ABSTRACT

The Effects of Creatine Monohydrate Supplementation on Creatine Transporter Activity and Creatine Metabolism in Resistance Trained Males

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Creatine is a nutritional supplement that is used for its potential performance enhancing (ergogenic) benefits. It constitutes an important component of the immediate energy system, by which ATP is regenerated during intense physical activity. Oral creatine supplementation has been shown to provide numerous benefits, including increases in lean muscle mass, muscular strength, and enhanced performance in various athletic capacities. The creatine transporter is a transmembrane protein that mediates the entry of creatine from the circulation into the muscle cell. Little is understood about the importance of the creatine transporter in controlling the uptake and regulation of creatine within human skeletal muscle. This study attempts to characterize the specific variations in creatine receptor activity and concurrent creatine metabolism in human skeletal muscle in response to a regimen of oral creatine supplementation including a one week loading phase, a four week maintenance phase, and a four week washout phase. Supplementation induced significant increments in total body mass ($p = 0.03$) and lean body mass ($p = 0.01$). A moderate effect size ($d = 0.51$) was found for strength increase, which suggests that the study was underpowered to detect a significant difference in strength increase.

There appeared to be no effect of supplementation on intramuscular creatine; however, these data were subject to large measurement error and are not likely accurate. There was no apparent effect on creatine transporter mRNA or creatine transporter content when measured after the loading phase, during and after the maintenance phase, and after the washout phase.

The Effects of Creatine Monohydrate Supplementation on Creatine Transporter Activity and Creatine Metabolism in Resistance Trained Males

by

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A Thesis

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

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Cr – creatine

Pl – placebo

CrT – creatine transporter

mRNA – message ribonucleic acid

ATP – adenosine triphosphate

DEXA – dual energy x-ray absorptiometry

RT-PCR – real-time polymerase chain reaction

WBC – white blood cell

RBC – red blood cell

MCV – mean cell volume

MCH – mean cell hematocrit

MCHC – mean cell hematocrit concentration

LDL – low density lipoprotein

HDL – high density lipoprotein

GGT – gamma-glutamyl transferase

LDH – lactate dehydrogenase

ALT – alanine aminotransferase

PVDF – polyvinyl difluoride

TBS – Tris-buffered saline

TTBS – Tween-Tris-buffered saline

BUN – blood urea nitrogen

ALP – alkaline phosphatase

ASP – aspartate aminotransferase

β-GPA – β-guanidinoproprionic acid

ELISA – enzyme linked immunosorbant assay

TMB – 3,3',5,5'-tetramethylbenzidine

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CHAPTER ONE

Introduction

Statement of the Problem

Creatine is a naturally occurring amino acid derivative that is endogenously synthesized primarily by the liver from arginine, glycine, and methionine, and it is essential in the regulation of muscular energy stores. It contributes to the generation of adenosine triphosphate (ATP), which is necessary for muscular contraction (Bessmann, 1985). In the past few decades, the exogenous ingestion of creatine has been used as a potentially performance enhancing (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 (Volek, 1996). Extensive reviews of the literature, including hundreds of various studies involving creatine supplementation and performance, for the most part (~ 70%) reveal a significant ergogenic benefit, particularly in exercise tasks that are primarily dependent on phosphocreatine content (Kreider, 2003; Williams, 1998). Generally, a creatine supplementation protocol includes a loading phase of 20 g of creatine per day (4 x 5 g) for 3-7 days, which has been demonstrated to significantly increase intramuscular creatine and phosphocreatine stores (Harris, 1992; Kreider, Willoughby, Greenwood, Tarnopolsky, & Parise, 2003). In humans, the increase of intramuscular creatine content following supplementation shows a considerable amount of variability, if an increase is induced at all (Greenhaff, 1994). This discrepancy may hint at the existence of “responders” and “non-responders”, whereby some individuals

may have little to no response to creatine supplementation, negligible elevation in intramuscular creatine, and no resultant ergogenic effect (Syrotuik, 2004).

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 regulation of the creatine transporter protein is important in controlling intramuscular creatine levels (Loike, 1998). This becomes apparent in certain creatine deficient pathologies where the creatine transporter may be defective or absent, such that supplementation is unable to restore creatine to ordinary levels (Cecil, 2001).

Strangely, chronic creatine supplementation has been demonstrated to cause a reduction of the creatine transporter protein in rats, while chronic supplementation of β-guanidinoproprionic acid (β-GPA), a creatine analog that competitively inhibits the creatine transporter, resulted in an increase in creatine transporter protein (Guerrero-Ontiveros, 1998). This suggests that intramuscular creatine content may regulate the amount of creatine transporter protein present in muscle. Creatine transport has also been demonstrated to be affected by the sodium concentration across the cell membrane in culture (Odoom, 1996). More recently, creatine transport has been identified in the mitochondria (Walzel, 2002), which suggests that creatine may not only exist in the cytosol of the muscle fibers, but that there may be an intermitochondrial pool of creatine as well. The regulation of total creatine metabolism within the muscle is still poorly understood. This knowledge is essential in determining the mechanism(s) that may

regulate muscle creatine uptake, as well as comprehending the basis of the ergogenic effects of creatine supplementation.

