing a temporary ATP source until other bioenergetic systems reach maximal rates. PCr also functions as an acid-base buffer to prevent declines in cell pH since the CK reaction utilizes a hydrogen ion. Muscular contraction can be negatively affected when pH levels markedly decrease (Echegaray & Rivera, 2001).

While PCr provides energy at a very high rate, there is a limited supply found within the muscle. During physical activities of long duration, PCr concentration within the phosphagen system cannot continuously supply energy for an extended period of time. It has been observed that aerobic training may have various affects on PCr concentrations such as: 1) an increase in resting PCr levels; and 2) a decline in the rate of PCr depletion during an absolute power output, but not at relative percentage of maximum (Baechle & Earle, 2000). During prolonged, fatiguing exercise, it appears that fatigue is not primarily due to PCr depletion. Febbraio and Dancy (1999) concluded that since the total adenine nucleotide (TAN) pool did not decrease following exercise to exhaustion, PCr levels also did not significantly decline at fatigue.

### *Introduction to Creatine Supplementation*

Creatine was initially discovered in 1835 by the French scientist, Chevreul, and was later followed by various studies in the early 1900's which included animal (Chanutin, 1927) and human supplementation (Chanutin, 1928) to identify the ultimate fate and function of creatine. In the United States, the major breakthrough observing that creatine supplementation may benefit athletic performance did not occur until the early 1990's (Harris, Soderlund & Hultman, 1992). Creatine supplementation, in relation to its use in athletic populations, is aimed at the ergogenic enhancement of high-intensity

performance measures, such as power, strength, muscle mass, or multiple sprints (Bemben & Lamont, 2005). Probably the most consistent finding in the literature is that when creatine supplementation is combined with resistance training, there is an observance of significant increases in lean body mass, maximal strength, and weight-lifting performance (Rawson & Volek, 2003). In addition, there is an increasing body of literature indicating that creatine supplementation may also be an effective clinical treatment for various neuromuscular (Felber, Skladal, Wyss, Kremser, Koller, & Sperl, 2000), neurological (Matthews, Yang, Jenkins, Ferrante, Rosen, Kaddurah-Daouk, *et al.*, 1998), and cardiovascular (Saks & Strumia, 1993) diseases, as well as a role as an anticancer agent (Martin, Winslow, & Kaddurah-Daouk, 1994). Lastly, there have been no clinically significant safety concerns or side effects reported with creatine supplementation (Farquhar & Zambraski, 2002; Greenwood, Kreider, Melton, Rasmussen, Lancaster, Cantler, *et al*., 2003; Kreider, Melton, Rasmussen, Greenwood, Lancaster, Cantler, *et al*., 2003; Poortmans & Francaux, 2000; Schilling, Stone, Utter, Kearney, Johnson, Coglianese, *et al.*, 2001).

The most common dosing protocol for creatine, which consistently observes significant increases in intracellular PCr concentration, begins with a loading phase of 20 g/day for 3-7 days, followed by a maintenance phase of 5 g/day for a period of several weeks (Bemben & Lamont, 2005). However, this absolute dosing may not be the best method since creatine uptake will most likely vary in regards to differences in muscle mass. Instead, a relative amount should be employed, based on either total body mass or fat-free mass, and adjusted accordingly throughout creatine supplementation (Burke *et al*., 2001; Hultman, Soderlund, Timmons, Cederblad & Greenhaff, 1996).

*Creatine Supplementation and Intracellular PCr Response*

Most research with creatine supplementation has been focused upon anaerobic exercise. Creatine supplementation has less ergogenic evidence during activities lasting longer than three minutes (Bemben & Lamont, 2005). For example, Van Loon et al. (2003), examined creatine supplementation prior to performing an endurance-based cycling protocol. The results provided evidence that "prolonged creatine supplementation in humans does not increase muscle or whole-body oxidative capacity and, as such, does not influence substrate utilization or performance during endurance cycling exercise." Following prolonged endurance training, Jones *et al*. (2002) investigated how $VO_2$ is affected during submaximal exercise while following a creatine loading and maintenance regimen. Results concluded that no significant difference was observed regarding $VO_2$ response or increases in performance. In contrast, during intermittent endurance exercise, Yquel et al. (2002) concluded that creatine supplementation assisted in power maintenance due to the pH buffering capabilities of the phosphagens. Collectively, it appears that positive ergogenic responses to creatine supplementation during aerobic activities are less when compared to anaerobic exercise, except possibly during acidic conditions with intermittent endurance exercise. However, a higher intracellular concentration of PCr may be helpful during the final stretch of an endurance event when exercise intensity is greatly increased.

Creatine supplementation has been studied in great detail during high-intensity anaerobic exercise such as resistance training, jumping, sprinting, and Wingate cycling protocols. While variability does exist, most research indicates a positive ergogenic effect with creatine supplementation (Kreider, 2003). Much of this improvement in

performance is linked to an increased pool of PCr, which is available to resynthesize ATP (Kurosawa, Hamaoka, Katsumura, Kuwamori, Kimura, Sako, *et al*., 2003). Additionally, there is evidence that creatine supplementation may also increase myosin heavy chain synthesis as well as increase muscle fiber diameter (Volek, Duncan, Mazzetti, Staron, Putukian, Gomez, *et al.,* 1999; Willoughby & Rosene, 2001).

Interestingly, some researchers have observed no significant physiological effects with creatine supplementation in similar high-intensity exercise bouts and thus raise the issue of responders versus non-responders (Harris *et al*., 1992). It is hypothesized that this variance may be due to regulatory mechanisms of a transport protein, CreaT1. CreaT1 is directly involved in the extracellular uptake of creatine to increase the pool of metabolically active creatine in muscle cells. Research directly examining how CreaT1 is expressed following creatine supplementation or resistance training has not been extensively studied, and it is imperative that future research examines CreaT1 activity in order provide a better understanding of creatine metabolism and how it is regulated in human skeletal muscle (Schoch *et al*., 2006).

### *Creatine Supplementation and GLUT-4 Expression*

Previous research with creatine supplementation has observed increases in glycogen storage, but it has been controversial as to whether this is due to an up-regulation of GLUT-4 expression and activity. In relation to creatine supplementation and the impact on glucoregulation, it has been demonstrated that when creatine was ingested with carbohydrates, the post-exercise repletion of glycogen was enhanced, and was most likely insulin-mediated (Robinson, Sewell, Hultman & Greenhaff, 1999). When creatine intake alone was compared to exogenous carbohydrate and creatine supplementation, the

co-ingestion of carbohydrates demonstrated similar effects, whereby an augmented ability to increase muscle glycogen concentration was observed (Green *et al*, 1996). In the absence of a carbohydrate source, an *in vitro* study was able to successfully exhibit increased insulin secretion due to high concentrations of extracellular creatine (Alsever, Georg, & Sussman, 1970), but when human creatine supplementation was examined, no significant difference in insulin secretion was observed (Green *et al*., 1996; Steenge *et al*., 1998). A recent study supports the theory that exogenous creatine may up-regulate muscle GLUT-4 expression with rehabilitation training, and implies that oxidative and transport proteins may be up-regulated concomitantly (Op't Eijnde, Urso, Richter, Greenhaff, & Hespel, 2001). In contrast, van Loon *et al*. (2004) concluded that creatine ingestion itself stimulates muscle glycogen storage, but does not affect muscle GLUT-4 expression (van Loon, Murphy, Oosterlaar, Cameron-Smith, Hargreaves, Wagenmakers, *et al*., 2004).

### *The Creatine Transporter*

As previously discussed, creatine is taken up by skeletal muscle fibers predominately via the sodium-chloride dependent creatine transporter, CreaT1 (Persky, Brazeau & Hochhaus, 2003). There are actually two isoforms of these membrane-spanning creatine transport proteins, CreaT1 and CreaT2, with the latter primarily active and present within the testes (Snow & Murphy, 2001). Since creatine ingested through supplementation is absorbed into skeletal muscle exclusively by means of CreaT1, this isoform is of the greater focus.

Although most studies have reported significant increases in intramuscular creatine levels with supplementation, variability does exist. It is hypothesized that much

of this variability is due to the process that controls both the influx and efflux of creatine across the cell membrane, and is likely due to a decrease in CreaT1 activity. The vast majority of creatine supplementation studies designed to determine CreaT1 regulation used animal models. Only fairly recently have studies began to examine CreaT1 expression and regulation in humans.

The information generated from human and animal studies suggest that an individual's initial biological profile may partially determine the extent of CreaT1 activity during supplementation, which ultimately affects creatine uptake. Accordingly, a potential theory was developed to explain the physiological observances of responders versus non-responders: 1) it appears that individuals that possess a greater concentration of intramuscular creatine prior to supplementation display a reduction in CreaT1 activity, thus intramuscular creatine uptake is decreased; and 2) in contrast, when intramuscular creatine stores are limiting, CreaT1 expression and activity is up-regulated, resulting in a enhanced ability to increase intramuscular creatine concentration. Collectively, creatine uptake appears to be inversely related to a "predetermined" intracellular creatine concentration. Since it would presumably be very difficult to alter this aspect of CreaT activity, it is imperative that creatine formulations that may potentially up-regulate individuals displaying a reduction in CreaT1 activity be examined (Schoch *et al*., 2006).

### *Insulin Response to Creatine Supplementation*

A significant amount of interest has developed in regards to the most effective means to enhance creatine uptake and retention. Adding a high glycemic index carbohydrate source to creatine monohydrate has been observed to enhance uptake rate, primarily through the effect of an insulin-mediated response (Green, Hultman,

Macdonald, Sewell & Greenhaff, 1996; Steenge, Lambourne, Casey, Macdonald & Greenhaff, 1998). Following a meal containing carbohydrates or protein, glucose levels rise, signaling the beta cells of the pancreas to release insulin. Insulin is responsible for the uptake of glucose, as well as amino acids, from the blood. Since creatine is structurally similar to amino acids, it seems feasible that insulin may also mediate extracellular creatine transport into skeletal muscle. Additional research has indicated that insulin stimulates muscle blood flow, which may provide another means resulting in enhanced creatine availability (Steenge *et al*., 1998).

### *Cinnulin$^{TM}$ – Cinnamon Extract*

Previous research has indicated that when creatine is consumed with simple carbohydrates, such as glucose, its absorption rate is significantly improved due to an increase in insulin secretion; this insulin response aids the clearance and absorption of both carbohydrate and creatine into skeletal muscle (Green *et al*., 1996). The theoretical rationale for combining Cinnulin$^{TM}$, a cinnamon extract, with creatine monohydrate is to potentially up-regulate insulin signaling responses within skeletal muscle, resulting in increased absorption of creatine, independent to the need of a carbohydrate source.

A fair amount of research has examined cinnamon extract (Anderson, Broadhurst, Polansky, Schmidt, Khan, Flanagan, *et al.*, 2004; Broadhurst, Polansky & Anderson, 2000; Khan, Safdar, Ali Khan, Khattak & Anderson, 2003; Qin *et al.*, 2003, 2004), and it appears plausible that this mechanism may occur, but has not been studied directly with creatine supplementation. For example, in vitro studies with isolated adipocytes, indicated that cinnamon extract potentiates insulin activity (Broadhurst et al., 2000; Imparl-Radosevich *et al*., 1998). More recently, Qin et al (2003) observed that cinnamon

extract enhanced insulin action within skeletal muscle in Wistar rats. Results from this study suggested that cinnamon extract improves insulin activity, at least in part due to the enhancement of the insulin-signaling pathway in skeletal muscle. When a ligand successfully activates the insulin receptor, a cascade of events follows which affects glucose and lipid homeostasis, as well as protein synthesis. Cinnulin$^{TM}$ has been indicated to directly up-regulate insulin signaling through the phosphatidylinositol signaling system (PI3K) via the insulin receptor and insulin receptor substrate (IRS-1) (Qin *et al*., 2003). IRS molecules function as docking proteins between the insulin receptor and an intricate system of intracellular signaling molecules (Sesti, Federici, Lauro, Sbraccia & Lauro, 2001). Accordingly, Cinnulin$^{TM}$ appears to have insulin mimicking effects, which may up-regulate insulin signaling. An up-regulation of PI3K activity would probably also result in the concurrent enhancement of glucose transport via the glucose transporter, GLUT-4, since these pathways intersect. Similarly, protein kinase B (Akt) activity may also be elevated if cinnamon extract sufficiently activates the insulin receptor.

Currently, there are no data describing the effects that creatine supplementation combined with Cinnulin$^{TM}$ may have on mRNA and protein expression of the CreaT1 gene. Since cinnamon extract has been observed to mimic insulin, it potentially may have the ability to up-regulate CreaT1 activity, and possibly provide a beneficial creatine formulation for previous non-responders to creatine supplementation. Furthermore, it appears that Cinnulin$^{TM}$ may have an added ability to improve body composition by potentially providing a thermogenic effect, possibly mediated through an elevated metabolic rate (Ziegenfuss, Hofheins, Mendel, Landis & Anderson, 2006). Although

there are several published studies on the effects of cinnamon extract supplementation, there are none examining the combination of this extract with creatine monohydrate.

# CHAPTER THREE

## Methods

### *Subjects*

Twenty-one males between the ages of 18 and 30 were recruited and completed this double-blinded 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 contraindication defined and outlined by the American College of Sports Medicine (ACSM) nor if they ingested any supplements claimed to have ergogenic properties within three months prior to the study [e.g., creatine, hydroxyl-betamethylbutyrate (HMB) or anabolic hormone precursors (androstenedione, dihydroepiandrosterone (DHEA), etc.]. The potential participant became eligible only after an informed consent form was read and signed, in addition to the completion of a successful medical screening examination. The results of this study are a preliminary subset of subjects (n = 21) from a larger study (n = 30).

### *Study Site*

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

### *Study Design*

Table 1 provides an outline of the study design. The study included baseline testing followed by double-blinded supplementation with various creatine formulations for 47-days. The supplementation regimen included both a loading phase of 0.3 g of supplement per kg of fat-free mass/day for five days dosed at 1 g of Cinnulin$^{TM}$ for every 20 g of creatine monohydrate, and a maintenance phase of 0.075 g/kg/day thereafter for six weeks, which was dosed at 250 mg of Cinnulin$^{TM}$ for every 5 g of creatine monohydrate. 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.

### *Independent and Dependent Variables*

The independent variables were the creatine supplements and the placebo. Dependent variables included the following: strength and power measurements; resting energy expenditure (REE); total-body and thigh lean tissue mass, body fat percentage; intracellular, extracellular, and total body water; serum creatine and creatinine; total intramuscular creatine concentration; CreaT1 and GLUT-4 mRNA and protein expression; insulin signaling responses of IRS-1, PI3K, and Akt; and clinical blood chemistry markers.

Table 1

*Creatine Study Design*

| | Loading Phase | Maintenance Phase | |
|---|---|---|---|
| T1<br>Day 0 | T2<br>Day 6 | T3<br>Day 27 | T4<br>Day 48 |
| Food Log | Food Log | Food Log | Food Log |
| REE | REE | REE | REE |
| 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 |

*Entry and Medical Screening Session*

Participants were recruited from advertisement flyers posted in various locations in Waco, Texas, primarily on the campus of Baylor University. The advertisements contained the details of the study, qualifications of entry, and provided the necessary contact information. Potential participants were then interviewed to determine if they qualified for participation. Participants believed to meet eligibility criteria were then invited to attend an entry/familiarization session.

*Familiarization Session*

Participants that met eligibility criteria were familiarized to the study design and protocol. Once reporting to the ESNL, medical history questionnaires and informed consent forms were given to complete, and a general physical examination was performed to further determine if the potential participant qualifies to partake in the study. Participants completed a familiarization one repetition maximum (1-RM) bench press, 1-RM leg press, and an anaerobic muscle performance Wingate test. The participants were then instructed to refrain from exercise for 24-hours, fast for eight hours, and record their dietary intake for four days prior to each testing session. Lastly, the participants were given an appointment time to meet for their official baseline and subsequent testing sessions.

*Strength and Assessment*

Participants performed four 1-RM muscular strength tests using an isotonic 45° leg press (Nebula Fitness, Inc., Versailles, OH) and bench press (Nebula Fitness, Inc., Versailles, OH) to determine potential changes that occur throughout the study resulting from supplementation. These tests were performed at each of the four testing sessions. Participants began the 1-RM leg press and bench press test by first completing five to ten repetitions at approximately 50% of their previously established 1-RM. Following a two minute rest, three to five repetitions were performed at approximately 70% of their 1-RM. From this time forward, the weight was increased gradually, until an official 1-RM was reached. The rest period between each successful lift was two minutes. Test-retest reliability for 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*

A 30-second Wingate anaerobic capacity test was performed on a computerized Lode cycle ergometer (Groningen, Nederland) to assess anaerobic capacity and power at each testing session. This test consisted of having each participant warm up at 30-revolutions per minute (rpm) for 120-seconds, followed by a maximal sprint on the cycle ergometer for 30-seconds against a standard 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, Body Composition, and REE Testing Procedures*

Total body mass (kg) was determined on a calibrated electronic scale with a precision of ± 0.02 kg (Detecto, Webb City, MO). Total body water was 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. Body water was measured through the placement of four electrodes on the body: one electrode placed on the

posterior surface of the right wrist, in between the radial and ulna styloid processes (wrist bones); another electrode placed on the posterior surface of the right hand at the distal base of the second metacarpal; the third electrode was placed on the anterior surface of the right foot; and the last electrode placed at the distal end of the first metatarsal. Participants lied on a table in a supine position and electrode wires were connected to the analyzer. After the subject was connected, age, gender, weight, and height were entered into the unit by the technician. After the unit measured the resistance, which takes approximately 30 seconds, the unit calculated total body water, intracellular water content, and extracellular water content.

Body composition/bone density was determined using a calibrated Discovery W dual-energy x-ray absorptiometry (DEXA) (Hologic, Waltham, MA) by licensed personnel with limited x-ray technology training. The DEXA body composition test involved having the participant lie in a supine position in a pair of shorts/t-shirt or a gown. A low dose of radiation scanned their entire body for approximately six minutes. The DEXA segmented 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. Additionally, a subsection was generated to isolate the thigh region for the determination of thigh muscle mass; this region included the knee joint line to the head of the femur, and was extended medially to the pubic symphysis. Radiation exposure from DEXA for the whole body scan is approximately 1.5 mR per scan. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation exposure was approximately 6 mR for the entire study.

REE assessments were made according to standard protocols utilizing the Parvo Medics TrueMax 2400 Metabolic Measurement System (Sandy, UT). This required the participants to assume a supine position on an exam table and to remain motionless without falling asleep. A see-through metabolic canopy was placed over the participant's neck and head, and metabolic measurements were obtained by collecting expired gases for 15-20 minutes. After at least 10-minutes of starting the test, the lowest five-minute kcal/day average with less than 5% variability was reported.

The participants underwent total body mass, BIA, DEXA, and REE testing at each of the four testing sessions. Additionally, the DEXA scans assisted in the modification of the supplement doses based upon fat-free mass.

### *Dietary Analysis*

Participants recorded their dietary intake for four days prior to each of the four testing sessions, in which total food consumed for three weekdays and one weekend was recorded, if applicable. The participants' diets were not standardized, but subjects were instructed not to change their dietary habits during the course of the study. The dietary recalls were evaluated with Food Processor dietary assessment software (ESHA Nutrition Research, Salem, OR) to determine the average total Calorie consumption and macronutrient composition in their diet.

### *Supplementation Protocol*

Participants were randomly assigned to ingest, in a double-blinded manner, three supplement formulations consisting of both capsules and powder, which were of similar color and texture. The placebo supplement (PLA) contained dextrose capsules (Gaspari

Nutrition, Lakewood, NJ) and dextrose powder (AST Sport Science, Colorado Springs, CO). The creatine supplement contained creatine monohydrate capsules and powder (Integrity Nutraceuticals, Sarasota, FL) (CR). The creatine combined with Cinnulin$^{TM}$ supplement contained creatine monohydrate powder (Integrity Nutraceuticals, Sarasota, FL) and Cinnulin$^{TM}$ capsules (Integrity Nutraceuticals, Sarasota, FL) (CCI). After baseline testing procedures and fat-free mass determination via DEXA, all three groups ingested their respective supplements 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 a relative daily dose of 0.075 g/kg fat free mass (~5 g/day) during the 42-day maintenance phase. Accordingly, for the loading phase, the CCI group ingested approximately 20 g of creatine monohydrate powder and 1 g (8 capsules) of Cinnulin$^{TM}$ each day, and 5 g of creatine and 250 mg of Cinnulin$^{TM}$ ( 2 capsules) per day during the maintenance phase. The CR group ingested a similar combination of creatine capsules and powder to equate the same relative doses during the loading and maintenance phases. Lastly, the PLA group received a combination of capsules and powder containing dextrose. As a result, in addition to the supplement in powder form, all groups also received the same number of capsules during the loading and maintenance phases.

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 (~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. At

the T2, T3, and T4 testing session, participants reported by questionnaire supplement toleration, the supplementation compliance, and any medical problems or symptoms they may have encountered (see Appendix E for questionnaire).

### *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 participants performed 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 curls) twice per week. Participants performed three sets of 10-repetitions with as much weight as they can lift in proper form for each set (typically 70 – 80% 1RM). Rest periods between exercises lasted no longer than three minutes and rest between sets were no longer than two minutes. Training was conducted at the Student Life Center (SLC) at Baylor University or another area gym, and was documented in training logs, and signed off to verify compliance and monitor progress.

### *Muscle Biopsies and Venous Blood Sampling*

Using a 5mm Bergstrom needle, percutaneous muscle biopsies (~50 mg) were obtained from the middle portion of the vastus lateralis muscle of the dominant leg at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. For the remaining three biopsies, attempts were made to extract the tissue from the same location as the initial biopsy by using the pre-biopsy scar, depth markings on the

needle, and a successive incision that was made approximately 0.5 cm to the former from medial to lateral. Following removal, muscle samples were immediately frozen in liquid nitrogen and stored at -80°C for later analysis. One muscle sample was taken at each of the four testing sessions.

Participants were required to fast for at least 8-hours prior to donating blood. Blood samples were obtained by standard/sterile procedures by personnel who are experienced in phlebotomy and are qualified to do so under guidelines established by the Texas Department of Health and Human Services. Venous blood samples were obtained from the antecubital vein into 10 mL collection tubes using a standard Vacutainer™ apparatus. A total of four blood samples were obtained, one at each testing session. Whole blood samples were immediately ready for analysis, whereas serum was centrifuged for 15-minutes and then frozen at -20°C.