Nearly the entire bulk of data describing creatine metabolism with respect to the creatine transporter has been generated from animal models. There has been little more than a trivial amount of work done involving the creatine transporter in human skeletal muscle (Tarnopolsky, 2001). Although the groundwork for understanding the creatine transporter's role in creatine metabolism has been laid, it is important to begin to address creatine metabolism in human skeletal muscle in much the same manner that has been done previously in animal models.

Purpose of Study

This study involved two groups of male participants. One group ingested a dextrose placebo while the other group ingested creatine monohydrate at approximately 17-20 grams/day for a one week loading phase and then at approximately 5-7 grams/day for a four week maintenance phase, followed by a four week washout period without any supplementation. In light of this, the purpose of this study was to provide initial data on the response of human skeletal muscle creatine transporter activity and creatine metabolism during the loading and maintenance phases of an oral creatine supplementation regimen, as well as during a four week washout period following the conclusion of creatine supplementation.

Hypotheses

H_{1A}: that urinary levels of free creatine will increase significantly during the loading phase of an oral creatine supplementation, remain elevated during the maintenance

phase, and decrease to baseline levels after four weeks removed from creatine supplementation.

H_{1B}: that urinary levels of creatinine will increase significantly during the loading phase of an oral creatine supplementation, remain elevated during the maintenance phase, and decrease to baseline levels after four weeks removed from creatine supplementation.

H_{1C}: that serum levels of free creatine will increase significantly during the loading phase of an oral creatine supplementation, remain elevated during the maintenance phase, and decrease to baseline levels after four weeks removed from creatine supplementation.

H_{1D}: that serum levels of creatinine will increase significantly during the loading phase of an oral creatine supplementation, remain elevated during the maintenance phase, and decrease to baseline levels after four weeks removed from creatine supplementation.

H_2: that intramuscular levels of free creatine will increase significantly during the loading phase of an oral creatine supplementation, remain elevated during the maintenance phase, and decrease to baseline levels after four weeks removed from creatine supplementation.

H_3: that there will be no significant changes in creatine transporter mRNA expression relative to baseline levels as a result of both the loading and maintenance phases of oral creatine supplementation.

H_4: that there will be no significant changes in creatine transporter protein expression relative to baseline levels as a result of both the loading and maintenance phases of oral creatine supplementation.

H_5: that leg press 1-RM will increase significantly as a result of both the loading and maintenance phases of oral creatine supplementation.

H_6: that there will be no significant changes in relative total body water or overall fluid distribution as a result of both the loading and maintenance phases of oral creatine supplementation.

H_7: that body weight and fat-free mass will increase significantly as a result of both the loading and maintenance phases of oral creatine supplementation.

Delimitations

This study was delimited to:

1. Nineteen healthy males, aged 18-23, who were involved in regular resistance-training for at least six months prior to the study, including at least one lower body exercise bout per week.
2. The participants were randomly assigned by age and weight to either a creatine monohydrate group or placebo group.
3. The participants in the creatine monohydrate group took their supplement for one week at a loading dose of 0.3 g/kg of lean body mass, followed by four weeks at a maintenance dose of 0.075 g/kg of lean body mass. The participants in the placebo group took the same amount of placebo as the supplement group took creatine monohydrate. All participants followed the same resistance-training regimen, which was verified by oral communication and activity logs.

4. Urine, blood, and muscle samples were taken from each participant at nine, six, and five different time points, respectively, to determine measures of creatine uptake, metabolism, and retention.
5. The study was conducted over a period of five months between February, 2006 and June, 2006.

Limitations

This study was limited by:

1. The sample size of this study was small (N=19) and so caution is advised when extrapolating data to a larger population.
2. Participant consumption of the supplement was not directly monitored.
3. Participants' weight training was not directly monitored.

Assumptions

1. It was assumed that participants were truthful regarding their prior training status.
2. It was assumed that participants did not alter their normal dietary intake during the study.
3. It was assumed that the participants did not take any additional nutritional supplements during the study.
4. It was assumed that the participants completely consumed the entire supplement that was given to them.
5. It was assumed that the creatine supplement increased creatine supply to the muscle tissue.

Definitions

Creatine – An organic molecule derived from amino acids that plays an important role in energy utilization during muscular contraction. Also used as a potentially ergogenic nutritional supplement.

Creatine transporter – The molecular gateway protein that allows creatine to enter muscle tissue from the circulation.

mRNA – Message ribonucleic acid. A coding molecule that provides genetic information for the cell to produce new proteins.

1-RM – One repetition maximum. The maximum amount of weight that an individual can lift for a particular movement.