Using a Dade Dimension clinical chemistry analyzer (Newark, DE) and an Abbott Cell Dyn 3500 hematology analyzer (Abbott Park, IL), blood and serum samples were assayed for general clinical chemistry markers (i.e., glucose, total protein, blood urea nitrogen, creatinine, BUN/creatinine ratio, uric acid, aspartate aminotransferase, alanine aminotransferase, lactic dehydrogenase, gamma-glutamyl transferase, albumin, calcium, total bilirubin, alkaline phosphatase, triglycerides, cholesterol, HDL, LDL) while whole blood samples were assayed for standard cell blood counts with percentage differentials [i.e., hemoglobin, hematocrit, red blood cell counts, MCV, MCH, MCHC, white blood cell counts (neutrophils, lymphocytes, monocytes, eosinophils, basophils)]. The DADE clinical chemistry analyzer was calibrated daily using liquid assay multiqual (BIO-RAD, Hercules, CA). The protocol for this analyzer involved adding approximately 70 µl

aliquots of serum from a BD Vacutainer® serum seperator collection tube (SST™) to a sample cup to prepare for the subsequent anaylses. This anaylzer uses a sonicating probe to mix the sample. Once daily, two levels of a quality control of known concentrations were assesed. These clinical blood chemistry assays helped to evaluate the effects of the proposed creatine supplement formulations on general markers of tissue degradation, immune function, and clinical safety.

*Serum IGF-1 and Insulin Assays*

The quantitative measurement of serum insulin was determined by using a "one-step" sandwich-type ELISA kit [Diagnostic Systems Laboratories, Webster, TX, (DSL-10-1600)]. 100 µl of horse radish peroxidase (HRP)-labeled anti-insulin antibody was added to the wells of the pre-coated insulin microplate containing 25 µl of the standards, controls, and unknown serum samples. After a one hour incubation period (shaken at 500-700 rpm) and five washes in a buffered saline solution containing a nonionic detergent, each well was then incubated in the presence of a 100 µl mixture of hydrogen peroxide and tetramethylbenzidine (TMB). Lastly the reaction was halted by the addition of an acidic stopping solution (100 µl 0.2 M sulfuric acid), and absorbances were read at 450 nm within thirty minutes. Absorbances are directly proportional to the concentration of insulin present in serum. Quality control data from the manufacturer of this kit reports that the intra-assay precision determined from the average of 12 replicates each with three human serum samples yielded a coefficient of variation of 2.6%, 2.2%, and 1.3%, respectively. Inter-assay precision determined from the average of 18 separate assays over 11-days with three human serum samples yielded a coefficient of variation of 5.2%, 5.8%, and 6.2%, respectively.

Serum IGF-1 concentration was determined by using a "two-step" sandwich-type ELISA kit [Diagnostic Systems Laboratories, Webster, TX, (DSL-10-2800)]. The protocol was similar to insulin quantification, but an additional pretreatment step was needed to separate the IGF and IGF-binding proteins prior to the assay. Sample pretreatment involved the transfer of 20 µl of the unknown serum samples to a 2 ml microfuge tube. 990 µl of a sample buffer containing a non-mercury preservative was then added to the tube, vortexed, and incubated at room temperature for thirty minutes. 990 µl of a second sample buffer also containing a non-mercury preservative was added and mixed thoroughly. At this point the pretreated sample was ready for analysis. 20 µl of the standards, controls, and pretreated unknown serum samples were added to the pre-coated IGF-1 wells of the microplate. 100 µl of a protein-based buffer with a non-mercury preservative was then added. The microplates were subsequently incubated for two hours (shaken at 500-600 rpm), followed by five washing periods using a solution containing buffered saline with a nonionic detergent. 100 µl of anti-IGF-1 antibody conjugated to HRP in a protein-based buffer with a non-mercury preservative was then added to all wells. A thirty minute incubation period then occurred (shaken at 500-600 rpm), followed by five washing periods, using the same buffer solution as previously described. 100 µl of a TMB chromogen solution was added, followed by a ten minute incubation period (shaken at 500-600 rpm). Lastly, 100 µl of 0.2 M sulfuric acid was added to stop the reaction, and absorbances were read at 450 nm within thirty minutes. Absorbances are directly proportional to the concentration of IGF-1 present in serum. Quality control data from the manufacturer of this kit reports that the intra-assay precision determined from the average of 12 replicates each with three human serum

samples yielded a coefficient of variation of 8.6%, 4.5%, and 6.3%, respectively. Inter-assay precision determined from the average of eight separate assays with three human serum samples yielded a coefficient of variation of 6.8%, 6.0%, and 3.3%, respectively.

### *Muscle and Serum Creatine Analysis*

Muscle tissue samples were analyzed spectrophotometrically for total creatine by the diacetyl/α-napthtol reaction (McBride & Gregory, 2002). Using similar methods, serum samples were measured for creatine concentration. Serum samples were immediately ready for creatine analysis, while muscle tissue had to first be prepared.

Approximately 10-15 mg of muscle tissue was cut and placed in an a microfuge tube, and then placed in a vacuum centrifuge (Savant ISS110 SpeedVac$^{TM}$ Concentrator, Thermo Scientific, Milford, MA) to be spun for 18-24 hours. After sufficient muscle drying, the samples were then placed in an ultra-low freezer at -80°C. Dried muscle was powdered by grinding on a porcelain plate with a pestle. Connective tissue was removed and discarded, whereas powdered muscle was placed into pre-weighed microfuge tubes. Powdered muscle was extracted in a 0.5 M perchloric acid/1 mM EDTA solution at a relative ratio of 800 µl per 10 mg powdered muscle on ice for 15-minutes, while periodically vortexing. Samples were then spun at 15,000 rpm at 4˚C for 5-minutes. The supernatant was transferred into a microfuge tube and neutralized with 2.1 M $KHCO_3$ / 0.3 M MOPS solution at a ratio of 1:5 and then centrifuged again at 15,000 rpm for 5-minutes. The resulting supernatant was then stored at -80˚C for future use. In order to determine muscle total creatine concentration, 40 µl of the supernatant from the above reaction was combined with 140 µl $ddH_2O$ and 20 µl 0.4 N HCl and heated at 65˚C for

10-minutes to hydrolyze phosphate groups. The solution was then neutralized with 40 µl of 2.0 N NaOH and analyzed as described above.

### *CreaT1 and GLUT-4 mRNA Expression*

#### *Skeletal Muscle Total RNA Isolation*

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, followed by the homogenization of the muscle samples using a pestle. 100 µl of chloroform was then added, shaken, and allowed to stand at room temperature for 15-minutes. Samples were centrifuged for 15-minutes at 12,000 rpm, resulting in the appearance of three distinct phases: 1) lower (pink) organic phase containing protein; 2) middle (gray) interphase phase containing DNA; and 3) upper (clear) aqueous phase containing RNA. The clear aqueous phase was transferred to a new microfuge tube by using a sterile transfer pipet, followed by the addition of 250 µl 100% isopropanol and allowed to sit at room temperature for 10-minutes (the remaining interphase and organic phase was saved and stored at -80°C). Samples were then spun at 12,000 rpm for 10-minutes, resulting in the formation of an RNA pellet. The supernatant was discarded, followed by the addition of 500 µl 75% ethanol, and vortexed to wash the pellet. The sample was centrifuged at 7,500 rpm for five minutes, followed by discarding the supernatant (process repeated twice). The pellet was then allowed to air dry for 10-minutes at room temperature prior to adding 50 µl nuclease free water. Lastly, the RNA samples were stored at -80°C until later analysis.

*Reverse Transcription and cDNA Synthesis*

2 μg of total skeletal muscle RNA were reverse-transcribed to synthesize cDNA using the iSscript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). A reverse transcription reaction mixture [2 μg of cellular RNA, 5x reverse transcription buffer (20 mM Tris-HCl, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$; 100 μg of bovine serum albumin/ml), a dNTP mixture containing 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.8 μM $MgCl_2$, 0.5 μg/μl of oligo(dT)15 primer, and 25 μl/μg of MMLV RNAase H+ reverse transcriptase enzyme (Bio-Rad, Hercules, CA, USA)] was incubated at 25°C for 5 min, 42°C for 30 min, heated to 85°C for 10 min, and then quick-chilled on ice. The cDNA concentration was determined by using an $OD_{260}$ equivalent to 50 μg/μl and the starting cDNA template concentration was standardized by adjusting all samples to 200 ng prior to amplification (Willoughby, Stout & Wilborn, 2007).

*Oligonucleotide Primers for PCR*

The mRNA sequences of human skeletal muscle CreaT1 (Accession # L31409), GLUT-4 (Accession # M20747), and β-actin (Accession # NM_001101) published in the NCBI Entrez Nucleotide database (www.ncdi.nlm.hih.gov) were used to construct oligonucleotide PCR primers using Beacon Designer software (Bio-Rad, Hercules, CA, USA). The sense and anti-sense primers were synthesized (Integrated DNA Technologies, Coralville, IA). These primers were demonstrated to amplify fragments of 141, 135, and 135 bp for CreaT1, GLUT-4 and β-actin, respectively. β-actin was used within the reaction due to its consideration as a constitutively expressed "housekeeping gene," and the fact that it has been shown to be an appropriate external reference standard

in real-time PCR. With real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise (Mahoney, Carey, Fu, Snow, Cameron-Smith, Parise, et al., 2004), β-actin was used for detecting the relative change in the quantity of mRNA.

*Real-Time PCR Amplification and Quantitation*

200 ng of cDNA were added to each of the 25 μl PCR reactions for β-actin, CreaT1, and GLUT-4 using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Specifically, each PCR reaction contained the following mixtures: [10x PCR buffer, 0.2 μM dNTP mixture, 2.0 μM of a cocktail containing both the sense and antisense RNA oligonucleotide primers, 2 mM $MgCl_2$, 1.0 ug/μl of hot-start iTaq DNA polymerase, SYBR Green I dye, and nuclease-free $dH_2O$]. Each PCR reaction was amplified using real-time quantitative PCR (iCycler IQ Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA). The amplification profile was run for 40-cycles employing a denaturation step at 95°C for 30-seconds, primer annealing at 58°C for 30-seconds, and extension at 72°C for 30-seconds. Fluorescence was measured after each cycle, resulting from the incorporation of SYBR green dye into the amplified PCR product. To help control for differences in amplification efficiency during thermocycling, all PCR reactions were prepared from the same stock solution. The specificity of the PCR was demonstrated with absolute negative controls using separate PCR reactions containing no cDNA template or primers, and single gene products were confirmed using DNA melt curve analysis (Willoughby *et al*., 2007). The relative expression of mRNA was assessed by determining the ratio between the $C_T$ values of CreaT1 and GLUT-4 and the $C_T$ values for β-actin for each muscle sample obtained at each of the four testing sessions.

*Total Muscle Protein Isolation*

Total protein remaining from the total RNA isolation procedure was isolated with isopropanol, ethanol, and 0.1 M guanidine hydrochloride. Specifically, 750 µl isopropanol was added and allowed to sit at room temperature for ten minutes, followed by a ten 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 twenty 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 ten minutes. 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 microfuge tube (Willoughby *et al*., 2007).

*Creatine Transporter and GLUT-4 Muscle Protein Expression*

The muscle protein expression of CreaT1 and GLUT-4 was assessed through the use of ELISAs. Polyclonal antibodies specific for either CreaT1 or GLUT-4 were purchased from Alpha Diagnostics (San Antonio, TX). Initially, 1 µg/ml of CreaT1 and GLUT-4 antibodies were diluted in coating buffer ($Na_2CO_3$, $NaHCO_3$, and $ddH_2O$, pH 9.6) and added to their respective mitcotiter 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, $ddH_2O$) was added to every well. The blocking buffer was added to prevent 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 the 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 dilution buffer (10X phosphate buffered saline, Tween-20, bovine serum albumin, $ddH_2O$), 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 and GLUT-4 in the samples, were measured at a wavelength of 450 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.

### *Insulin Signaling Analysis*

Total muscle protein content (phosphorylated and non-phosphorylated) of the insulin signaling intermediates, Akt and IRS-1, was determined by using ELISA kits (Biosource, Camarillo, CA). Muscle PI3K activity levels were also determined by ELISA (Echelon Biosciences, Salt Lake, UT).

The protocol for determining Akt protein content in muscle was determined by using an ELISA kit (#KHO0101). This kit detects and quantifies both phosphorylated and non-phosphorylated Akt protein. 100 µl standards, samples (1:10 dilution with $ddH_2O$),

and controls were added to their respective wells coated with a monoclonal antibody specific for Akt, incubated at room temperature for two hours, and washed four times (w/ 15-second soak) with wash buffer. 100 µl of biotin rabbit-anti-Akt antibody was added to each well, incubated for one hour, and washed again four times with the wash buffer (w/ 15-second soak). Next, 100 µl anti-Rabbit IgG-HRP working conjugate was added to each well, incubated for 30-minutes, and washed four times with wash buffer (w/ 15-second soak). 100 µl stabilized chromogen (TMB) was added and incubated for thirty minutes. Finally, 100 µl stop solution was added, followed by reading absorbances at 450 nm. Relative Akt protein content was determined by dividing the calculated concentration by muscle weight. Quality control data from the manufacturer of this kit reported that the intra-assay precision determined from the average of 16 replicates each from three samples of known Akt concentration yielded a coefficient of variation of 6.9%, 9.9%, and 6.3%, respectively. Inter-assay precision determined from assaying three samples of known Akt concentration 48-times in multiple assays yielded a coefficient of variation of 9.7%, 9.6%, and 8.7%, respectively.

Muscle IRS-1 protein content was determined by using an ELISA kit (#KHO0511). This kit detects and quantifies both phosphorylated and non-phosphorylated IRS-1 protein. 100 µl standards, samples (1:10 dilution with dd$H_2O$), and controls were added to their respective wells coated with a monoclonal antibody specific for IRS-1, incubated at room temperature for two hours, and washed four times (w/ 15-second soak) with wash buffer. 100 µl of biotin rabbit-anti-IRS-1 antibody was added to each well, incubated for one hour, and washed again four times with the wash buffer (w/ 15 second soak). Next, 100 µl anti-Rabbit IgG-HRP working conjugate was added to

each well, incubated for 30-minutes, and washed four times with wash buffer (w/ 15 second soak). 100 µl TMB was added and incubated for thirty minutes. Lastly, 100 µl stop solution was added, followed by reading absorbances at 450 nm. Relative IRS-1 protein content was determined by dividing the calculated concentration by muscle weight. Quality control data from the manufacturer of this kit reported that the intra-assay precision determined from three samples of known IRS-1 concentration assayed in replicates of 16, yielded a coefficient of variation of 4.7%, 8.2%, and 7.6%, respectively. Inter-assay precision determined from assaying three samples of known IRS-1 concentration 36-times in multiple assays yielded a coefficient of variation of 5.1%, 9.3%, and 6.4%, respectively.

In order to determine muscle PI3K activity, a competitive ELISA kit [Echelon Biosciences, Salt Lake City, UT, (K-1000)] was used. An inverse relationship between the signal and amount of $PI(3,4,5)P_3$ produced was observed to quantify PI3K activity. There were three distinguishable steps, which included a kinase reaction, incubation phase, and detection period. In the kinase reaction, 204 µl TBS (150mM NaCl, 10mM Tris pH 7.5) was added to the supplied $PI(4,5)P_2$ substrate tubes. For each well required, 5 µl 10X reaction buffer (40mM $MgCl_2$, 200mM Tris pH 7.4, 100mM NaCl, 250 µM ATP), 10 µl substrate solution, and 35 µl $ddH_2O$ was combined to formulate the reaction mixture. The reaction was allowed to occur for one hour at room temperature, prior to adding 2.5 µl 100mM EDTA (per well needed) to stop the reaction. Within the incubation phase, the standard curve was serial diluted with various concentrations of reconstituted $PI(3,4,5)P_3$. 50 µl of each standard was added to their respective well, followed by the addition of 50 µl TBS. For blank controls, 100 µl TBS was added. 50 µl of each unknown

sample was then added to their respective wells, followed by the addition of 50 µl reaction mixture. The last step in the incubation period included the addition of 50 µl diluted $PI(2,3,5)P_3$ (1:200 in TBS-0.05% Tween) detector, and incubation at room temperature for one hour. Following incubation, 100 µl of the reacted mixtures were transferred to a $PI(3,4,5)P_3$-coated microplate, incubated for thirty minutes at room temperature, and followed by three washes with TBS-0.05% Tween. 100 µl of the secondary detection reagent was added to all wells and incubated for thirty minutes, followed by three washing periods. 100 µl TMB was added to each well, and color production was allowed to proceed for three minutes. The reaction was halted by the addition of 50 µl stop solution (0.2 M sulfuric acid). Absorbances were read at 450nm to determine absolute concentration and were relatively quantified by dividing by muscle weight. The manufacturer of this kit did not provide quality control data in relation to intra- and inter-assay precision.

### *Statistical Analysis*

A one-way univariate analysis of variance (ANOVA) was utilized to compare the means of every baseline criterion variable in order to determine if significant differences were present between the three groups at the onset of the study. If a significant difference between the groups were discovered at baseline in any of the dependent variables, a Bonferroni pair-wise comparison test was utilized to determine which groups were significantly different. Statistical analyses were performed by utilizing a [treatment groups (3) x time point (4)] repeated-measures multivariate analysis of variance (MANOVA) mixed methods with repeated measures on the second factor to analyze the delta variables. Delta values were determined by subtracting the baseline value (T1) from

each subsequent testing session (T2, T3, and T4) in order to determine changes over the course of the study. Eight sets of MANOVAs were analyzed and were grouped into the following categories: dietary; body composition and resting energy expenditure; performance; serum IGF-1, insulin, and creatine; muscle mRNA; muscle protein; whole blood clinical chemistry; and serum clinical chemistry. This approach was taken based on dependent variables that were likely to be related to one another. In addition, the use of a MANOVA analysis also prevents the chance of committing a Type I error that could result from the use of repeated univariate ANOVA procedures; in contrast, this will increase the likelihood of performing type II error, particularly with a low n-size. Following the initial MANOVA, separate ANOVAs for each criterion variable were utilized as follow-up tests. Significant differences in mean values for any main effects discovered in the individual ANOVAs were determined using a Bonferroni follow-up test to control for alpha inflation. Significant interactions that were discovered in the ANOVA were determined using independent samples T-tests. The index of effect size utilized was partial Eta squared ($\eta^2$), which estimates the proportion of variance in the dependent variable that can be explained by the independent variable. Partial Eta squared effect sizes were determined to be: weak = 0.17, medium = 0.24, strong = 0.51, very strong = 0.70 (O'Connor, Stip, Pelissier, Aardema, Guay, Gaudette, *et al*., 2007). All statistical procedures were performed using SPSS 15.0 software (Chicago, IL) and a probability level of $< 0.05$ was adopted throughout the study.

# CHAPTER FOUR

## Results

### *Initial Assessment for Baseline Differences of all Criterion Variables*

A one-way univariate ANOVA was calculated on all dependent variables to determine if significant differences existed at baseline between the three groups. If a significant difference existed at baseline in any of the dependent variables, a test of bivariate comparisons was conducted utilizing the Bonferroni test to determine which groups were significantly different at baseline. A significant difference existed at baseline for total body muscle mass ($p = 0.01$). To adjust for these differences at baseline, all skeletal muscle variables were analyzed as delta values (testing session – baseline), as previously described.

### *Subject Demographics*

Twenty-nine participants were recruited for the study and completed informed consent forms, out of which twenty-one completed the 47-day research study. Table 2 displays the baseline averages (±SD) of each group's height, age, and weight. Statistical analysis demonstrated that there were no significant differences between baseline height ($p = 0.25$), weight ($p = 0.17$), or age ($p = 0.63$). However, since there was variability between groups for total body muscle mass, which is considered to be a major dependent variable for this study, all statistical analyses were conducted using delta changes.

Table 2

*Subject Baseline Demographics*

| PLA Group | |
|---|---|
| N size | 7 |
| Height (cm) | 176.13 (3.0) |
| Weight (kg) | 74.19 (10.9) |
| Age (years) | 19.43 (.79) |
| **CCI Group** | |
| N size | 7 |
| Height (cm) | 180.21 (9.7) |
| Weight (kg) | 80.10 (16.3) |
| Age (years) | 20.14 (2.61) |
| **CR Group** | |
| N size | 7 |
| Height (cm) | 174.00 (5.4) |
| Weight (kg) | 91.44 (21.4) |
| Age (years) | 20.29 (1.4) |

*Note*: Values are reported as means (±SD).

*Dietary Analysis and Supplementation Compliance*

Participants were required to keep food records for four days prior to each testing session to determine to determine average daily kcal and macronutrient consumption and potential changes over time. There were no significant main effects for Time ($p = 0.86$) in relation to changes in total Calories (kcal/day), protein (g/day), carbohydrates (g/day), or fats (g/day), but there was a Group main effect trend for total Calories consumed ($p < 0.10$, effect size = 0.074) (Table 3). However, the reported food logs were not to be considered the most reliable of measurements, as the young college-aged participants virtually have had no experience in reporting this type of dietary data. Access to a metabolic kitchen was not possible for the purposes of this study. Supplement compliance was assessed by completed supplement logs. Based upon what was reported,

all of the participants were consuming their assigned supplement according to the protocol. There were no side effects reported, or adverse effects for any group during the course of the study.

Table 3

*Calorie and Macronutrient Intake*

| | Total Calories (kcal/day) | Protein (g/day) | Carbohydrate (g/day) | Fat (g/day) |
|---|---|---|---|---|
| PLA | | | | |
| T1 | 1620 (588) | 93 (34) | 213 (73) | 75 (35) |
| T2 | 1829 (447) | 99 (30) | 246 (67) | 82 (23) |
| T3 | 1865 (370) | 106 (44) | 241 (50) | 88 (24) |
| T4 | 1838 (584) | 101 (25) | 224 (70) | 93 (37) |
| CCI | | | | |
| T1 | 1795 (214) | 88 (22) | 237 (21) | 84 (20) |
| T2 | 1896 (183) | 104 (21) | 249 (42) | 88 (8) |
| T3 | 1610 (229) | 84 (32) | 213 (34) | 75 (19) |
| T4 | 1714 (306) | 85 (29) | 224 (29) | 81 (28) |
| CR | | | | |
| T1 | 1374 (298) | 92 (17) | 281 (82) | 70 (19) |
| T2 | 1393 (444) | 102 (26) | 299 (103) | 81 (24) |
| T3 | 1389 (384) | 107 (21) | 299 (90) | 74 (18) |
| T4 | 1409 (311) | 98 (14) | 307 (76) | 71 (24) |

*Note*: Data are represented as means (±SD)

### *Total Muscle Creatine Uptake*

Baseline dry weight (DW) muscle creatine concentration, independent of group was 112.6 mmol/kg DW ± 16.4, which is consistent with the typically reported values of ~120 mmol/kg DW. The coefficient of variation between duplicate wells in the total muscle creatine assay was 1.01%, and the standard curve correlation coefficient between the three plates was 0.997. Statistical analyses were designed to measure delta values

over time. There was no significant Group x Time interaction (p = 0.39, effect size = 0.391), but there was a significant Time main effect for muscle creatine uptake (p = 0.001, effect size = 0.521), observing increases at T2 (p = 0.001), T3 (p = 0.001), and T4 (p = 0.001). Furthermore, a Group main effect was located (p = 0.01, effect size = 0.154), in which the CCI group (p = 0.01) and CR group (p = 0.02) increased total creatine uptake greater than PLA. Figure 1 displays the delta changes for muscle creatine uptake over the course of the study, whereas Table 4 displays the absolute values for each group.

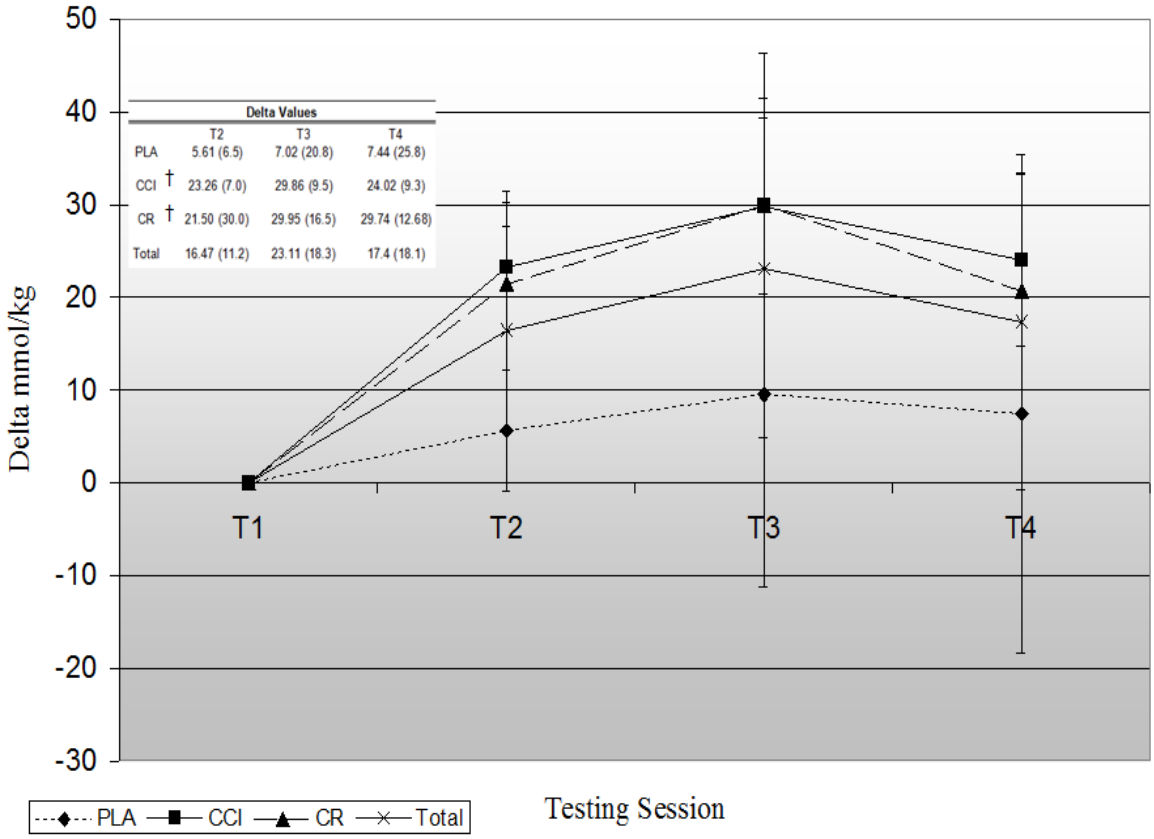


*Figure 1*. Changes in Muscle Total Creatine. Delta values were reported as mean (± SD). † indicates a significant group main effect in which CCI (p 0.01) and CR (p = 0.02) was greater than PLA.

Table 4

*Intramuscular Total Creatine Concentration*

| Testing Session | CCI Group Mean (±SD) | CR Group Mean (±SD) | PLA Group Mean (±SD) |
|---|---|---|---|
| T1 | 109.7 (12.6) | 116.6 (16.6) | 111.4 (20.7) |
| T2 | 132.5 (11.2) | 138.1 (11.2) | 117.0 (16.6) |
| T3 | 139.5 (5.6) | 146.6 (9.4) | 121.0 (10.4) |
| T4 | 133.7 (8.3) | 137.4 (7.4) | 118.8 (11.1) |

*Note*: Delta values are reported in mmol/kg DW and are means (±SD).

*Muscle Total Creatine Hypothesis Conclusion*

- The $H_1$ hypothesis stating that intramuscular total creatine will significantly increase following creatine supplementation is accepted.

*Body Composition and Resting Energy Expenditure Variables*

The following variables were grouped together for statistical analysis based upon delta values: body weight; body fat percentage; total-body lean tissue mass; thigh lean tissue mass; total, intracellular, and extracellular body water; and REE. There were significant multivariate main effects for Group ($p = 0.001$, effect size = 0.292) and Time ($p = 0.001$, effect size = 0.404), but no significant Group x Time interaction ($p = 0.102$, effect size = 0.146). Body composition and resting energy expenditure were examined for potential changes in body weight (Figure 2), total-body lean tissue mass (Figure 3), thigh lean tissue mass (Figure 4), body fat percentage (Figure 5), and REE over the course of the study. Each variable combined with alpha levels for Group, Time, and Group x Time interaction are presented in Table 5.

Table 5

*Body Composition and REE Results*

| Variable | Session | PLA Group | CCI Group | CR Group | Time Effect p value | Group Effect p value | Group x Time Effect p value |
|---|---|---|---|---|---|---|---|
| Body Weight (kg) | T1 | 75.7 | 80.1 | 91.4 | - | - | - |
| | T2 | 74.5 | 81.7 | 92.5 | - | - | - |
| | T3 | 77.2 | 83.0 | 89.0 | - | - | - |
| | T4 | 75.2 | 81.4 | 91.4 | * 0.03 | 0.08 | 0.26 |
| Total Body Lean Tissue Mass (kg) | T1 | 53.4 | 57.2 | 65.6 | - | - | - |
| | T2 | 53.8 | 59.0 | 66.8 | - | - | - |
| | T3 | 56.3 | 59.9 | 65.5 | - | - | - |
| | T4 | 54.9 | 60.6 | 67.5 | * 0.001 | 0.02 | 0.58 |
| Thigh Lean Tissue Mass (kg) | T1 | 7.86 | 8.36 | 9.35 | - | - | - |
| | T2 | 7.98 | 8.76 | 9.53 | - | - | - |
| | T3 | 8.33 | 9.01 | 9.29 | - | - | - |
| | T4 | 8.13 | 8.98 | 9.78 | * 0.001 | * 0.01 | 0.58 |
| Body Fat (%) | T1 | 18.8 | 17.9 | 25.6 | - | - | - |
| | T2 | 16.8 | 17.1 | 24.8 | - | - | - |
| | T3 | 16.9 | 17.2 | 24.2 | - | - | - |
| | T4 | 16.1 | 16.5 | 23.2 | * 0.001 | 0.08 | 0.29 |

(*table continues*)

| Variable | Session | PLA Group | CCI Group | CR Group | Time Effect p value | Group Effect p value | Group x Time Effect p value |
|---|---|---|---|---|---|---|---|
| Total Body Water | T1 | - | - | - | - | - | - |
| (% change) | T2 | -0.10 | 5.67 | -0.25 | - | - | - |
| | T3 | 4.83 | 8.02 | -1.34 | - | - | - |
| | T4 | 3.83 | 9.32 | 4.16 | * 0.001 | * 0.001 | 0.06 |
| Intracellular Water | T1 | - | - | - | - | - | - |
| (% change) | T2 | 3.83 | 6.07 | -0.15 | - | - | - |
| | T3 | 8.12 | 9.64 | -0.94 | - | - | - |
| | T4 | 9.46 | 10.75 | 7.29 | * 0.001 | * 0.01 | 0.43 |
| Extracellular Body Water | T1 | - | - | - | - | - | - |
| (% change) | T2 | -0.71 | 5.09 | -0.40 | - | - | - |
| | T3 | 5.43 | 5.60 | -1.93 | - | - | - |
| | T4 | 0.95 | 7.20 | -0.52 | * 0.04 | * 0.001 | 0.10 |
| REE | T1 | 1879 | 1888 | 1993 | - | - | - |
| (kcal/day) | T2 | 1845 | 1948 | 2112 | - | - | - |
| | T3 | 1923 | 1962 | 1941 | - | - | - |
| | T4 | 1757 | 1952 | 1860 | 0.28 | 0.26 | 0.55 |

*Note*: Dependent variables are presented as absolute values for measures of body mass, body fat percentage, and REE. Percent change is reported for measures of body water. Statistical analysis is based upon delta values. Significance is indicated by an asterisk.

*Body Weight*

There was not a significant Group x Time interaction (p = 0.26, effect size = 0.108). However, a significant main effect for Group was observed (p = 0.027, effect size = 0.104), in which CCI body weight delta values were significantly greater than CR (p = 0.04), with an increasing trend when compared to PLA (p < 0.10). There were no significant main effects for Time (p < 0.10, effect size = 0.096), but an increasing trend was observed (p < 0.10).

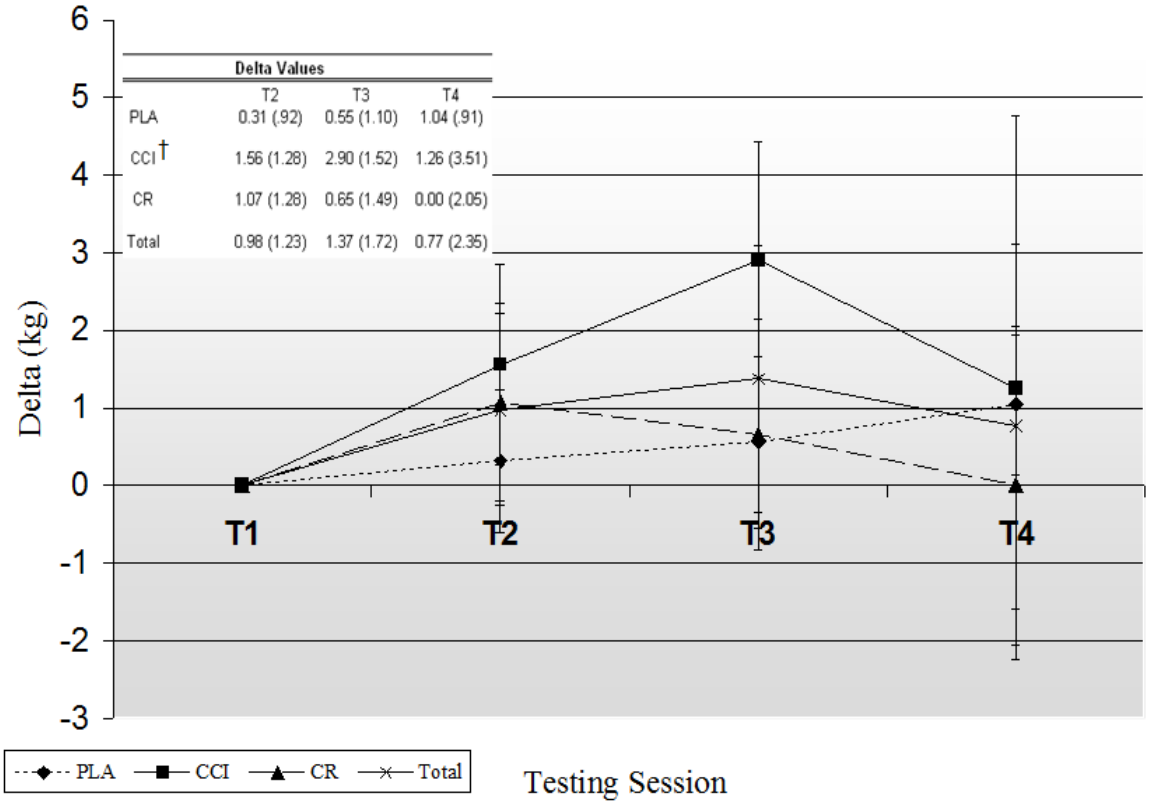


*Figure 2*. Changes in body weight. Body weight delta values were reported as mean (± SD). Group main effect significance is indicated as † in which CCI was significantly greater than CR (p = 0.04).

*Total-Body Lean Tissue Mass*

No significant Group x Time interaction was observed in DEXA determined total body lean tissue mass (p = 0.58, effect size = 0.067). A significant main effect for Group was observed (p = 0.02, effect size = 0.108); however, post-hoc results showed only a

trend, in which CCI was greater when compared to PLA (p = 0.052) and CR (p = 0.057). A significant main effect for Time was located (p = 0.001, effect size = 0.506), indicating that total-body lean tissue mass was increased at T2 (p = 0.001), T3 (p = 0.001), and T4 (p = 0.001); furthermore, total-body lean tissue mass was significantly greater at T4 when compared to T2 (p = 0.005).

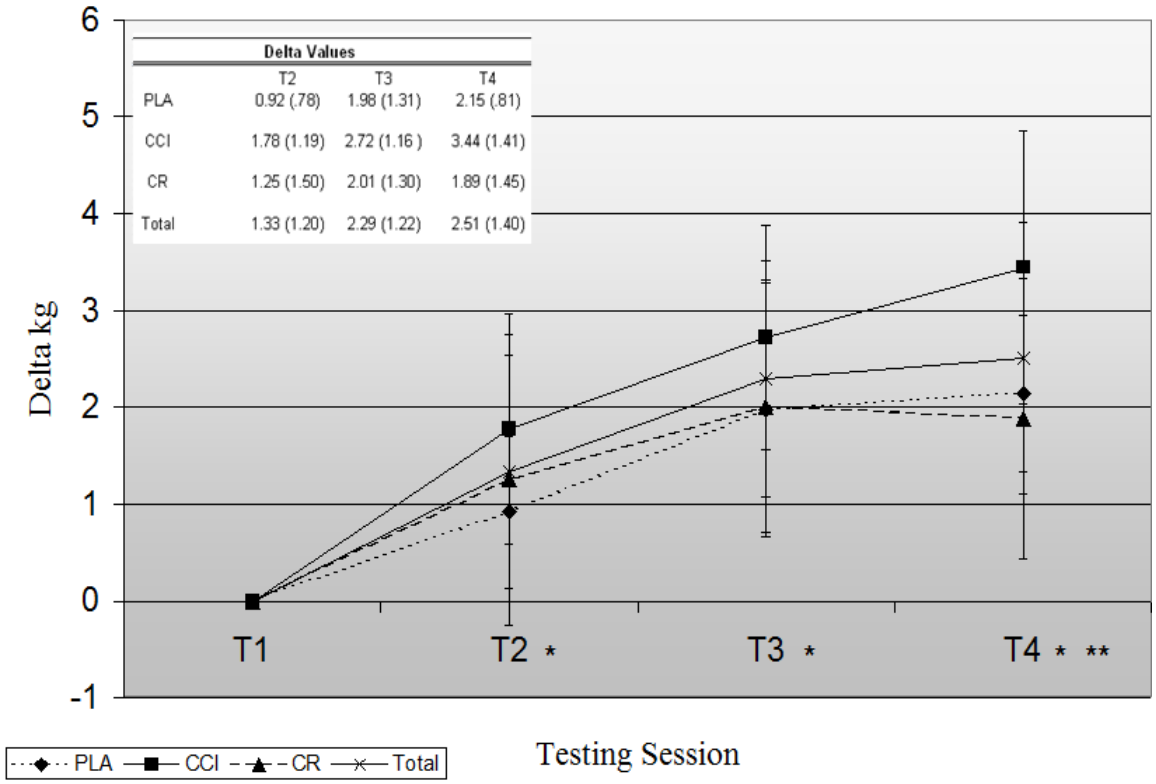


*Figure 3*. Changes in total-body lean tissue mass. Delta values were reported as mean (± SD). * indicates a significant main effect for Time showing that lean tissue mass was increased at T2 (p = 0.001), T3 (p = 0.001), and T4 (p = 0.001). Furthermore, ** indicates that total-body lean tissue mass was significantly greater at T4 when compared to T2 (p = 0.005).

*Thigh Lean Tissue Mass*

No significant Group x Time interaction was observed in DEXA determined thigh lean tissue mass (p = 0.58, effect size = 0.067). A significant main effect for Group was located (p = 0.01, effect size = 0.132), in which CCI was greater than PLA (p = 0.01) but

only a trend when compared to CR (p = 0.069). A significant main effect for Time was observed (p = 0.001, effect size = 0.407), showing thigh lean tissue mass to be increased at T2 (p = 0.001), T3 (p = 0.001), and T4 (p = 0.001).

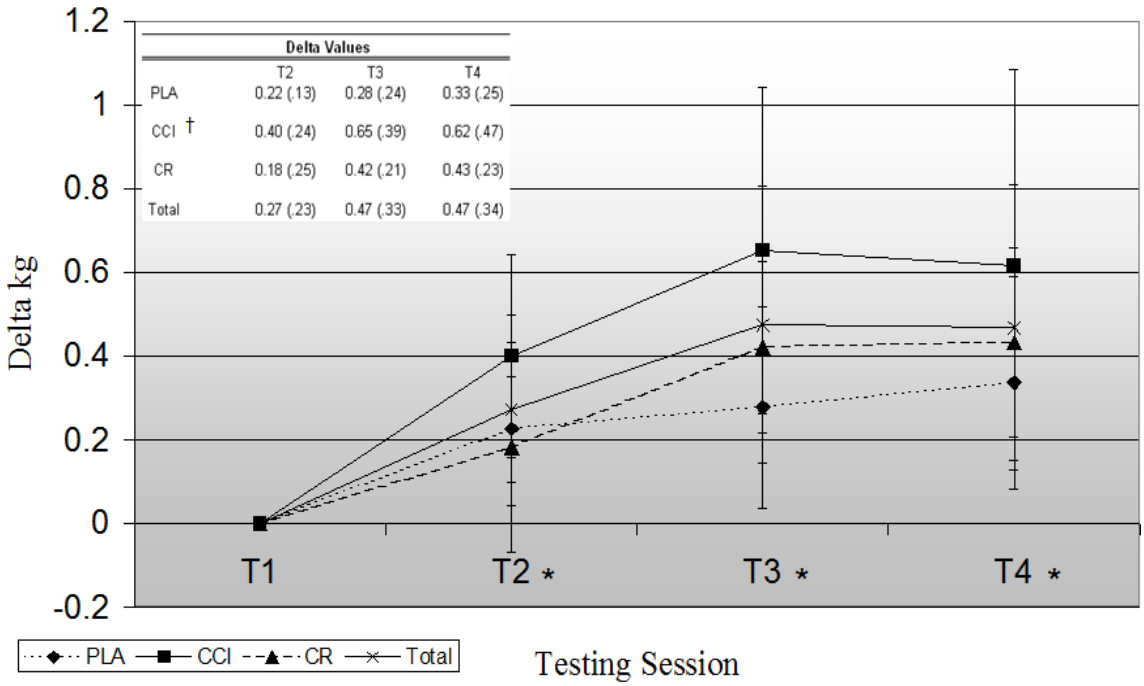


*Figure 4*. Changes in thigh lean tissue mass. Delta values were reported as mean (± SD). * indicates a significant main effect for Time showing that lean tissue mass was increased at T2 (p = 0.001), T3 (p = 0.001), and T4 (p = 0.001). Main effect for Group is signified as †, indicating a significant difference between CCI and PLA (p = 0.01).

*Body Fat Percentage*

There was no significant Group x Time interaction for body fat percentage delta values (p = 0.29, effect size = 0.102). No significant main effect for Group was located (p < 0.10, effect size = 0.073); however, there was a trend for a decreased body fat percentage in the CR group compared to CCI (p < 0.10). A significant main effect for Time occurred (p = 0.001, effect size = 0.400), as body fat percentage was significantly decreased at T2 (p = 0.001), T3 (p = 0.001), and T4 (p = 0.001). Furthermore, the decrease in body fat percentage at T4 was significantly different than T2 (p = 0.04).

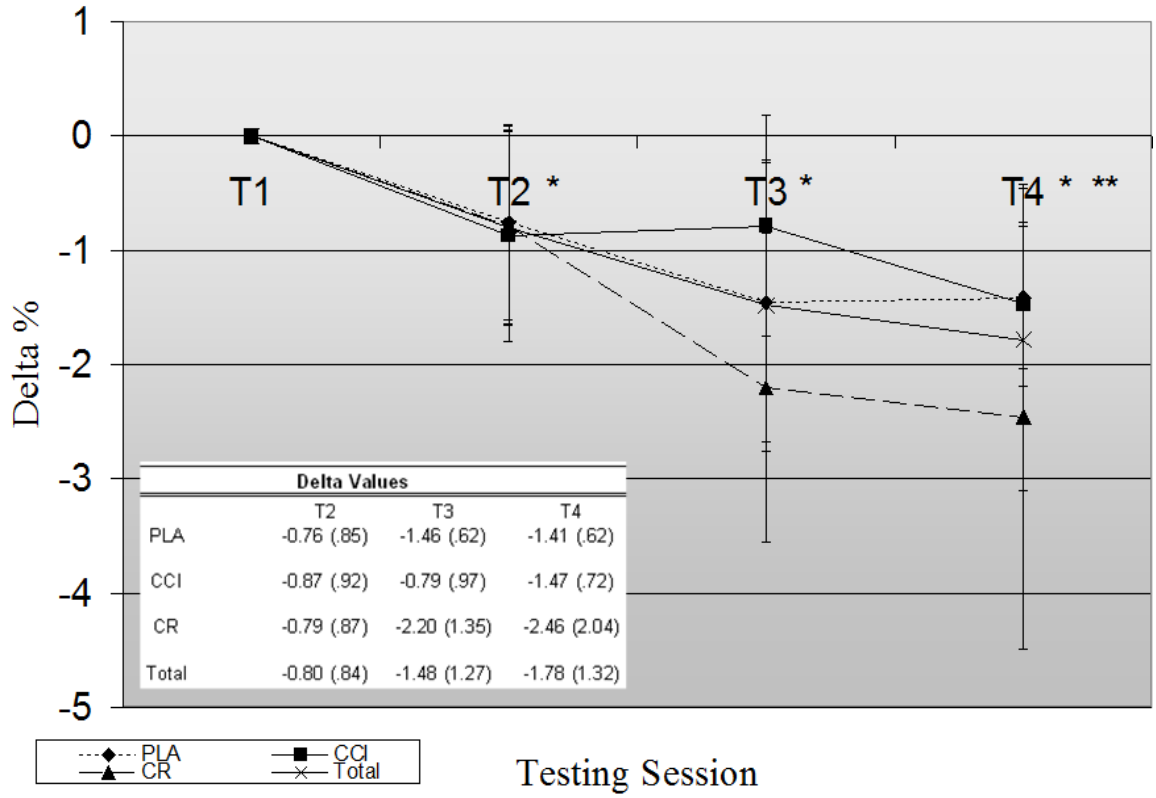


*Figure 5*. Changes in body fat percentage. Body fat percentage delta values were reported as mean (± SD). * indicates a significant main effects for Time as body fat percentage was significantly decreased at T2 (p = 0.001), T3 (p = 0.001), and T4 (p = 0.001). ** indicates that the decrease at T4 was significantly different from T2 (p = 0.041).

*Body Water Retention*

There were no significant Group x Time interactions for total body water (p = 0.06, effect size = 0.163), intracellular water (p = 0.43, effect size = 0.083), or extracellular water (p = 0.10, effect size = 0.144). Figures 6, 7, and 8 display the changes in the distribution of body water over the course of time for total, intracellular, and extracellular water content, respectively.

*Total Body Water*. A significant Group main effect was observed (p = 0.001, effect size = 0.332), in which the CCI group was greater than PLA (p = 0.001) or CR (p = 0.001) for total body water content. Time main effects (p = 0.001, effect size = 0.374)

included a significantly increased total body water at T3 (p = 0.003) and T4 (p = 0.001), as well as an increased T4 total body water content when compared to T2 (p = 0.002).

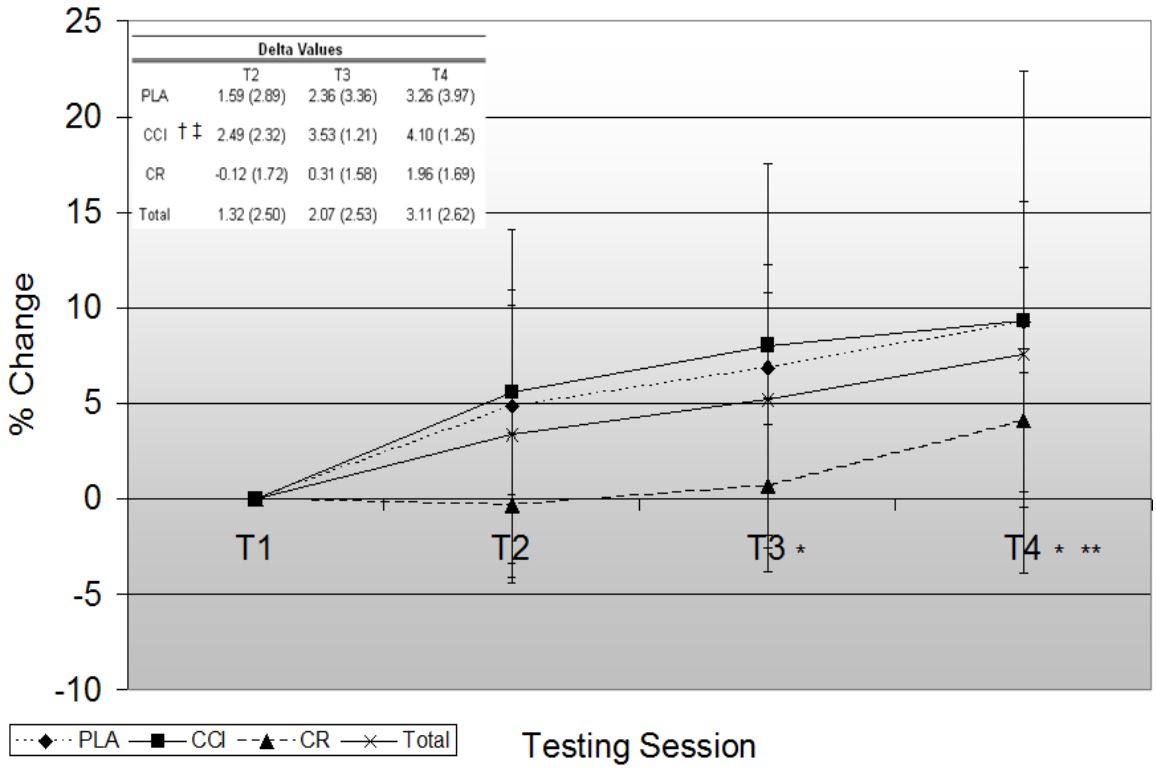


*Figure 6*. Changes in total body water. Delta values were reported as means in liters (± SD). * indicates a significant increase at T3 (p = 0.003) and T4 (p = 0.001). ** indicates significant increase compared to T2 (p = 0.002). † and ‡ indicate significant group main effects when compared to PLA (p = 0.001) and CR (p = 0.001), respectively.

*Intracellular Body Water.* A significant Group main effect was located (p = 0.01, effect size = 0.135), in which intracellular body in the CCI group was greater when compared to CR (p = 0.01). Time main effects were observed (p = 0.001, effect size = 0.325), in which intracellular water content was increased at T3 (p = 0.001) and T4 (p = 0.001); furthermore, T4 intracellular water content was significantly increased compared to T2 (p = 0.01).

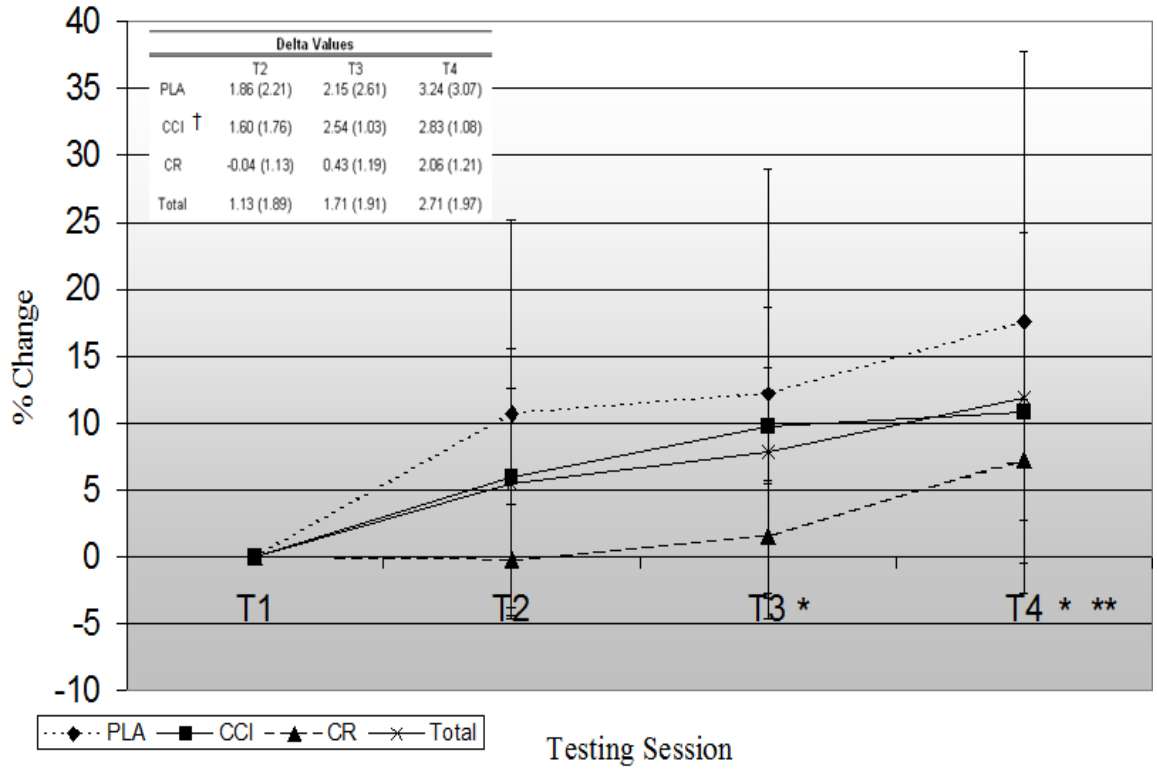


*Figure 7*. Changes in intracellular body water. Delta values were reported as means in liters (± SD). * indicate significant increases at T3 (p = 0.001) and T4 (p = 0.001); ** indicates a significant increase at T4 compared to T2 (p = 0.01). † indicates a significant Group main effect in relation to CR (p = 0.01).

*Extracellular Body Water.* A Group main effect was located (p = 0.001, effect size = 0.292), in which CCI had a greater extracellular body water content compared to PLA (p = 0.001) and CR (p = 0.001), respectively. No significant Time main effects were observed through statistical analysis (p = 0.041, effect size = 0.117).

*Resting Energy Expenditure*

Delta changes in REE over the course of the study revealed no significant differences or trends regarding main effects for Group (p = 0.26, effect size = 0.040), main effects for Time (p = 0.28, effect size = 0.056), or Group x Time interaction (p = 0.55, effect size = 0.070).

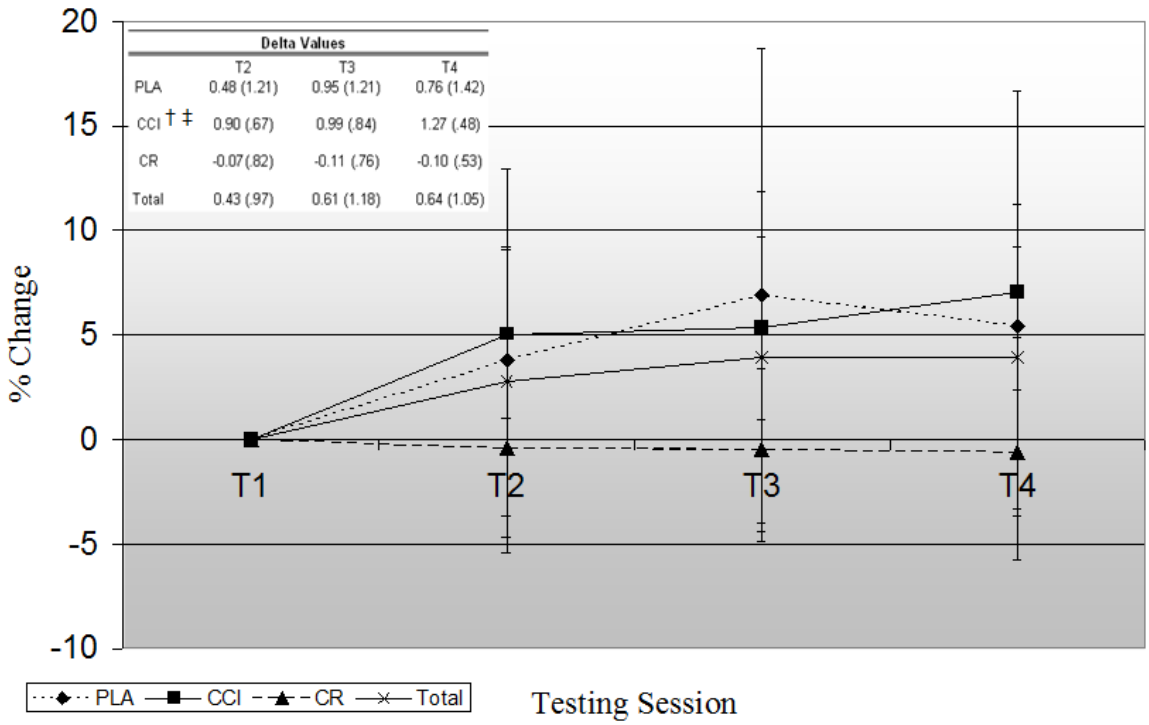


*Figure 8*. Changes in extracellular body water. Delta values were reported as means in liters (± SD). † and ‡ indicate significant Group main effects in relation to CR ($p = 0.001$) and PLA ($p = 0.001$), respectively.

*Body Composition and REE Hypothesis Conclusion*

- The $H_2$ hypothesis stating that there will be a significant improvement in body composition following creatine supplementation is accepted only for the CCI group. The CCI group demonstrated significant mean main effects for Group for increasing thigh lean tissue mass with trends in relation to total-body lean tissue mass. The CR group displayed a trend for decreased body fat percentage, but was not significant.

- The $H_9$ hypothesis stating that there will be a significant increase in measures of body water retention following creatine supplementation is partially accepted, since the CR group did not significantly increase body water, although there was a non-significant increase in total and intracellular water content. The CCI group significantly increased all measures of body water, thus the $H_8$ hypothesis is rejected.

- The $H_7$ hypothesis stating that there will be no significant differences in resting energy expenditure between groups is accepted.

### *Performance Variables*

Performance measures (strength and power) were assessed at each of the four testing sessions and all of these variables were grouped for statistical analysis based upon delta values. Although there were individual significant multivariate main effects for Group ($p = 0.002$, effect size = 0.287) and Time ($p = 0.001$, effect size = 0.385), statistical analysis revealed no significant multivariate Group x Time interaction ($p = 0.60$, effect size = 0.140) for any of the measures for 1-RM bench press, 1-RM leg press, relative peak power, relative mean power, and anaerobic rate to fatigue. Figures 9 and 10 display the delta results for relative 1-RM bench press and leg press, respectively. Table 6 displays the absolute values for bench press and leg press performance variables, whereas Table 7 displays Wingate anaerobic power measurements.

#### *Relative 1-RM Bench Press*

1-RM relative bench press resulted in no significant Group main effects ($p = 0.76$, effect size = 0.007), but Time main effects were observed over the course of the study ($p = 0.001$, effect size = 0.525). Relative bench press measures were significantly increased at T3 ($p = 0.001$) and T4 ($p = 0.001$). Furthermore, relative bench press values were significantly increased at T3 ($p = 0.001$) and T4 ($p = 0.001$) compared to T2, and T4 measures were increased when compared to T3 ($p = 0.01$).

Table 6

*Bench Press and Leg Press Results*

| Variable | Session | PLA Group | CCI Group | CR Group | Time Effect p value | Group Effect p value | Group x Time Effect p value |
|---|---|---|---|---|---|---|---|
| 1-RM | T1 | 82.7 | 81.8 | 100.0 | - | - | - |
| Bench Press | T2 | 87.1 | 83.0 | 95.1 | - | - | - |
| (kg) | T3 | 92.3 | 90.6 | 106.8 | - | - | - |
| | T4 | 92.4 | 93.8 | 112.1 | * 0.001 | 0.24 | 0.11 |
| Relative | T1 | 1.08 | 1.02 | 1.08 | - | - | - |
| 1-RM | T2 | 1.12 | 1.04 | 1.03 | - | - | - |
| Bench Press | T3 | 1.15 | 1.11 | 1.15 | - | - | - |
| (kg/kg) | T4 | 1.18 | 1.17 | 1.21 | * 0.001 | 0.76 | 0.32 |
| 1-RM | T1 | 266.8 | 261.4 | 308.0 | - | - | - |
| Leg Press | T2 | 301.1 | 273.1 | 301.0 | - | - | - |
| (kg) | T3 | 318.6 | 301.0 | 348.9 | - | - | - |
| | T4 | 309.5 | 306.5 | 344.3 | * 0.001 | * 0.01 | 0.39 |
| Relative | T1 | 3.49 | 3.25 | 3.35 | - | - | - |
| Leg Press | T2 | 3.87 | 3.38 | 3.30 | - | - | - |
| (kg/kg) | T3 | 3.95 | 3.65 | 3.77 | - | - | - |
| | T4 | 3.93 | 3.80 | 3.73 | * 0.001 | * 0.03 | 0.35 |

*Note*: Absolute values are reported as mean (±SD). Statistical analysis was performed on delta values. Significances are indicated with an asterisk.

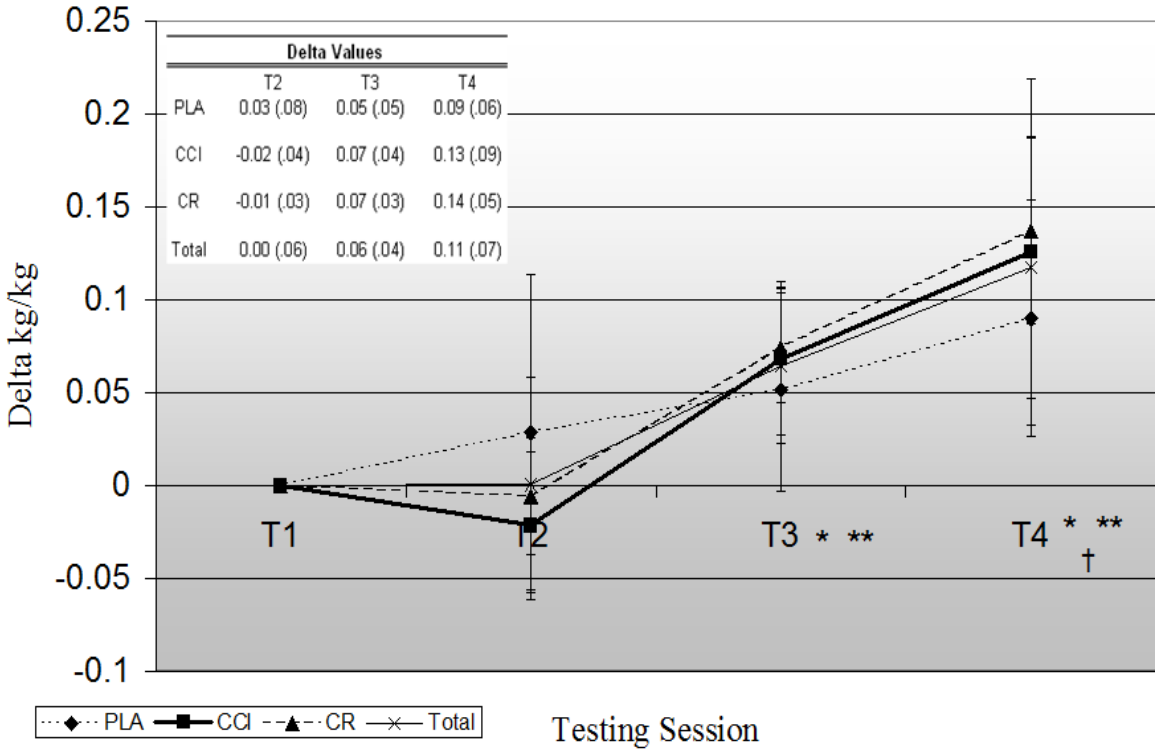


*Figure 9*. Changes in relative 1-RM bench press. Delta values were reported as mean (± SD). * indicates a significant increase in relative 1-RM at T3 ($p = 0.001$) and T4 ($p = 0.001$). ** indicates a significant increase at T3 ($p = 0.001$) and T4 ($p = 0.001$) compared to T2. † indicates a significant increase in relation to T3 ($p = 0.01$).

*Relative 1-RM Leg Press*

1-RM relative leg press presented a significant Group main effect ($p = 0.026$, effect size = 0.096), in which strength was significantly greater in the CCI group compared to CR ($p = 0.04$), with a trend in relation to PLA ($p < 0.10$). Time main effects were observed ($p = 0.001$, effect size = 0.373), in which relative leg press significantly increased at T3 ($p = 0.001$) and T4 ($p = 0.001$). Furthermore, 1-RM relative leg press measures were increased at T3 ($p = 0.01$) and T4 ($p = 0.01$) when compared to T2.

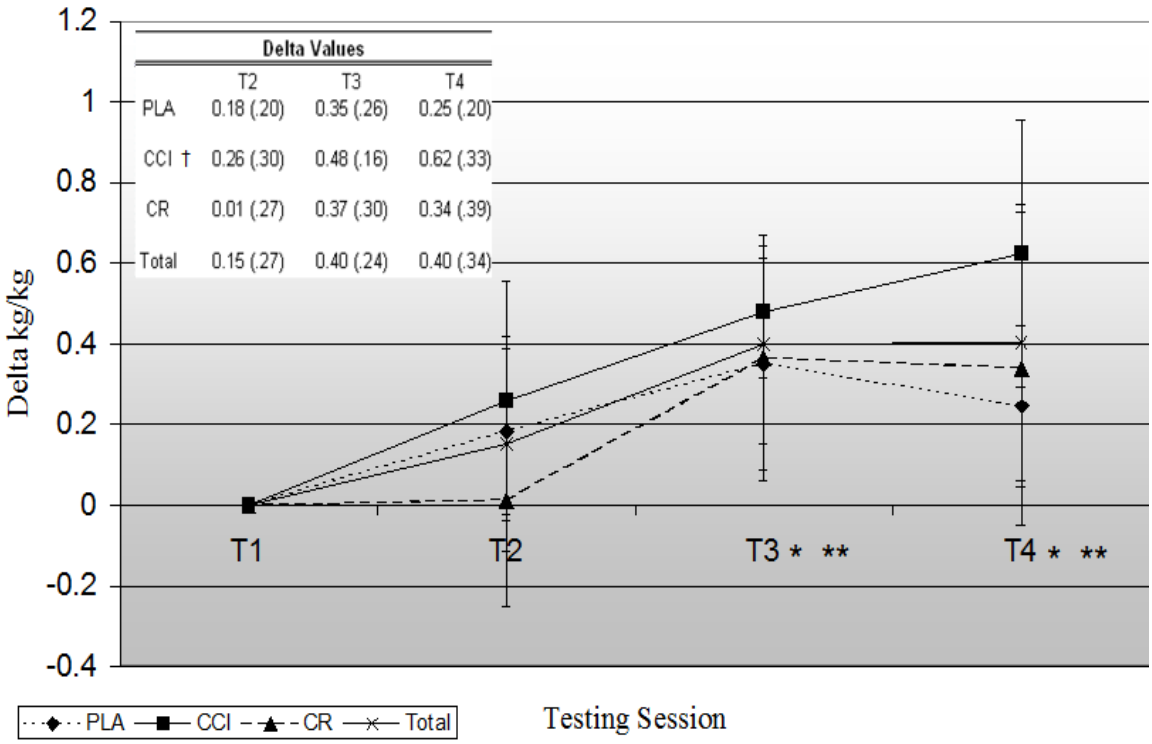


*Figure 10*. Changes in relative 1-RM leg press. Delta values were reported as mean (± SD). * indicates a significant increase in relative 1-RM at T3 ($p = 0.001$) and T4 ($p = 0.001$). ** indicates a significant increase at T3 ($p = 0.01$) and T4 ($p = 0.01$) compared to T2. † indicates a significant increase compared to CR ($p = 0.04$).

*Wingate Anaerobic Power*

Anaerobic performance variables were assessed through the use of a Wingate test. No significant Group x Time interactions or main effects for Group were located for any of the Wingate dependent variables, but significant main effects for Time were revealed (see Table 4). Relative peak power increased at T4 ($p = 0.02$). Furthermore, relative peak power increased at T4 compared to T2 ($p = 0.01$), with a trend in relation to T3 ($p = 0.058$, effect size = 0.159). A trend was observed in which relative mean power was increased at T4 ($p < 0.10$, effect size = 0.095). Rate to fatigue was significantly increased at T4 ($p = 0.01$) and was also increased at T4 compared to T2 ($p = 0.04$).

*Performance Variables Hypothesis Conclusion*

- The $H_2$ hypothesis stating that there will be a significant improvement in muscle strength and power compared to placebo is partially accepted. The hypothesis is accepted in relation to the CCI group for 1-RM leg press, but is rejected for the other measures of strength and power. The $H_2$ hypothesis is rejected for the CR group since no significances were found in relation to strength and power.

## *Serum Variables*

*Serum IGF-1, Insulin, Creatine, and Creatinine*

Serum variables (IGF-1, insulin, and creatine) were grouped together and analyzed using delta values. There were no significant multivariate main effects for Group x Time interaction ($p = 0.84$, effect size = 0.054) or Time ($p = 0.62$, effect size = 0.033), but a trend regarding multivariate main effects for Group was observed ($p < 0.10$, effect size = 0.080). Serum concentration changes for insulin, IGF-1, and creatine are presented in Figures 11, 12, and 13, respectively. The mean correlation coefficient of variation between duplicates for serum insulin, IGF-1, and creatine plates was 15.32%, 3.25%, and 12.5%, respectively. The standard curve correlation coefficient between the plates used for insulin, IGF-1, and creatine was 0.997, 0.998, and, 0.999 respectively.

There were no significances or trends in regards to Group ($p = 0.54$, effect size = 0.017) or Time ($p = 0.40$, effect size = 0.040) main effects for insulin, nor were there any significant Group ($p = 0.59$, effect size = 0.015) or Time ($p = 0.40$, effect size = 0.040) main effects for IGF-1. However, serum creatine displayed a significant main effect for Group ($p = 0.01$, effect size = 0.133), in which PLA concentration was greater than CCI ($p = 0.01$). Table 8 displays serum creatine raw data.

Table 7

*Absolute Wingate Results*

| Variable | Session | PLA Group (±SD) | CCI Group (±SD) | CR Group (±SD) | Time Effect p value | Group Effect p value | Group x Time p value |
|---|---|---|---|---|---|---|---|
| Peak Power/Mass | T1 | 15.8 (1.9) | 16.3 (2.1) | 14.1 (3.0) | - | - | - |
| (W/kg) | T2 | 16.8 (2.8) | 16.1 (1.8) | 13.2 (2.2) | - | - | - |
| | T3 | 15.9 (2.0) | 16.3 (1.3) | 14.7 (2.5) | - | - | - |
| | T4 | 17.7 (2.2) | 17.8 (1.9) | 15.2 (3.4) | * 0.006 | 0.394 | 0.584 |
| Mean Power/Mass | T1 | 8.3 (0.6) | 8.7 (0.6) | 7.7 (1.2) | - | - | - |
| (W/kg) | T2 | 8.4 (1.1) | 8.7 (0.6) | 7.7 (1.0) | - | - | - |
| | T3 | 8.4 (0.9) | 8.9 (0.5) | 8.1 (1.0) | - | - | - |
| | T4 | 8.6 (1.0) | 9.3 (0.6) | 8.0 (1.1) | 0.064 | 0.872 | 0.863 |
| Rate to Fatigue (W/Sec) | T1 | 30.2 (3.8) | 36.3 (3.9) | 34.2 (7.3) | - | - | - |
| | T2 | 38.9 (9.3) | 35.1 (6.5) | 32.3 (8.1) | - | - | - |
| | T3 | 36.5 (6.2) | 37.4 (5.6) | 38.1 (6.5) | - | - | - |
| | T4 | 41.0 (8.3) | 41.1 (6.1) | 40.8 (5.4) | * 0.010 | 0.270 | 0.799 |

*Note*: This data represents the absolute Wingate performance values over the course of the study. Data are expressed as absolute means (± SD). Significant delta Time effects, Group effects, and/or Group x Time effects contain an asterisk.

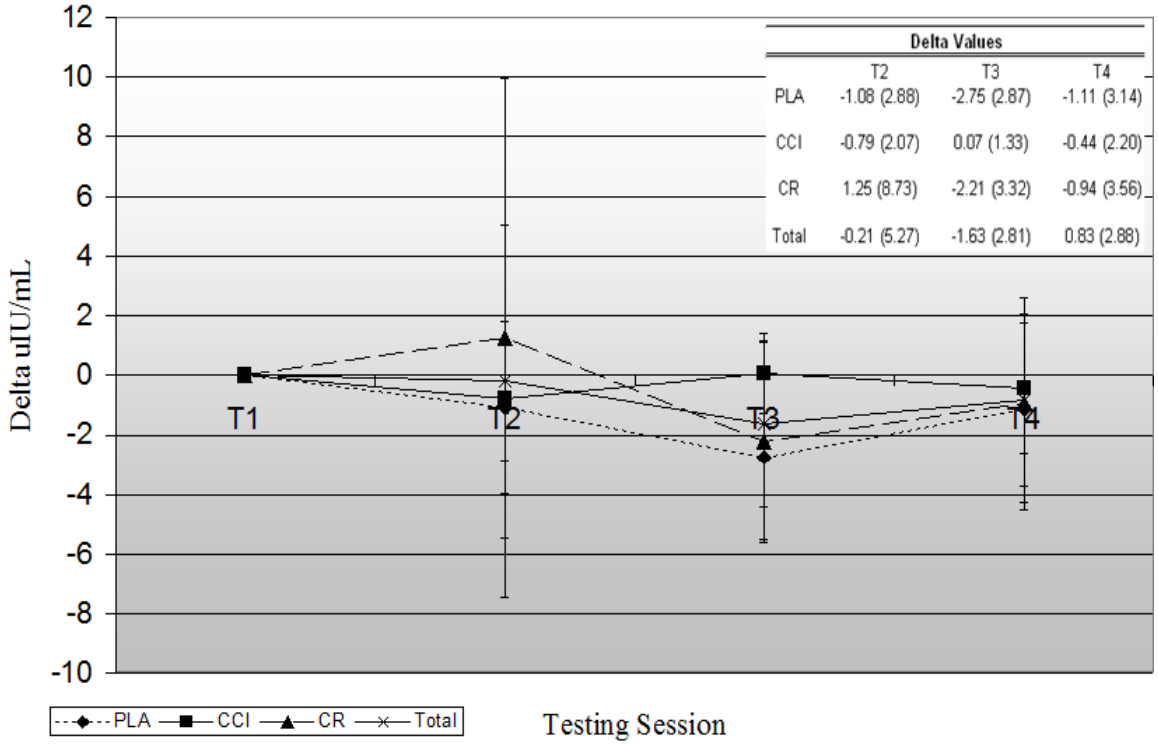


*Figure 11*. Changes in serum insulin. Delta values were reported as mean (± SD).

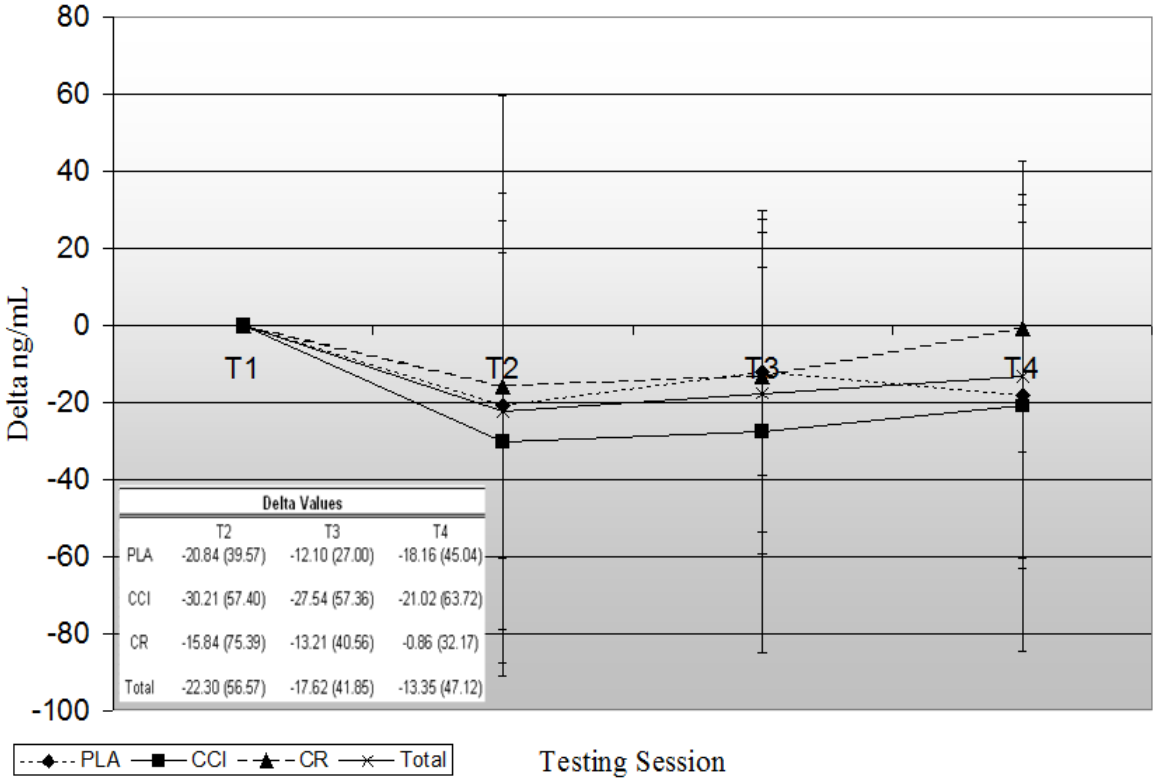


*Figure 12*. Changes in serum IGF-1. Delta values were reported as mean (± SD).

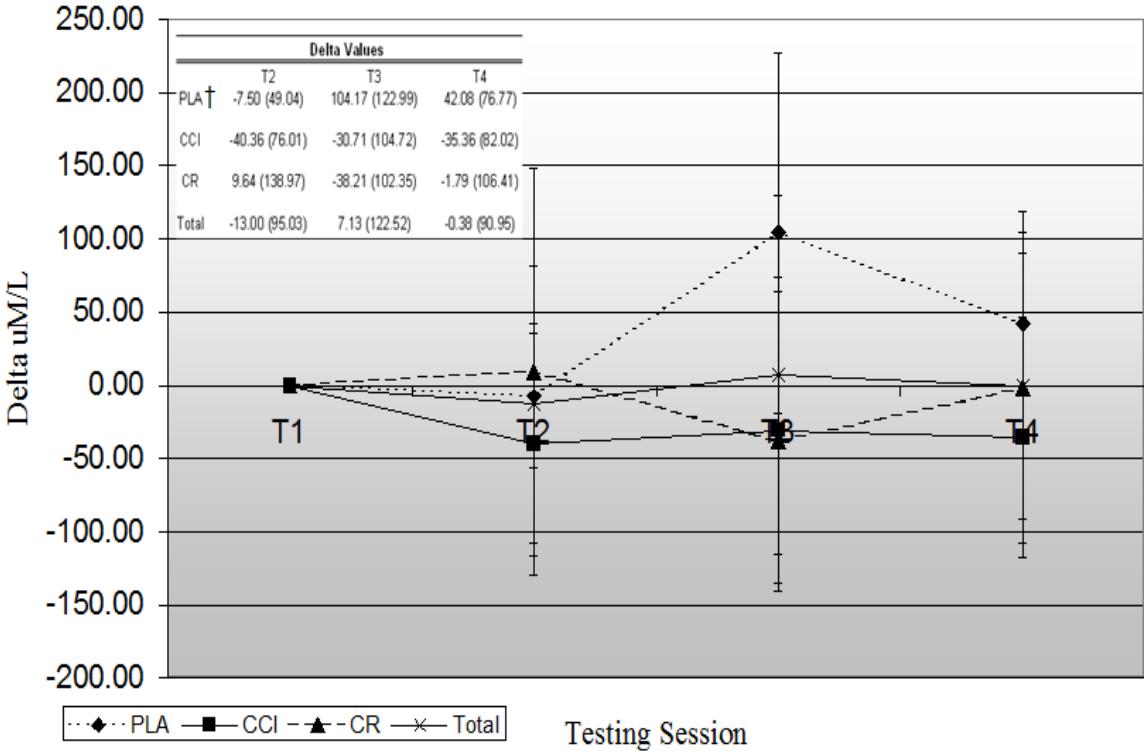


*Figure 13*. Changes in serum creatine concentration. Delta values were reported as mean (± SD). The symbol, †, indicates a significant group main effect compared to CCI ($p = 0.01$).

Table 5

*Absolute Serum Creatine Concentration*

| | Serum Creatine Assay (μM/L) | | | |
|---|---|---|---|---|
| | T1 | T2 | T3 | T4 |
| PLA | 308.5 (53.5) | 301.3 (28.5) | 412.0 (189.7) | 350.8 (61.7) |
| CCI | 395.0 (64.9) | 346.3 (26.7) | 362.1 (72.6) | 357.5 (48.3) |
| CR | 349.6 (85.8) | 355.4 (76.9) | 302.5 (43.0) | 354.6 (62.0) |

*Note*: Data is expressed as mean (±SD).

*Serum Creatinine*

Serum creatinine was a notable variable that did not result in any significant differences. Creatinine was statistically analyzed as delta values and grouped with the other blood clinical chemistry markers. Specifically, there were no main effects for Time

(p = 0.34, effect size = 0.126), Group (p = 0.11, effect size = 0.059), or Group x Time interaction (p = 0.73, effect size = 0.069). Figure 14 displays the changes that occurred over the course of the study, and is presented in absolute values to emphasize that creatinine values were within normal clinical ranges. Table 9 displays absolute values.

Table 6

*Absolute Serum Creatinine Concentration*

| | Serum Creatinine (mg/dL) | | | |
|---|---|---|---|---|
| | T1 | T2 | T3 | T4 |
| PLA | 1.08 (.08) | 1.20 (.14) | 1.04 (.16) | 1.09 (.13) |
| CCI | 1.01 (.07) | 1.10 (.08) | 1.17 (.15) | 1.17 (.10) |
| CR | 0.93 (.17) | 1.10 (.22) | 1.13 (.17) | 1.11 (.13) |

*Note*: Data are reported as mean (± SD).

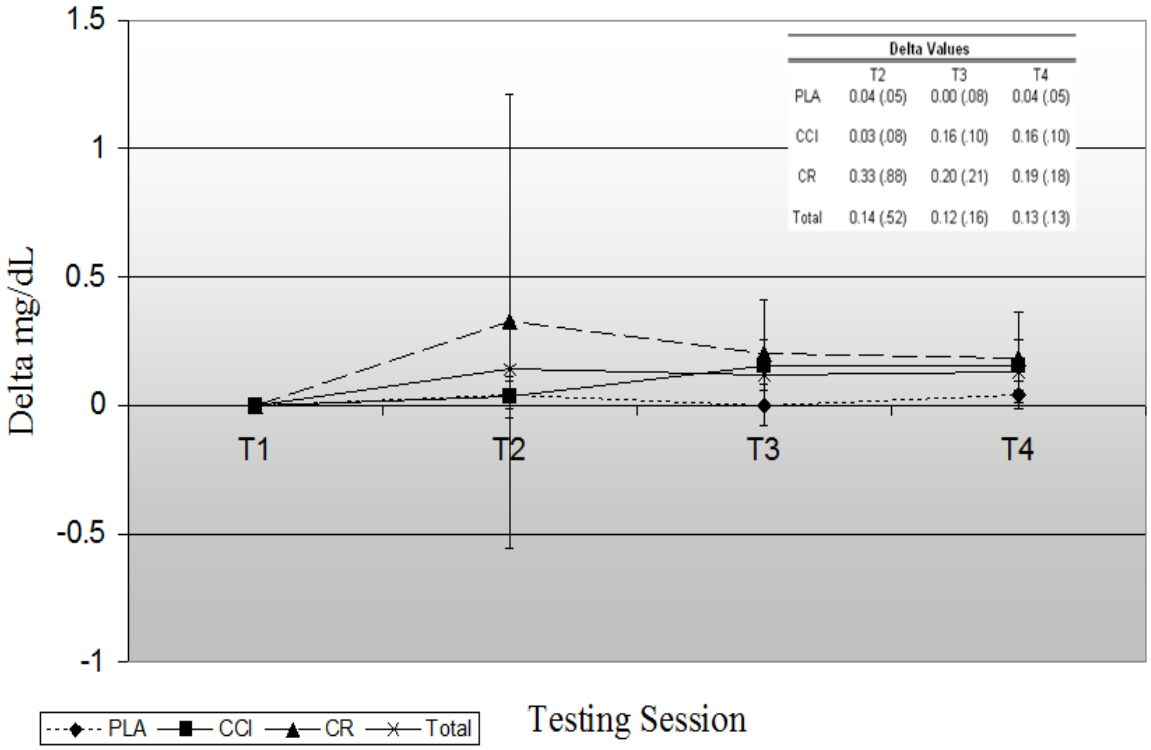


*Figure 14*. Serum creatinine changes.

*Serum Variables Hypotheses Conclusion*

- The $H_1$ hypothesis stating that following CR and CCI supplementation, serum creatine concentration will significantly increase when compared to placebo is rejected.

- The $H_5$ hypothesis stating that there will be no significant differences in serum insulin and IGF-1 between groups is accepted.

- The $H_8$ hypothesis, in relation to serum creatinine, is accepted since there were no significant alterations outside of normal clinical ranges following creatine supplementation.

### *CreaT1 and GLUT-4 Skeletal Muscle mRNA Expression*

CreaT1 and GLUT-4 mRNA expression variables were grouped together and analyzed using delta values. No significant multivariate Group x Time interaction ($p = 0.61$, effect size = 0.069) or main effects for Time ($p = 0.65$, effect size = 0.030) were observed for CreaT1 or GLUT-4 expression, but a significant multivariate Group main effect ($p = 0.01$, effect size = 0.091) was located, in which GLUT-4 expression was greater in the CR group compared to PLA ($p = 0.03$). Other than this, there were no other Group or Time main effects for either variable. Figures 15 and 16 demonstrate the delta expression for the CreaT1 and GLUT-4 genes over the course of time, respectively. Table 10 displays absolute mRNA expression for both genes relative to β-actin.

*Skeletal Muscle mRNA Expression Hypotheses Conclusion*

- The $H_3$ hypothesis stating that there will be no significant differences in relation to muscle expression of CreaT1 mRNA expression in the CCI or CR group compared to placebo is accepted.

- The $H_4$ hypothesis stating that there will be no significant differences in relation to muscle expression of GLUT-4 mRNA compared to placebo is accepted. The hypothesis is accepted in relation to CCI, and also accepted for the CR group since a significant increase in GLUT-4 mRNA expression was not observed when compared to PLA.

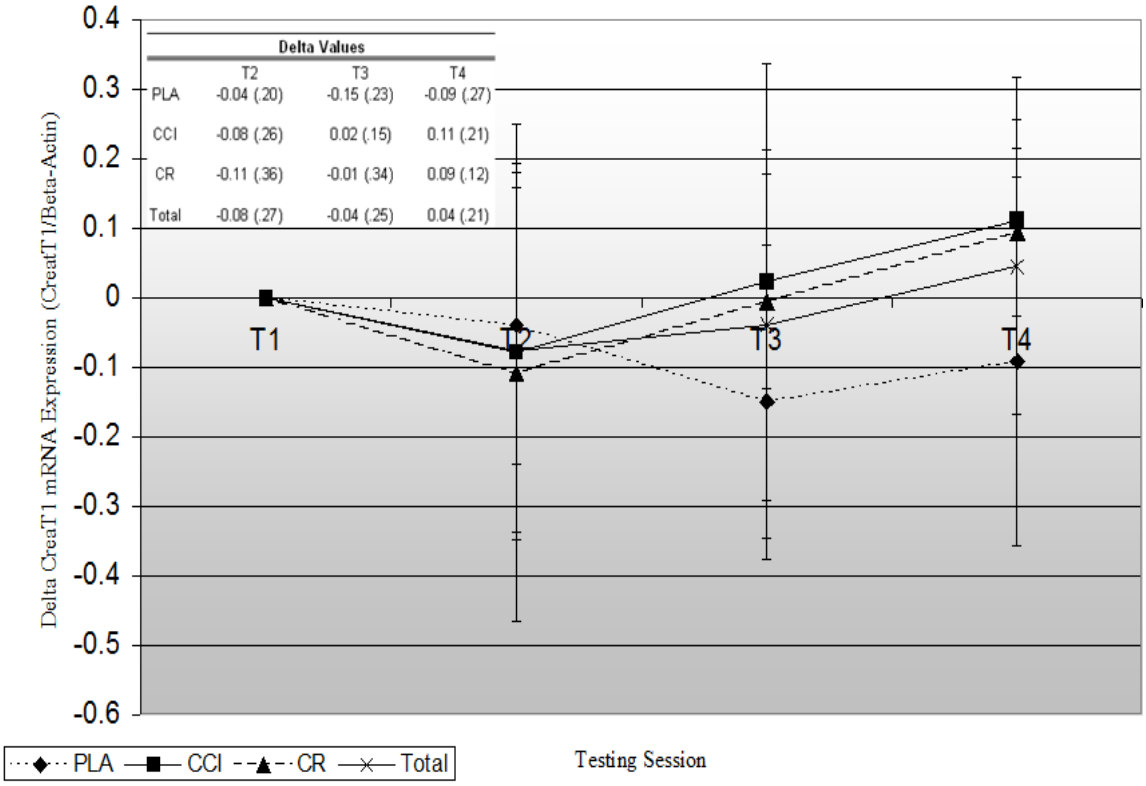


*Figure 15*. Changes in CreaT1 mRNA expression relative to beta-actin. Delta values were reported as mean (± SD).

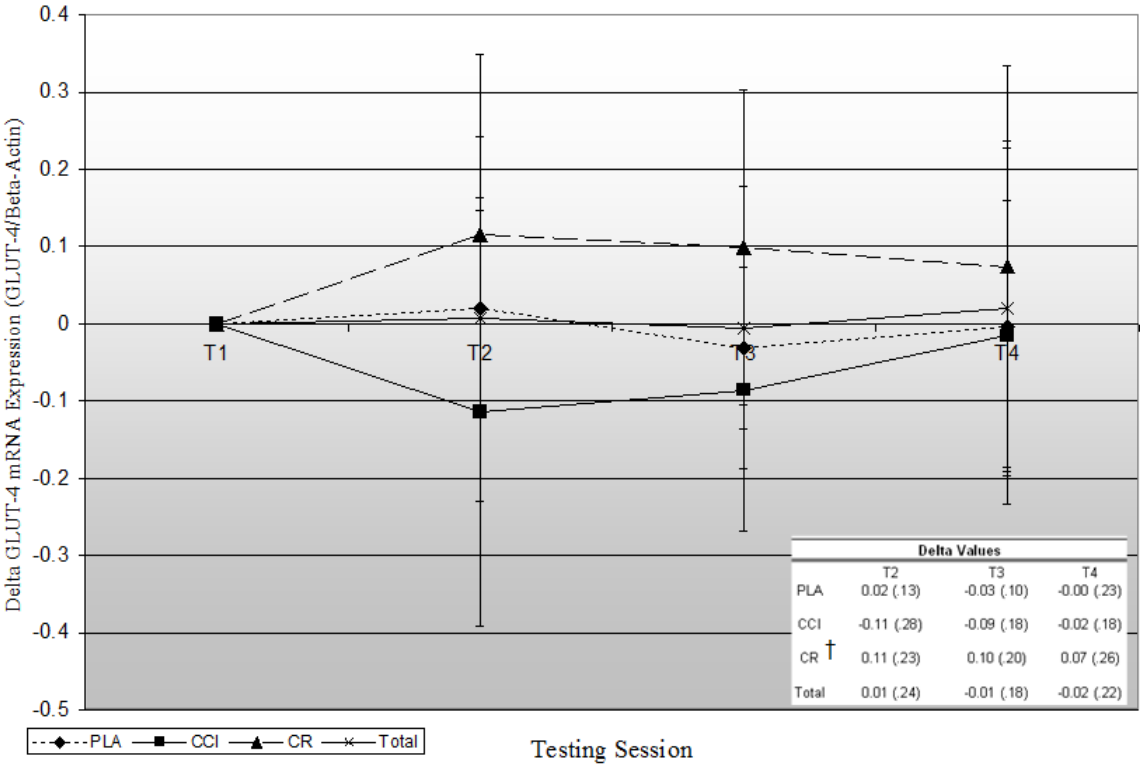


*Figure 16*. Changes in GLUT-4 mRNA expression relative to beta-actin. Delta values were reported as mean (± SD). The symbol, †, indicates a significant group main effect in comparison to CCI ($p = 0.03$).

Table 7

*Absolute CreaT1 and GLUT-4 mRNA Expression Relative to β-actin*

| Variable | Group | Session | Ratio | ± SD |
|---|---|---|---|---|
| CreaT1 mRNA | PLA | T1 | 1.37 | 0.21 |
| | | T2 | 1.32 | 0.11 |
| | | T3 | 1.24 | 0.11 |
| | | T4 | 1.27 | 0.25 |
| | CCI | T1 | 1.07 | 0.14 |
| | | T2 | 1.00 | 0.26 |
| | | T3 | 1.10 | 0.15 |
| | | T4 | 1.18 | 0.12 |
| | CR | T1 | 1.11 | 0.20 |
| | | T2 | 0.97 | 0.19 |
| | | T3 | 1.11 | 0.29 |
| | | T4 | 1.19 | 0.23 |
| GLUT-4 mRNA | PLA | T1 | 1.19 | 0.06 |
| | | T2 | 1.21 | 0.09 |
| | | T3 | 1.17 | 0.07 |
| | | T4 | 1.19 | 0.19 |
| | CCI | T1 | 1.16 | 0.12 |
| | | T2 | 1.03 | 0.23 |
| | | T3 | 1.06 | 0.11 |
| | | T4 | 1.13 | 0.09 |
| | CR | T1 | 0.93 | 0.19 |
| | | T2 | 1.03 | 0.19 |
| | | T3 | 1.04 | 0.13 |
| | | T4 | 1.00 | 0.18 |

*Note*: Ratios are determined by dividing the $C_T$ values between the specified gene and β-actin.

### *Skeletal Muscle Protein Variables*

The following delta variables were grouped together for statistical analysis: IRS-1 total protein; Akt total protein; PI3K activity; muscle creatine uptake; GLUT-4 protein; and CreaT1 protein. There were multivariate main effects for Time ($p = 0.001$, effect size = 0.294) and Group ($p = 0.001$, effect size = 0.239), but no Group x Time interaction ($p = 0.614$, effect size = 0.087).

*IRS-1 and Akt Protein Expression and PI3K Activity*

Statistical analysis revealed no significant Group x Time interactions for IRS-1 ($p = 0.92$, effect size = 0.031), Akt ($p = 0.33$, effect size = 0.104), or PI3K activity ($p = 0.87$, effect size = 0.039). Furthermore, there were no Time main effects for IRS-1 ($p = 0.57$, effect size = 0.033), Akt ($p = 0.38$, effect size = 0.049), or PI3K activity ($p = 0.52$, effect size = 0.036). However, Akt content had a statistically significant Group main effect ($p = 0.01$, effect size = 0.131), in which CCI was greater than PLA ($p = 0.01$). Figures 17, 18, and 19 display the delta changes for Akt, IRS-1, and PI3K activity, respectively, whereas Table 11 displays the absolute values and statistical results. The mean correlation coefficient of variation between duplicates for muscle IRS-1, Akt, and PI3K plates was 8.8%, 14.2%, and 13.0%, respectively. The standard curve correlation coefficient between the plates used for IRS-1, Akt, and PI3k was 0.996, 0.993, and 0.970, respectively.

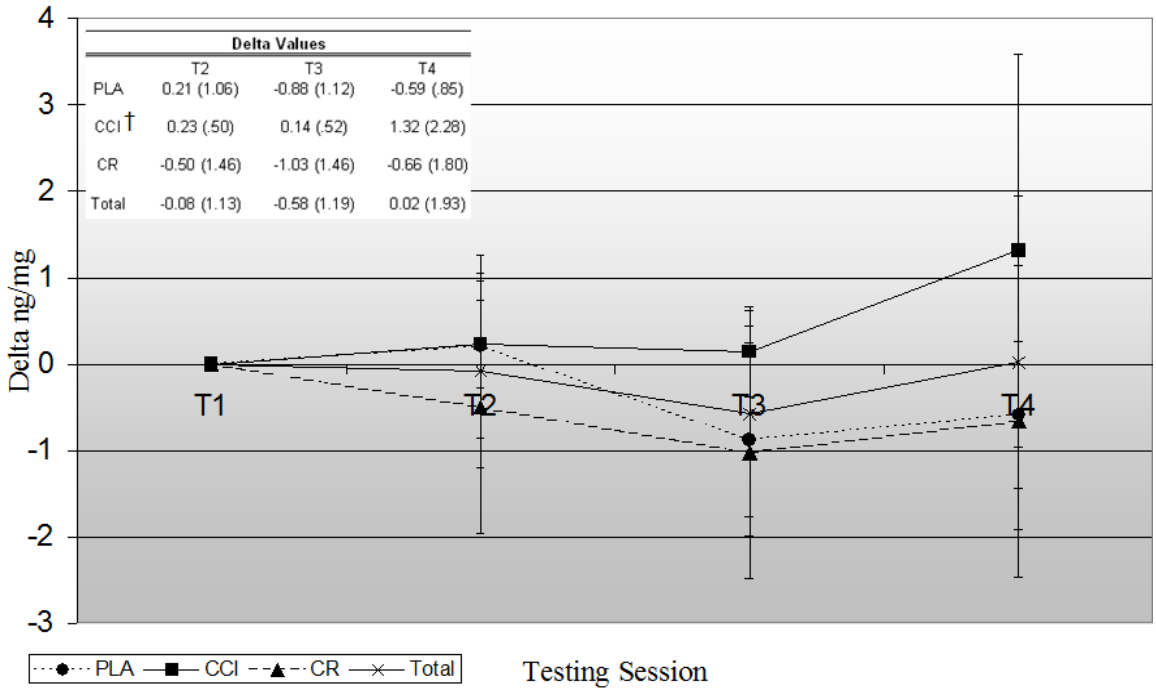


*Figure 17*. Changes in total Akt protein expression. Delta values were reported as mean (± SD). † indicates a significant group main effect when compared to CR ($p = 0.01$).

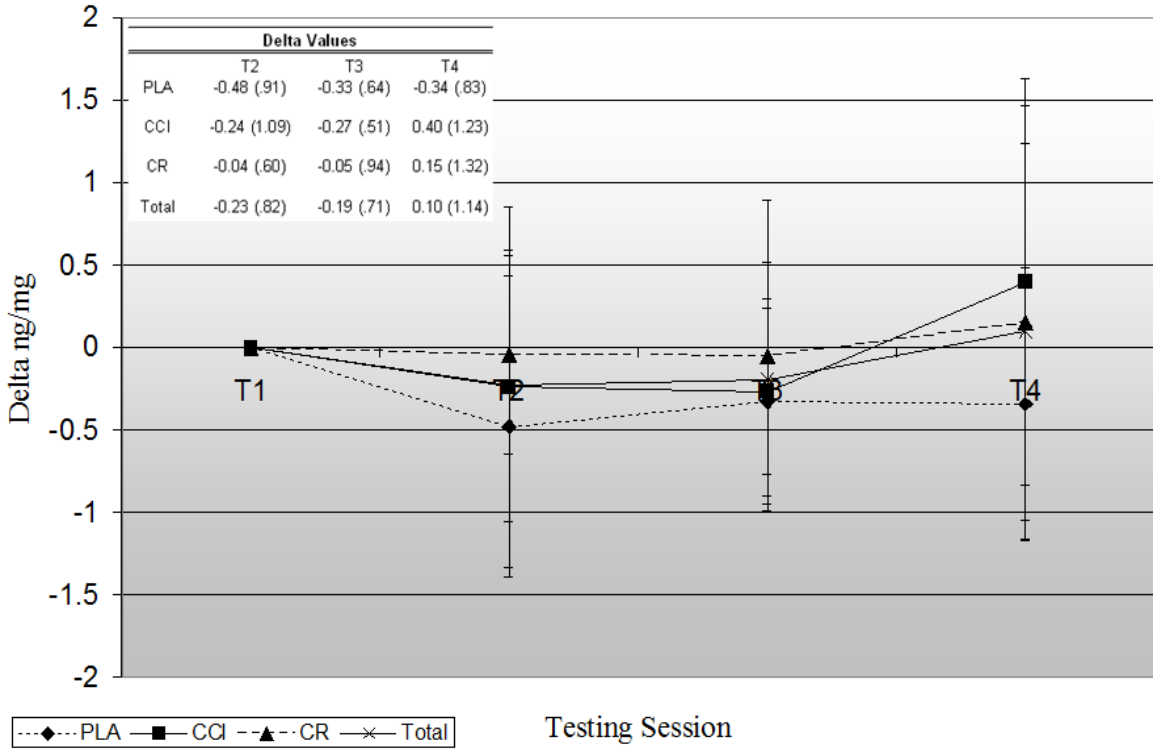


*Figure 18*. Changes in total IRS-1 protein expression. Delta values were reported as mean (± SD).

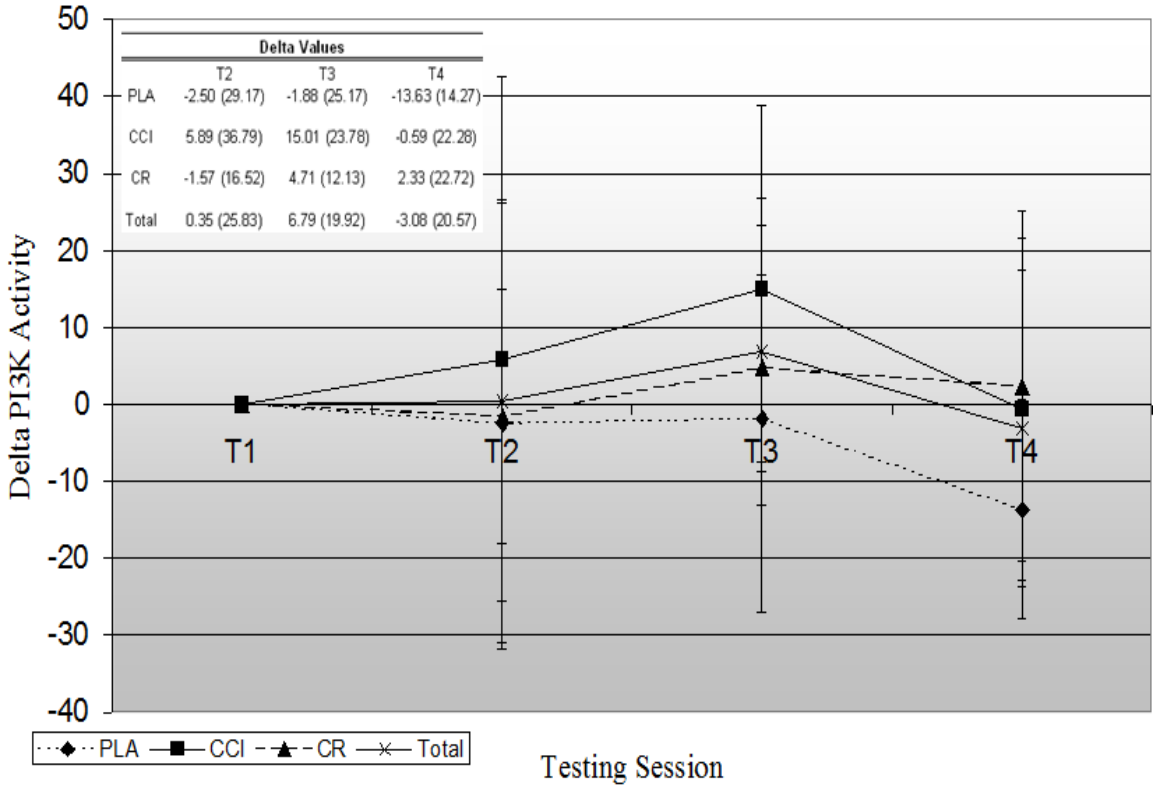


*Figure 19*. Changes in PI3K activity. Delta values were reported as mean (± SD).

Table 11

*Relative IRS-1, Akt, and PI3K Results*

| Variable | Session | PLA Group | CCI Group | CR Group | Time Effect p value | Group Effect p value | Group x Time Effect p value |
|---|---|---|---|---|---|---|---|
| Total IRS-1 (ng/mg) | T1 | 0.97 (.83) | 0.86 (.88) | 1.12 (1.01) | - | - | - |
| | T2 | 0.55 (.30 | 0.81 (.43) | 1.03 (.62) | - | - | - |
| | T3 | 0.45 (0.19) | 0.70 (.55) | 1.04 (.65) | - | - | - |
| | T4 | 0.79 (.53) | 1.30 (.61) | 0.96 (.63) | 0.57 | 0.40 | 0.92 |
| Total Akt (ng/mg) | T1 | 1.66 (0.79) | 0.58 (1.07) | 2.05 (2.19) | - | - | - |
| | T2 | 1.86 (1.45) | 1.47 (1.68) | 1.34 (1.71) | - | - | - |
| | T3 | 0.75 (.30) | 1.09 (1.23) | 0.87 (.94) | - | - | - |
| | T4 | 1.50 (1.17) | 2.01 (2.82) | 1.04 (.70) | 0.38 | * 0.01 | 0.33 |
| PI3K Activity | T1 | 34.83 (9.4) | 32.84 (18.3) | 22.08 (15.8) | - | - | - |
| | T2 | 29.74 (23.9) | 32.21 (22.8) | 20.01 (5.8) | - | - | - |
| | T3 | 27.11 (19.9) | 44.07 (22.2) | 26.77 (15.2) | - | - | - |
| | T4 | 23.36 (9.47) | 30.38 (18.1) | 18.64 (8.9) | 0.52 | 0.26 | 0.87 |

*Note*: Relative values are reported as mean (±SD). Statistical analysis was performed on delta values. Significance is indicated by an asterisk.

*GLUT-4 and CreaT1 Protein Expression*

There was not a significant Group x Time interaction for GLUT-4 protein expression (p = 0.77, effect size = 0.051) or CreaT1 protein expression (p = 0.87, effect size = 0.038). There were no main effects for Time (p = 0.56, effect size = 0.033) or Group (p = 0.16, effect size = 0.059) for GLUT-4 expression. Likewise, for CreaT1 expression, there were no main effects for Time (p = 0.56, effect size = 0.033) or Group (p = 0.30, effect size = 0.039). Figures 20 and 21 exhibit the changes in GLUT-4 and CreaT1 protein expression, respectively, whereas Table 12 displays absolute protein expression for both genes. The mean correlation coefficient of variation between duplicates for GLUT-4 and CreaT1 protein plates was 9.60% and 9.94%, respectively. There was no standard curve for this assay, thus the correlation coefficient for the curve could not be calculated.

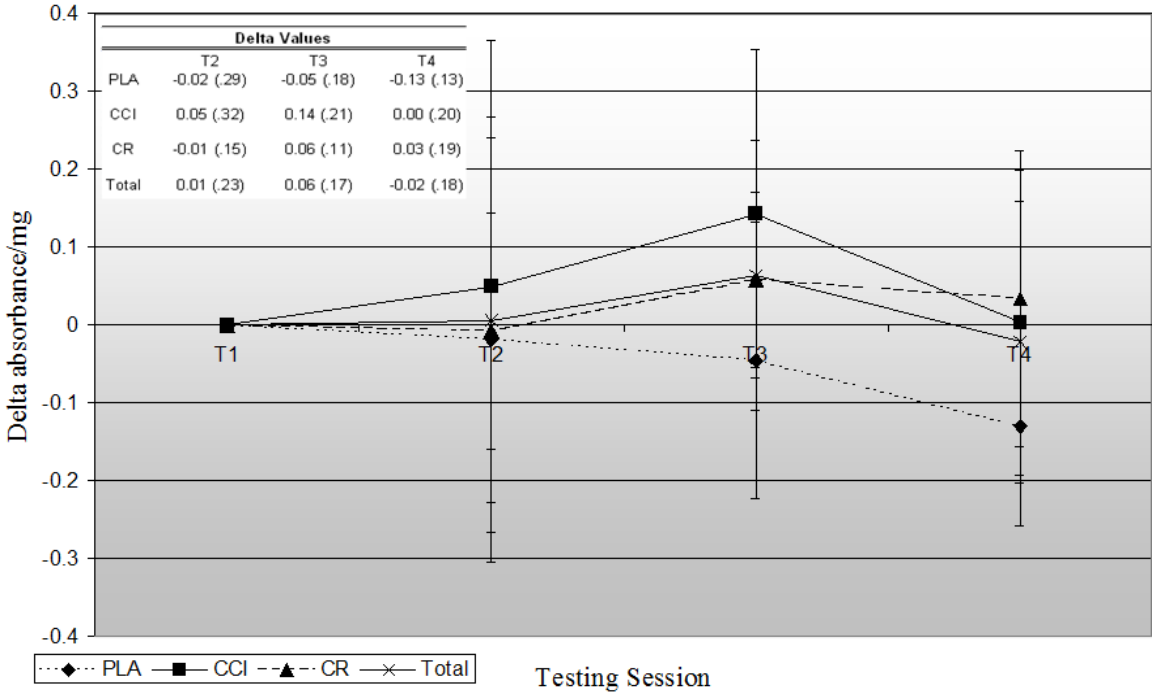


*Figure 20*. Changes in GLUT-4 protein content. Delta values were reported as mean (± SD).

Table 12

*Relative GLUT-4 and CreaT1 Protein Results*

| Variable | Session | PLA Group | CCI Group | CR Group | Time Effect p value | Group Effect p value | Group x Time Effect p value |
|---|---|---|---|---|---|---|---|
| GLUT-4 | T1 | 0.316 (.07) | 0.281 (.15) | 0.204 (.14) | - | - | - |
| (Abs/mg) | T2 | 0.276 (.23) | 0.283 (.19) | 0.193 (.06) | - | - | - |
| | T3 | 0.242 (.17) | 0.394 (.19) | 0.263 (.14) | - | - | - |
| | T4 | 0.213 (.09) | 0.273 (.15) | 0.189 (.07) | 0.56 | 0.16 | 0.77 |
| CreaT1 | T1 | 0.361 (.11) | 0.337 (.17) | 0.214 (.14) | - | - | - |
| (Abs/mg) | T2 | 0.306 (.22) | 0.316 (.19) | 0.197 (.05) | - | - | - |
| | T3 | 0.277 (.21) | 0.435 (.21) | 0.270 (.14) | - | - | - |
| | T4 | 0.251 (.09) | 0.297 (.18) | 0.189 (.06) | 0.56 | 0.30 | 0.87 |

*Note*: Values are absorbances at 450 nm relative to muscle weight reported as mean (±SD). Statistical analysis was performed on delta values.

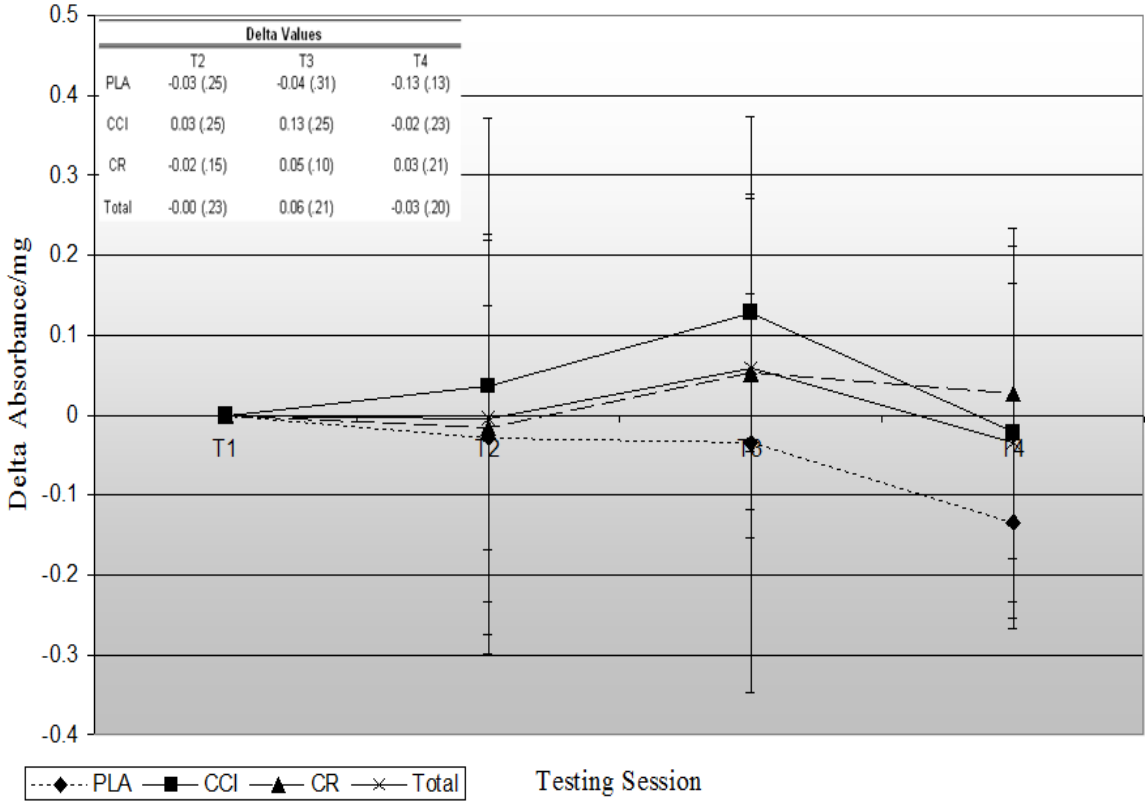


*Figure 21*. Changes in CreaT1 protein content. Delta values were reported as mean (± SD).

*Skeletal Muscle Protein Variables Hypotheses Conclusion*

- The $H_5$ hypothesis stating that there will be no significant differences in the total content of the insulin signaling pathway (IRS-1, PI3K, Akt) is partially accepted. The hypothesis is accepted in relation to IRS-1 and PI3K, but rejected for Akt, since the CCI group displayed a significantly greater total Akt protein content when compared to CR.

- The $H_3$ and $H_4$ hypotheses respectively stating that there will be no significant increase in relation to muscle expression of CreaT1 and GLUT-4 protein following CR and CCI supplementation when compared to PLA is accepted.

*Whole Blood Clinical Chemistry*

The data presented in Table 13 display the changes in whole blood markers over the course of the study. There was not a significant multivariate Group x Time interaction for any of these values ($p = 0.21$, effect size = 0.180), but there were significant

multivariate main effects for Group ($p = 0.003$, effect size = 0.302) and Time ($p = 0.03$, effect size = 0.356).

There were no significant Group main effects for white blood cell count ($p = 0.59$), neutrophils ($p = 0.37$), lymphocytes ($p = 0.14$), eosinophils ($p = 0.36$), basophils ($p = 0.56$), red blood cells ($p = 0.47$), or hematocrit ($p = 0.26$). However, a Group main effect trend occurred, in which the CR group had a greater hemoglobin content compared to PLA ($p = 0.074$, effect size = 0.076). No significant Time main effects were observed for lymphocytes ($p = 0.47$), monocytes ($p = 0.35$), eosinophils ($p = 0.12$), or basophils ($p = 0.82$). However, red blood cell count decreased at T2 ($p = 0.04$) and T3 ($p = 0.004$). Hemoglobin content also decreased at T3 ($p = 0.002$). Furthermore, there were Time main effect trends in which the following occurred: white blood cell counts decreased at T2 ($p < 0.10$, effect size = 0.105) and T3 ($p < 0.10$, effect size = 0.105); neutrophil values decreased at T3 ($p = 0.061$, effect size = 0.097); and hematocrit decreased at T2 ($p = 0.059$, effect size = 0.151). Since all of these markers of clinical blood chemistry remained within the clinical range, and because there were no significant Group x Time interactions, it is concluded that the various creatine formulations do not adversely affect these whole blood clinical markers.

*Whole Blood Clinical Chemistry Hypothesis Conclusion*

- The $H_8$ hypothesis, in relation to whole blood markers, stating that there will be no significant alterations in clinical chemistry outside of normal ranges following creatine supplementation is accepted.

Table 8

*Whole Blood Clinical Chemistry Markers*

| Variable | Group | Session | Mean | ± SD | Variable | Group | Session | Mean | ± SD |
|---|---|---|---|---|---|---|---|---|---|
| WBC | PLA | T1 | 6.13 | 1.21 | RBC | PLA | T1 | 5.44 | 0.32 |
| K/uL | | T2 | 6.13 | 1.41 | M/uL | | T2 | 5.27 | 0.29 |
| | | T3 | 4.72 | 0.77 | | | T3 | 5.17 | 0.24 |
| | | T4 | 5.63 | 1.58 | | | T4 | 5.46 | 0.45 |
| | CCI | T1 | 6.25 | 1.78 | | CCI | T1 | 5.29 | 0.46 |
| | | T2 | 6.27 | 1.97 | | | T2 | 5.04 | 0.22 |
| | | T3 | 5.84 | 1.16 | | | T3 | 5.10 | 0.34 |
| | | T4 | 6.15 | 1.73 | | | T4 | 5.04 | 0.30 |
| | CR | T1 | 7.20 | 1.58 | | CR | T1 | 5.18 | 0.43 |
| | | T2 | 7.21 | 2.19 | | | T2 | 5.04 | 0.39 |
| | | T3 | 5.88 | 0.70 | | | T3 | 4.94 | 0.39 |
| | | T4 | 6.87 | 2.56 | | | T4 | 5.11 | 0.39 |
| NEU | PLA | T1 | 3.49 | 1.02 | HGB | PLA | T1 | 15.11 | 1.37 |
| K/uL | | T2 | 3.37 | 1.21 | g/dL | | T2 | 14.83 | 1.22 |
| | | T3 | 2.25 | 0.67 | | | T3 | 14.40 | 0.99 |
| | | T4 | 2.85 | 1.11 | | | T4 | 15.21 | 1.65 |
| | CCI | T1 | 3.52 | 1.41 | | CCI | T1 | 15.57 | 0.87 |
| | | T2 | 3.36 | 1.53 | | | T2 | 14.80 | 0.55 |
| | | T3 | 3.13 | 0.31 | | | T3 | 14.77 | 0.71 |
| | | T4 | 3.58 | 1.50 | | | T4 | 14.74 | 0.93 |
| | CR | T1 | 4.35 | 1.39 | | CR | T1 | 15.01 | 1.07 |
| | | T2 | 3.95 | 1.29 | | | T2 | 14.71 | 0.90 |
| | | T3 | 2.93 | 0.62 | | | T3 | 14.41 | 0.84 |
| | | T4 | 3.94 | 2.16 | | | T4 | 14.73 | 0.91 |
| LYM | PLA | T1 | 1.76 | 0.29 | HCT | PLA | T1 | 48.49 | 3.98 |
| K/uL | | T2 | 1.84 | 0.42 | % | | T2 | 46.93 | 3.33 |
| | | T3 | 1.76 | 0.45 | | | T3 | 46.16 | 2.65 |
| | | T4 | 1.97 | 0.47 | | | T4 | 48.73 | 4.71 |
| | CCI | T1 | 1.86 | 0.60 | | CCI | T1 | 48.97 | 3.21 |
| | | T2 | 2.00 | 0.66 | | | T2 | 46.80 | 1.40 |
| | | T3 | 1.81 | 0.62 | | | T3 | 47.57 | 2.86 |
| | | T4 | 1.72 | 0.44 | | | T4 | 46.54 | 2.29 |
| | CR | T1 | 1.84 | 0.68 | | CR | T1 | 48.03 | 3.51 |
| | | T2 | 2.32 | 0.97 | | | T2 | 46.74 | 2.77 |
| | | T3 | 2.09 | 0.58 | | | T3 | 46.06 | 2.89 |
| | | T4 | 2.13 | 0.63 | | | T4 | 48.83 | 3.84 |

(*Table Continues*)

| Variable | Group | Session | Mean | ± SD | Variable | Group | Session | Mean | ± SD |
|---|---|---|---|---|---|---|---|---|---|
| MONO | PLA | T1 | 0.61 | 0.23 | MCV | PLA | T1 | 88.99 | 3.83 |
| K/uL | | T2 | 0.62 | 0.20 | fL | | T2 | 88.93 | 3.52 |
| | | T3 | 0.46 | 0.15 | | | T3 | 89.31 | 3.75 |
| | | T4 | 0.54 | 0.23 | | | T4 | 89.16 | 3.00 |
| | CCI | T1 | 0.53 | 0.22 | | CCI | T1 | 92.81 | 3.54 |
| | | T2 | 0.51 | 0.14 | | | T2 | 92.86 | 3.14 |
| | | T3 | 0.53 | 0.18 | | | T3 | 93.40 | 3.33 |
| | | T4 | 0.52 | 0.11 | | | T4 | 92.43 | 3.42 |
| | CR | T1 | 0.73 | 0.36 | | CR | T1 | 92.91 | 4.47 |
| | | T2 | 0.65 | 0.21 | | | T2 | 92.90 | 4.37 |
| | | T3 | 0.58 | 0.12 | | | T3 | 93.44 | 4.54 |
| | | T4 | 0.57 | 0.18 | | | T4 | 95.70 | 7.07 |
| EOS | PLA | T1 | 0.21 | 0.10 | MCH | PLA | T1 | 27.76 | 1.60 |
| K/uL | | T2 | 0.24 | 0.16 | pg | | T2 | 28.10 | 1.60 |
| | | T3 | 0.19 | 0.10 | | | T3 | 27.86 | 1.55 |
| | | T4 | 0.20 | 0.11 | | | T4 | 27.86 | 1.45 |
| | CCI | T1 | 0.28 | 0.26 | | CCI | T1 | 29.53 | 1.30 |
| | | T2 | 0.33 | 0.35 | | | T2 | 29.39 | 1.27 |
| | | T3 | 0.29 | 0.24 | | | T3 | 29.01 | 1.41 |
| | | T4 | 0.26 | 0.22 | | | T4 | 29.29 | 1.28 |
| | CR | T1 | 0.21 | 0.11 | | CR | T1 | 29.10 | 1.67 |
| | | T2 | 0.21 | 0.12 | | | T2 | 29.27 | 1.96 |
| | | T3 | 0.20 | 0.08 | | | T3 | 29.31 | 1.57 |
| | | T4 | 0.16 | 0.07 | | | T4 | 28.86 | 1.69 |
| BASO | PLA | T1 | 0.05 | 0.03 | MCHC | PLA | T1 | 31.17 | 0.64 |
| K/uL | | T2 | 0.06 | 0.03 | g/dL | | T2 | 31.57 | 0.64 |
| | | T3 | 0.06 | 0.02 | | | T3 | 31.17 | 0.49 |
| | | T4 | 0.07 | 0.04 | | | T4 | 31.24 | 0.85 |
| | CCI | T1 | 0.07 | 0.03 | | CCI | T1 | 31.81 | 0.39 |
| | | T2 | 0.06 | 0.02 | | | T2 | 31.66 | 0.48 |
| | | T3 | 0.08 | 0.02 | | | T3 | 31.06 | 0.85 |
| | | T4 | 0.07 | 0.02 | | | T4 | 31.67 | 0.64 |
| | CR | T1 | 0.07 | 0.02 | | CR | T1 | 31.29 | 0.61 |
| | | T2 | 0.08 | 0.04 | | | T2 | 31.47 | 0.81 |
| | | T3 | 0.08 | 0.03 | | | T3 | 31.37 | 0.50 |
| | | T4 | 0.07 | 0.04 | | | T4 | 30.20 | 1.03 |

*Serum Clinical Chemistry Markers*

There were significant multivariate main effects for Time ($p = 0.01$), Group ($p = 0.001$), and Group x Time interaction ($p = 0.02$), but all of these variables remained within normal clinical ranges. Most of the Group significances took place in the CR group when compared to CCI or PLA. Specifically, total cholesterol ($p = 0.04$) and LDL

($p = 0.03$) measures were significantly greater in the CR group when compared to PLA. BUN/creatinine ($p = 0.001$), calcium ($p = 0.002$), albumin ($p = 0.041$), total bilirubin ($p = 0.02$), and alkaline phosphatase ($p = 0.01$) were greater in the CR group when compared to CCI. The PLA group had a significant Group main effect in which BUN was significantly greater when compared to CCI ($p = 0.001$). The complete panel of serum chemistry data is presented in appendix D.

*Serum Clinical Chemistry Hypothesis Conclusion*

- The $H_8$ hypothesis, in relation to serum blood markers, stating that there will be no significant alterations in clinical chemistry outside of normal ranges following creatine supplementation is accepted.

# CHAPTER FIVE

## Discussion

The purpose of this study was to determine the extent to which a seven-week resistance training program in conjunction with a supplementation regimen utilizing a one-week loading phase followed by a six-week maintenance phase with creatine monohydrate and creatine monohydrate combined with Cinnulin$^{TM}$ affected the following variables: muscle strength and mass; body composition and resting energy expenditure; muscle creatine uptake; serum insulin and IGF-1; CreaT1 and GLUT-4 mRNA and protein expression; total content of IRS-1 and Akt; activity of PI3K involved in the insulin signaling pathway; and clinical blood chemistry markers.

### *Serum and Muscle Creatine Concentration*

An interesting finding was discovered when comparing the CCI group to placebo. We observed that serum creatine concentration was significantly greater in the PLA group compared to CCI ($p = .014$), with no effect in the creatine supplementation groups. It is possible that some of the participants within the PLA group were not fasted, since an increase in serum creatine is not a normal or expected result. Conversely, the CCI ($p = 0.01$) and CR ($p = 0.02$) groups demonstrated a greater total intramuscular creatine content in comparison to PLA (Figure 1). Since there is no previous research specifically examining any form of cinnamon extract combined with creatine, and based upon the present observations, it is concluded that Cinnulin$^{TM}$ combined with creatine monohydrate is no more effective than creatine monohydrate alone, towards increasing

total muscle creatine concentration. The conclusion that creatine supplementation increases total creatine concentration has been supported by numerous studies (Greenhaff *et al*. 1994; Harris *et al.*, 1992; Hultman *et al*, 1996; Kreider, Ferreira, Wilson, Grindstaff, Plisk, Reinardy, *et al*., 1998; Willoughby & Rosene, 2003).

*Body Composition and REE*

Since this study integrated a strength and conditioning program, it was expected that all groups would improve their overall body composition. Results from the present study demonstrate significant improvements over the course of the study which support these improvements in relation to fat loss and muscle mass increases. Interestingly, the CR group displayed a trend for a greater body fat percentage decrease when compared to CCI ($p < 0.10$, effect size = 0.073), but no significance or trend was observed when compared to PLA ($p = 0.757$). Through closer examination, it was observed that the CR group, although not significant, had a greater baseline body fat percentage (25.6% ± 9.4) when compared to CCI (17.9% ± 7.2) or PLA (17.6% ± 7.6), and also reported a lower Caloric intake (kcal/day) based on reported food logs. As a result, this may partially explain the greater fat loss that occurred through training in the CR group. The CCI group lost virtually the same amount of body fat percentage as PLA (-1.5% ± 0.72 versus -1.6% ± 0.52, respectively) and was consuming the same relative dose of creatine monohydrate as the CR group. If the latter speculation is true, it may be concluded that neither creatine monohydrate, nor creatine monohydrate combined with Cinnulin$^{TM}$, decrease body fat more than exclusively following a standardized resistance-training program. Consistent with these findings, Kutz and Gunter (2002) observed that creatine supplementation did not affect body fat percentage, but increased body mass and total body water, following a

four-week resistance-training program. Contrary to most research findings with creatine supplementation, the CR group did not significantly increase overall body weight when compared to placebo ($p = 1.00$). Even though delta values from baseline were used for the statistical analysis, this response in body mass may partially be explained by the fact that, although not significant different ($p = 0.17$), the CR group had a greater baseline body mass (91.4 kg ± 21.2) when compared to CCI (80.1 kg ± 16.3) or PLA (74.2 kg ± 10.9). Subjects were matched as best as possible, but due to the study being conducted simultaneously with recruitment it was difficult to properly match participants without compromising randomization. In contrast, there was a significant difference among groups demonstrating that the body weight for the CCI group significantly increased more than CR ($p = 0.04$), with an smaller increasing trend when compared to PLA ($p = .098$, effect size = .104). Over time, independent of group assignment, there was an increasing trend for body weight increases ($p = 0.083$, effect size = 0.096), which is most likely due to the training program itself. For example, independent of group, body weight increased 0.77 kg ± 1.68 from pre- (T1) to post-test (T4).

A purpose of this study was to also evaluate the potential alterations in total-body and thigh lean tissue mass. Results demonstrated that Cinnulin$^{TM}$ combined with creatine monohydrate was more effective than placebo towards increasing thigh lean tissue mass ($p = 0.01$), with a trend in comparison to creatine monohydrate alone ($p = 0.069$, effect size = 0.132). Furthermore, total-body lean tissue mass was close to being statistically greater in the CCI group when compared to CR ($p= 0.057$, effect size = 0.108) and PLA ($p= 0.052$, effect size = 0.052). Due to the small sample size it is likely that increases in total-body lean tissue mass will be observed once additional participants complete this

study. Willoughby *et al*. (2001) observed that creatine supplementation when combined with resistance training significantly increased numerous parameters in relation to skeletal muscle, which included thigh volume, myofibrillar protein content, fat-free mass, and Type I, IIa, and IIx myosin heavy chain (MHC) mRNA and protein expression. However, in the present study, the CR group did not have a statistically significant increase in lean tissue mass, which may be accounted for by the significantly greater baseline total-body lean tissue mass when compared to the other groups ($p = 0.02$). Future statistical reanalysis using ANCOVA may assist in the elimination of this baseline difference. Previous research specifically examining Cinnulin$^{TM}$ observed small but significant improvements in body composition, specifically an increase in lean mass and a decrease in fat mass (Zeigenfuss *et al*., 2006). It was chosen to examine resting energy expenditure over the course of the study to determine if Cinnulin$^{TM}$ exhibits a thermogenic effect; however, no change in resting energy expenditure was observed for any of the groups ($p = 0.263$) or over the course of time ($p = 0.283$). However, the Zeigenfuss *et al.* (2006) study examined subjects with pre-diabetes and metabolic syndrome; therefore, that population subgroup may benefit more from cinnamon extract supplementation to support fat loss. Based upon the present results, it is concluded that CCI supplementation was more effective than CR or PLA towards increasing lean tissue mass, but not towards increasing resting energy expenditure or decreasing fat mass.

Resulting from body water statistical analyses, it should be noted that all groups increased total body water and intracellular water retention over time. There were significant differences among groups demonstrating that CCI significantly increased all of the following measures of body water: greater total body water than CR ($p = .001$) and

PLA (p = .001); greater intracellular water content compared to CR (p = .001); and greater extracellular water content compared to PLA (p = .002) and CR (p = .001). Interestingly, research has demonstrated that creatine supplementation increases total body water but does not alter overall fluid distribution (Powers, Arnold, Weltman, Perrin, Mistry, Kahler, *et al*., 2003). Strangely, the CR group actually portrayed a decreased extracellular water content when compared to CCI (-.83 L ± .16, p = 0.001), whereas CCI and PLA increased all measures of fluid retention (Figures 6, 7, and 8). The most considerable result was most likely due to the strength training program itself since fluid retention generally increased independent of creatine supplementation, over the course of the training period. Kutz and Gunter (2003) observed that creatine supplementation combined with resistance training significantly increases total body water with trained participants, but not in the placebo supplement group. Since an increase in total body water was observed in the PLA group, a possible explanation may be that non-resistance trained or recreationally active males (which were used in our study) may significantly increase fluid retention to a greater extent when compared to a trained population group.

### *Performance Measures*

In order to determine muscle performance, it was chosen to examine muscle power output and relative muscle strength. Previous research examining creatine monohydrate supplementation towards these variables has repeatedly reported significant improvements (Bemben & Lamont, 2005; Rawson & Volek, 2003). Interestingly, combining creatine monohydrate with Cinnulin$^{TM}$ resulted in the greatest improvement in relative maximal leg press strength. Specifically, the CCI group significantly increased their relative 1-RM leg press greater than CR (p = 0.043). There were no significant

differences among groups for the other measures of performance; however, over the course of the study relative bench press and leg press strength, Wingate relative peak power, and Wingate rate to fatigue were improved significantly. Therefore, it is concluded that the prescribed strength training program, alone, appeared to impart the greatest effect towards improving strength and power measures, but Cinnulin$^{TM}$ combined with creatine monohydrate may have some differential effect. Future studies with a larger sample size as well as examining resistance-trained participants are needed before a final conclusion can be made.

### *Insulin Signaling*

Since this is the first study to combine Cinnulin$^{TM}$ and creatine monohydrate, there is no previous research to directly base the current observations or conclusions upon. The primary rationale for combining Cinnulin$^{TM}$ with creatine monohydrate is to potentially provide a similar mechanism in which creatine consumed with simple carbohydrates has been shown to significantly increase muscle creatine absorption rate (Green *et al*., 1996). Previously research conducted by Kreider *et al*. (1998) demonstrated that a glucose/creatine supplement promoted greater increases in fat-free mass, isotonic lifting volume, and sprint performance during intense resistance training combined with agility training. Since cinnamon extract has been observed to activate the insulin receptor via up-regulated insulin signaling (Qin *et al.*, 2003), it was claimed that large doses of carbohydrate may not be needed to increase insulin-mediated intramuscular creatine uptake.

IGF-1 and insulin are able to activate the insulin receptor; therefore, it was chosen to examine serum levels of these hormones to determine the magnitude in which these

hormones are affected by supplementation. Previous animal and human research with cinnamon extract has indicated potential capabilities in the up-regulation of insulin signaling, so the decision was made to examine various signaling intermediates of the insulin pathway. Once activated, the insulin receptor activates IRS-1, which then subsequently activates the downstream pathway intermediates, PI3K and Akt. In the present study, since IGF-1 appeared to decrease over time (Figure 12), and because Akt was moderately elevated in the CCI group (Figure 17), it is conceivable that alterations in the insulin signaling cascade are primarily due to the insulin receptor activating IRS-1, irrespective to serum IGF-1.

Since the purpose of the study was to assess training and/or supplement related adaptations, the decision was made to primarily examine alterations in protein content of the insulin signaling intermediates rather than the activation/phosphorylation state. Statistical analysis revealed no significant Group x Time interactions for IRS-1 ($p = 0.92$), Akt ($p = 0.33$), or PI3K activity ($p = 0.87$). Furthermore, there were no Time main effects for IRS-1 ($p = 0.57$), Akt ($p = 0.38$), or PI3K activity ($p = 0.52$). However, Akt protein content was significantly increased in the CCI group compared to CR or PLA ($p = 0.01$). As a result, since only a Group main effect was observed with no accompanying Group x Time interaction, it is concluded that Cinnulin$^{TM}$ combined with creatine monohydrate does not significantly up-regulate insulin signaling.

GLUT-4 mRNA and protein expression followed a similar observation as it did not significantly increase over time for the CCI group ($p = 0.26$) (Figure 16). Previous creatine monohydrate research has indicated an increase in glycogen content, but it has been controversial as to whether GLUT-4 up-regulation is involved. In relation to

creatine supplementation and the impact on glucose regulation, a recent study supports the theory that exogenous creatine may up-regulate muscle GLUT-4 expression, and implies that oxidative and transport proteins may be up-regulated concomitantly (Op't Eijnde, Urso, Richter, Greenhaff, & Hespel, 2001). On the contrary, van Loon *et al*. (2004) concluded that creatine ingestion itself stimulates muscle glycogen storage, but does not affect muscle GLUT-4 expression (van Loon, Murphy, Oosterlaar, Cameron-Smith, Hargreaves, Wagenmakers, *et al*., 2004). The results from the present study display a combination of both theories, as GLUT-4 mRNA expression was significantly increased in the CR group when compared to PLA ($p = 0.049$), but when GLUT-4 protein expression was examined no significances were found between groups ($p = 0.157$); however, this may be accounted for by the low sample size of the current study. Regardless, it is concluded that creatine monohydrate supplementation alone may play a role in GLUT-4 regulation, but future research should examine a longer supplementation period to determine if GLUT-4 protein expression increases as a result of increases in GLUT-4 mRNA expression observed to occur in the CR group. Even though an increase was not observed in GLUT-4 mRNA expression and protein expression in the CCI group, a significant elevation was observed in downstream insulin signaling intermediate, Akt. Even though GLUT-4 is translocated through the activation of the insulin receptor and IRS-1, which then proceed to activate PI3K and Akt, the increased protein expression of the sole intermediate, Akt, probably did not provide a sufficient stimulus to increase GLUT-4 activity in the CCI group.

It was observed that the CCI group did not significantly increase the expression and activity of various insulin signaling intermediates (PI3K activity and IRS-1 total

protein), but did increase the total protein content of Akt ($p = 0.01$). In addition, no up-regulation in relation to GLUT-4 mRNA or protein expression was observed in the CCI group. Ideally, a pattern in relation to the complete up-regulation of the insulin signaling cascade should be observed before it can be concluded that Cinnulin$^{TM}$ is efficacious along those parameters. Future studies should attempt to directly compare Cinnulin$^{TM}$ combined with creatine monohydrate to glucose combined with creatine monohydrate in order to determine if the former is more efficacious.

### *Creatine Transporter mRNA and Protein Content*

No significant alterations in CreaT1 mRNA or protein expression were observed with supplementation. It is therefore concluded that Cinnulin$^{TM}$ combined with creatine, creatine monohydrate alone, and placebo, do not significantly alter the activity of CreaT1. Future studies specifically examining the CCI group are needed to determine if the small sample size was a major contributor towards not observing significance.

### *Conclusions*

The present study has demonstrated that a seven week resistance training program, coupled with loading and maintenance phase supplementation with Cinnulin$^{TM}$ and creatine monohydrate, is no more effective than creatine monohydrate alone for increasing muscle creatine uptake. However, Cinnulin$^{TM}$ did improve some measures of muscle strength and lean tissue mass greater than creatine monohydrate supplementation alone, when compared to placebo. However, all groups improved in virtually all performance measures, and it is therefore concluded that the strength training program, itself, is primarily responsible for these enhancements. Over the course of the study, it

should be noted that dietary intake did not significantly change from baseline and subjects were instructed to follow identical upper- and lower-body resistance-training. As a result, the significant differences among groups are concluded to primarily occur due to different creatine supplement formulations. However, because the CR group displayed a lower reported mean dietary kcal/day intake, and due to subject matching discrepancies, it is possible that this is a viable explanation for the smaller increase in lean tissue mass and performance when compared to prior creatine monohydrate research. Since this study is a smaller subset of the total study, the smaller sample size may have under-powered the present study and affected the results, by potentially increasing the likelihood of Type II error. Data analysis will need to be repeated following the addition and completion of additional participants to determine if the same conclusions can be made. Regardless, this is the first investigation demonstrating that Cinnulin$^{TM}$ combined with creatine monohydrate elicits greater mean increases in relative 1-RM leg press, thigh lean tissue mass, body water retention, and total Akt protein content when compared to creatine monohydrate alone, or placebo.

# 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. CinnulinTM 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$^{TM}$ 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., CSCS.*** 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.

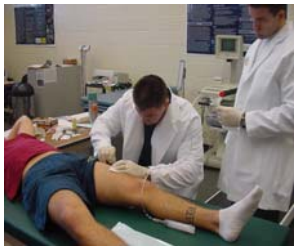
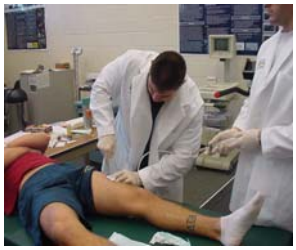
***Creatine Transporter mRNA Expression.*** Muscle tissue samples will be analyzed for creatine transporter mRNA transcription levels by quantitative real-time polymerase chain reaction (RT-PCR). The tissue will be homogenized with Tri Reagent (Sigma, St. Louis, MO) and total RNA will be isolated by 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 wavlength 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

# APPENDIX D

Serum Clinical Chemistry

| Variable | 1 = PLA<br>2 = CCI<br>3 = CR | Testing Session | Mean | Std. Deviation |
|---|---|---|---|---|
| Triglycerides (mg/dL) | 1 | 1 | 61.333 | 37.702 |
| | | 2 | 97.500 | 2.121 |
| | | 3 | 56.286 | 26.819 |
| | | 4 | 74.286 | 58.114 |
| | | Total | 67.136 | 40.777 |
| | 2 | 1 | 95.429 | 61.259 |
| | | 2 | 69.000 | 27.875 |
| | | 3 | 92.571 | 50.026 |
| | | 4 | 100.571 | 68.239 |
| | | Total | 92.792 | 54.850 |
| | 3 | 1 | 130.143 | 114.021 |
| | | 2 | 93.600 | 34.078 |
| | | 3 | 82.857 | 22.282 |
| | | 4 | 95.000 | 24.658 |
| | | Total | 100.923 | 62.626 |
| | Total | 1 | 97.350 | 80.448 |
| | | 2 | 87.000 | 29.086 |
| | | 3 | 77.238 | 36.912 |
| | | 4 | 89.952 | 52.219 |
| | | Total | 87.889 | 55.238 |
| Cholesterol (mg/dL) | 1 | 1 | 137.500 | 31.867 |
| | | 2 | 128.500 | 4.950 |
| | | 3 | 129.857 | 28.916 |
| | | 4 | 143.286 | 28.359 |
| | | Total | 136.091 | 27.348 |
| | 2 | 1 | 149.714 | 35.382 |
| | | 2 | 133.333 | 29.143 |
| | | 3 | 149.857 | 40.822 |
| | | 4 | 141.429 | 29.022 |
| | | Total | 145.292 | 33.021 |
| | 3 | 1 | 161.714 | 44.836 |
| | | 2 | 163.600 | 38.863 |
| | | 3 | 168.429 | 29.720 |
| | | 4 | 171.714 | 42.649 |
| | | Total | 166.577 | 37.274 |
| | Total | 1 | 150.250 | 37.380 |
| | | 2 | 147.500 | 33.968 |
| | | 3 | 149.381 | 35.725 |
| | | 4 | 152.143 | 35.232 |

| | | | | |
|---|---|---|---|---|
| | | Total | 150.167 | 35.095 |
| HDL (mg/dL) | 1 | 1 | 46.500 | 6.221 |
| | | 2 | 42.500 | 9.192 |
| | | 3 | 48.143 | 10.761 |
| | | 4 | 49.857 | 9.371 |
| | | Total | 47.727 | 8.719 |
| | 2 | 1 | 48.429 | 9.727 |
| | | 2 | 46.333 | 7.024 |
| | | 3 | 50.143 | 14.439 |
| | | 4 | 45.714 | 8.381 |
| | | Total | 47.875 | 10.250 |
| | 3 | 1 | 47.714 | 14.997 |
| | | 2 | 48.400 | 7.635 |
| | | 3 | 46.571 | 6.214 |
| | | 4 | 50.429 | 10.147 |
| | | Total | 48.269 | 9.974 |
| | Total | 1 | 47.600 | 10.570 |
| | | 2 | 46.600 | 7.199 |
| | | 3 | 48.286 | 10.541 |
| | | 4 | 48.667 | 9.107 |
| | | Total | 47.972 | 9.571 |
| LDL (mg/dL) | 1 | 1 | 79.833 | 30.714 |
| | | 2 | 78.000 | 15.556 |
| | | 3 | 73.286 | 26.862 |
| | | 4 | 82.143 | 26.879 |
| | | Total | 78.318 | 25.743 |
| | 2 | 1 | 83.143 | 27.070 |
| | | 2 | 75.333 | 24.826 |
| | | 3 | 86.143 | 32.800 |
| | | 4 | 78.714 | 21.708 |
| | | Total | 81.750 | 25.748 |
| | 3 | 1 | 99.714 | 36.390 |
| | | 2 | 102.400 | 38.533 |
| | | 3 | 107.143 | 30.411 |
| | | 4 | 107.429 | 35.104 |
| | | Total | 104.308 | 32.935 |
| | Total | 1 | 87.950 | 31.275 |
| | | 2 | 89.400 | 31.820 |
| | | 3 | 88.857 | 31.955 |
| | | 4 | 89.429 | 29.999 |
| | | Total | 88.847 | 30.523 |
| Gamma-Glutamyl Transferase (U/L) | 1 | 1 | 32.500 | 9.375 |
| | | 2 | 36.000 | 8.485 |
| | | 3 | 31.000 | 7.439 |
| | | 4 | 36.143 | 10.946 |
| | | Total | 33.500 | 8.927 |
| | 2 | 1 | 43.571 | 29.257 |
| | | 2 | 28.333 | 7.572 |
| | | 3 | 43.429 | 25.903 |

| | | | | |
|---|---|---|---|---|
| | | 4 | 37.143 | 17.363 |
| | | Total | 39.750 | 22.574 |
| | 3 | 1 | 41.571 | 18.220 |
| | | 2 | 40.000 | 17.103 |
| | | 3 | 39.000 | 14.640 |
| | | 4 | 39.286 | 16.730 |
| | | Total | 39.962 | 15.691 |
| | Total | 1 | 39.550 | 20.528 |
| | | 2 | 35.700 | 13.384 |
| | | 3 | 37.810 | 17.606 |
| | | 4 | 37.524 | 14.566 |
| | | Total | 37.917 | 16.854 |
| Lactic Dehydrogenase (U/L) | 1 | 1 | 116.500 | 19.470 |
| | | 2 | 109.000 | 7.071 |
| | | 3 | 125.571 | 26.538 |
| | | 4 | 121.857 | 14.565 |
| | | Total | 120.409 | 19.515 |
| | 2 | 1 | 122.429 | 16.277 |
| | | 2 | 132.000 | 7.810 |
| | | 3 | 131.143 | 22.974 |
| | | 4 | 142.714 | 28.941 |
| | | Total | 132.083 | 22.218 |
| | 3 | 1 | 142.429 | 59.352 |
| | | 2 | 136.200 | 35.379 |
| | | 3 | 126.429 | 23.656 |
| | | 4 | 122.429 | 25.105 |
| | | Total | 131.538 | 37.408 |
| | Total | 1 | 127.650 | 37.757 |
| | | 2 | 129.500 | 26.379 |
| | | 3 | 127.714 | 23.320 |
| | | 4 | 129.000 | 24.552 |
| | | Total | 128.319 | 28.165 |
| Uric Acid (mg/dL) | 1 | 1 | 5.367 | 1.325 |
| | | 2 | 5.850 | 0.212 |
| | | 3 | 5.329 | 0.953 |
| | | 4 | 5.529 | 1.097 |
| | | Total | 5.450 | 1.023 |
| | 2 | 1 | 5.471 | 1.131 |
| | | 2 | 4.100 | 1.153 |
| | | 3 | 5.514 | 1.182 |
| | | 4 | 5.471 | 1.272 |
| | | Total | 5.313 | 1.207 |
| | 3 | 1 | 5.771 | 2.278 |
| | | 2 | 5.460 | 1.884 |
| | | 3 | 6.157 | 1.816 |
| | | 4 | 5.943 | 1.626 |
| | | Total | 5.862 | 1.817 |
| | Total | 1 | 5.545 | 1.592 |
| | | 2 | 5.130 | 1.551 |
| | | 3 | 5.667 | 1.347 |

| | | | | |
|---|---|---|---|---|
| | | 4 | 5.648 | 1.298 |
| | | Total | 5.553 | 1.415 |
| Glucose | 1 | 1 | 89.500 | 8.643 |
| (mg/dL) | | 2 | 73.500 | 21.920 |
| | | 3 | 86.714 | 6.157 |
| | | 4 | 92.143 | 3.805 |
| | | Total | 88.000 | 9.087 |
| | 2 | 1 | 90.714 | 2.289 |
| | | 2 | 88.333 | 2.082 |
| | | 3 | 89.714 | 7.521 |
| | | 4 | 89.714 | 6.184 |
| | | Total | 89.833 | 5.198 |
| | 3 | 1 | 87.571 | 12.713 |
| | | 2 | 88.600 | 5.595 |
| | | 3 | 90.286 | 6.701 |
| | | 4 | 90.143 | 6.842 |
| | | Total | 89.192 | 8.198 |
| | Total | 1 | 89.250 | 8.614 |
| | | 2 | 85.500 | 10.406 |
| | | 3 | 88.905 | 6.663 |
| | | 4 | 90.667 | 5.571 |
| | | Total | 89.042 | 7.576 |
| Blood Urea Nitrogen | 1 | 1 | 16.833 | 3.125 |
| (mg/dL) | | 2 | 15.000 | 0.000 |
| | | 3 | 15.143 | 4.451 |
| | | 4 | 16.143 | 2.673 |
| | | Total | 15.909 | 3.250 |
| | 2 | 1 | 19.571 | 5.192 |
| | | 2 | 15.000 | 2.000 |
| | | 3 | 15.000 | 2.887 |
| | | 4 | 17.000 | 4.163 |
| | | Total | 16.917 | 4.221 |
| | 3 | 1 | 11.571 | 1.813 |
| | | 2 | 15.400 | 5.983 |
| | | 3 | 15.000 | 6.218 |
| | | 4 | 14.857 | 2.340 |
| | | Total | 14.115 | 4.430 |
| | Total | 1 | 15.950 | 4.925 |
| | | 2 | 15.200 | 4.104 |
| | | 3 | 15.048 | 4.477 |
| | | 4 | 16.000 | 3.130 |
| | | Total | 15.597 | 4.151 |
| Creatinine | 1 | 1 | 1.083 | 0.075 |
| (mg/dL) | | 2 | 1.200 | 0.141 |
| | | 3 | 1.043 | 0.162 |
| | | 4 | 1.086 | 0.135 |
| | | Total | 1.082 | 0.130 |
| | 2 | 1 | 1.014 | 0.069 |
| | | 2 | 1.100 | 0.100 |
| | | 3 | 1.171 | 0.150 |

| | | | | |
|---|---|---|---|---|
| | | 4 | 1.171 | 0.095 |
| | | Total | 1.117 | 0.124 |
| | 3 | 1 | 0.929 | 0.170 |
| | | 2 | 1.480 | 0.870 |
| | | 3 | 1.129 | 0.170 |
| | | 4 | 1.114 | 0.135 |
| | | Total | 1.138 | 0.419 |
| | Total | 1 | 1.005 | 0.128 |
| | | 2 | 1.310 | 0.612 |
| | | 3 | 1.114 | 0.162 |
| | | 4 | 1.124 | 0.122 |
| | | Total | 1.114 | 0.269 |
| BUN/Creatinine Ratio | 1 | 1 | 15.633 | 3.301 |
| | | 2 | 12.550 | 1.485 |
| | | 3 | 14.529 | 3.818 |
| | | 4 | 15.129 | 3.594 |
| | | Total | 14.841 | 3.361 |
| | 2 | 1 | 19.386 | 5.348 |
| | | 2 | 13.667 | 1.665 |
| | | 3 | 13.000 | 3.068 |
| | | 4 | 14.600 | 3.958 |
| | | Total | 15.413 | 4.628 |
| | 3 | 1 | 12.700 | 2.182 |
| | | 2 | 14.500 | 5.558 |
| | | 3 | 13.657 | 6.463 |
| | | 4 | 13.643 | 3.379 |
| | | Total | 13.558 | 4.386 |
| | Total | 1 | 15.920 | 4.656 |
| | | 2 | 13.860 | 3.900 |
| | | 3 | 13.729 | 4.488 |
| | | 4 | 14.457 | 3.521 |
| | | Total | 14.568 | 4.206 |
| Calcium (mg/dL) | 1 | 1 | 9.183 | 0.527 |
| | | 2 | 9.250 | 0.071 |
| | | 3 | 9.129 | 0.565 |
| | | 4 | 9.400 | 0.432 |
| | | Total | 9.241 | 0.474 |
| | 2 | 1 | 9.171 | 0.411 |
| | | 2 | 8.833 | 0.379 |
| | | 3 | 8.886 | 0.445 |
| | | 4 | 8.900 | 0.387 |
| | | Total | 8.967 | 0.407 |
| | 3 | 1 | 8.429 | 1.055 |
| | | 2 | 9.020 | 0.277 |
| | | 3 | 8.900 | 0.443 |
| | | 4 | 8.943 | 0.341 |
| | | Total | 8.808 | 0.641 |
| | Total | 1 | 8.915 | 0.782 |
| | | 2 | 9.010 | 0.300 |
| | | 3 | 8.971 | 0.477 |

| | | | | |
|---|---|---|---|---|
| | | 4 | 9.081 | 0.435 |
| | | Total | 8.993 | 0.545 |
| Total Protein | 1 | 1 | 7.467 | 0.516 |
| (g/dL) | | 2 | 7.300 | 0.283 |
| | | 3 | 7.314 | 0.564 |
| | | 4 | 7.586 | 0.241 |
| | | Total | 7.441 | 0.435 |
| | 2 | 1 | 7.286 | 0.478 |
| | | 2 | 6.933 | 0.603 |
| | | 3 | 6.986 | 0.481 |
| | | 4 | 6.914 | 0.398 |
| | | Total | 7.046 | 0.467 |
| | 3 | 1 | 6.986 | 1.119 |
| | | 2 | 7.300 | 0.520 |
| | | 3 | 7.057 | 0.529 |
| | | 4 | 7.286 | 0.344 |
| | | Total | 7.146 | 0.677 |
| | Total | 1 | 7.235 | 0.760 |
| | | 2 | 7.190 | 0.491 |
| | | 3 | 7.119 | 0.519 |
| | | 4 | 7.262 | 0.424 |
| | | Total | 7.203 | 0.561 |
| Albumin | 1 | 1 | 4.667 | 0.344 |
| (g/dL) | | 2 | 4.700 | 0.000 |
| | | 3 | 4.557 | 0.355 |
| | | 4 | 4.729 | 0.221 |
| | | Total | 4.655 | 0.289 |
| | 2 | 1 | 4.729 | 0.180 |
| | | 2 | 4.667 | 0.321 |
| | | 3 | 4.471 | 0.275 |
| | | 4 | 4.386 | 0.248 |
| | | Total | 4.546 | 0.273 |
| | 3 | 1 | 4.214 | 0.654 |
| | | 2 | 4.340 | 0.195 |
| | | 3 | 4.257 | 0.223 |
| | | 4 | 4.471 | 0.350 |
| | | Total | 4.319 | 0.401 |
| | Total | 1 | 4.530 | 0.484 |
| | | 2 | 4.510 | 0.269 |
| | | 3 | 4.429 | 0.304 |
| | | 4 | 4.529 | 0.304 |
| | | Total | 4.497 | 0.354 |
| Total Bilirubin | 1 | 1 | 0.383 | 0.117 |
| (mg/dL) | | 2 | 0.550 | 0.212 |
| | | 3 | 0.386 | 0.146 |
| | | 4 | 0.386 | 0.227 |
| | | Total | 0.400 | 0.169 |
| | 2 | 1 | 0.443 | 0.113 |
| | | 2 | 0.433 | 0.208 |
| | | 3 | 0.386 | 0.121 |

| | | | | |
|---|---|---|---|---|
| | | 4 | 0.371 | 0.095 |
| | | Total | 0.404 | 0.120 |
| | 3 | 1 | 0.314 | 0.135 |
| | | 2 | 0.360 | 0.134 |
| | | 3 | 0.371 | 0.170 |
| | | 4 | 0.486 | 0.219 |
| | | Total | 0.385 | 0.174 |
| | Total | 1 | 0.380 | 0.128 |
| | | 2 | 0.420 | 0.169 |
| | | 3 | 0.381 | 0.140 |
| | | 4 | 0.414 | 0.188 |
| | | Total | 0.396 | 0.154 |
| Alkaline Phosphatase (U/L) | 1 | 1 | 76.333 | 27.053 |
| | | 2 | 95.500 | 0.707 |
| | | 3 | 75.143 | 20.908 |
| | | 4 | 79.000 | 20.412 |
| | | Total | 78.545 | 21.235 |
| | 2 | 1 | 84.571 | 25.324 |
| | | 2 | 80.000 | 26.153 |
| | | 3 | 83.286 | 21.289 |
| | | 4 | 85.714 | 20.878 |
| | | Total | 83.958 | 21.493 |
| | 3 | 1 | 65.000 | 18.028 |
| | | 2 | 70.000 | 21.897 |
| | | 3 | 68.000 | 13.964 |
| | | 4 | 72.857 | 13.741 |
| | | Total | 68.885 | 15.998 |
| | Total | 1 | 75.250 | 23.850 |
| | | 2 | 78.100 | 21.682 |
| | | 3 | 75.476 | 19.146 |
| | | 4 | 79.190 | 18.476 |
| | | Total | 76.861 | 20.356 |
| Aspartate Aminotransferase (U/L) | 1 | 1 | 21.333 | 7.633 |
| | | 2 | 11.500 | 4.950 |
| | | 3 | 24.714 | 6.849 |
| | | 4 | 26.000 | 11.504 |
| | | Total | 23.000 | 9.144 |
| | 2 | 1 | 20.857 | 3.132 |
| | | 2 | 38.000 | 7.810 |
| | | 3 | 29.714 | 6.945 |
| | | 4 | 27.286 | 7.825 |
| | | Total | 27.458 | 8.108 |
| | 3 | 1 | 25.000 | 9.363 |
| | | 2 | 32.200 | 7.396 |
| | | 3 | 36.286 | 17.727 |
| | | 4 | 26.714 | 8.616 |
| | | Total | 29.885 | 12.044 |
| | Total | 1 | 22.450 | 7.060 |
| | | 2 | 29.800 | 11.858 |

| | | | | |
|---|---|---|---|---|
| | | 3 | 30.238 | 12.099 |
| | | 4 | 26.667 | 8.980 |
| | | Total | 26.972 | 10.256 |
| Alanine Aminotransferase (U/L) | 1 | 1 | 26.333 | 3.670 |
| | | 2 | 22.500 | 0.707 |
| | | 3 | 28.143 | 7.841 |
| | | 4 | 33.286 | 16.510 |
| | | Total | 28.773 | 10.538 |
| | 2 | 1 | 27.857 | 3.716 |
| | | 2 | 28.000 | 1.000 |
| | | 3 | 33.714 | 7.675 |
| | | 4 | 29.857 | 5.014 |
| | | Total | 30.167 | 5.631 |
| | 3 | 1 | 27.143 | 3.338 |
| | | 2 | 28.400 | 2.881 |
| | | 3 | 29.429 | 3.259 |
| | | 4 | 29.143 | 4.259 |
| | | Total | 28.538 | 3.432 |
| | Total | 1 | 27.150 | 3.438 |
| | | 2 | 27.100 | 3.143 |
| | | 3 | 30.429 | 6.727 |
| | | 4 | 30.762 | 9.909 |
| | | Total | 29.153 | 6.913 |

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:<br>0 = none<br>1 = minimal (1-2 per/wk)<br>2 = slight (3-4 per/wk)<br>3 = occasional (5-6 per/wk)<br>4 = frequent (7-8 per/wk)<br>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:<br>0 = none<br>1 = minimal<br>2 = slight<br>3 = moderate<br>4 = severe<br>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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