CHAPTER TWO

Literature Review

The primary purpose of administering an oral creatine supplementation is to increase intramuscular concentrations of creatine and phosphocreatine. The paradigm dosing strategy for creatine supplementation involves consumption of 20-25 g/d for a period of 3-7 days, which has been shown numerous times to significantly increase intramuscular creatine content (Greenhaff, 1994; Lemon, 1995; Balsom, 1995; Vandenberghe, 1996, Myburgh, 1996). This is often referred to as a loading phase. It appears that there is a finite upper limit to the total intramuscular creatine concentration of ~160 mmol/kg dry mass, and that once this limit is reached, additional supplementation will be excreted in the urine (Greenhaff, 1995). Similarly, a maintenance dose of 2 g/d following a standard loading phase was shown to maintain the elevated total creatine content induced by the loading (Hultman, 1996). Interestingly, a six week supplementation with 2 g/d without a loading phase failed to provide beneficial increases in total creatine and phosphocreatine levels (Thompson, 1996). The research indicates that a loading dose of 20-25 g/d for 3-7 days, followed by a maintenance dose of 2-3 g/d is adequate to significantly increase and maintain total intramuscular creatine levels. Furthermore, it has been shown that ~1 month after creatine supplementation of 20 g/d for six days, total intramuscular creatine content is not significantly different from pre-supplementation values (Hultman, 1996).

The majority of creatine studies have used absolute doses of creatine, although Hultman et al. (1996) recommended a loading dose of 0.3 g/kg body mass/d for 5-6 days, followed by a maintenance dose of 0.03 g/kg body mass/d. The rationale behind this approach is sound, as an absolute dose will elicit varying increases in serum and intramuscular creatine concentrations among separate individuals with different body masses. Following such a relative dosing protocol, Syrotuik and Bell still found a highly variable response among individuals in the ability to increase intramuscular creatine levels, which they describe as a phenomenon of responders and non-responders to the supplementation (2004). Furthermore, because 95% of bodily creatine is found in the skeletal muscle, a more uniform dosing strategy would be based upon skeletal muscle mass of the individual. In a recent unpublished study, Burke (2003, 2005) compared various relative doses that were based on lean body mass as determined by dual-energy x-ray absorptiometer, and found that a loading dose of 0.25 g/kg lean body mass/d and a maintenance dose of 0.0625 g/kg lean body optimized intramuscular creatine content and whole body creatine retention. Accordingly, it would be ideal to utilize a relative dosing strategy when attempting to characterize the biochemical response to creatine supplementation, in order to attempt to normalize the exposure of skeletal muscle to supra-physiological levels of creatine via an oral supplementation regimen. It should be noted that Burke administered the supplement with 500 mL of fruit drink, which presumably enhanced creatine uptake via insulin release (Steenge, 1998).

Creatine is an osmotically active compound, such that increases in total body creatine retention caused by oral creatine supplementation should result in increased water retention. This, in part, may explain how increases in total body mass are seen

after only a loading supplementation of 20-25 g/d for 5-7 days (Volek, 2001). In particular, it would be expected that increases in intramuscular creatine, which accounts for approximately 95% of total body creatine, would particularly augment intracellular water volume. However, it has been shown that a loading phase of 25 g/d for 7 days followed by a maintenance phase of 5 g/d for 21 days resulted in increases in intramuscular creatine and total body water, without altering proportional fluid distribution (Powers, 2003). Interestingly, increases in cell volume appear to be a proliferative, anabolic signal that may enhance protein synthesis (Haussinger, 1996), which suggests a method by which extended creatine supplementation may promote muscular hypertrophy.

All plasma membranes are impermeable to the diffusion of creatine and phosphocreatine. More than 90% of intramuscular creatine is shuttled across the sarcolemma against a concentration gradient via a Na^+/Cl^- dependent transporter known as the creatine transporter (CrT). Approximately 65% of the creatine that enters skeletal muscle will be actively transphosphorylated by creatine kinase to produce phosphocreatine, such that it is readily available to react to ATP depletion (Snow, 2003). Phosphocreatine cannot be transported by CrT, and as such, creatine kinase renders a portion of the intracellular creatine pool to be confined in the cytosol. Recently, Walzel et al. (2002) have reported a new and possibly unique CrT activity in the mitochondria, which suggests that there are likely two separate pools of intracellular creatine, regulated by different CrT activities. The authors suggest that serum creatine, at a concentration of 25-50 μM, moves through a plasma membrane CrT, contributing to a cytosolic creatine pool with a concentration of ~15 mM. Furthermore, a mitochondrial CrT allows for

cytosolic creatine to contribute to a mitochondrial pool of Cr with a concentration of ~20 mM. They report the plasma membrane CrT as a high affinity (low K_m) transporter, while the mitochondrial CrT is a low affinity (high K_m) transporter. It is possible that these separate creatine transporters are entirely different proteins, with variations in structure, and subject to different degrees of regulation. It is currently uncertain what purpose a mitochondrial pool of Cr may have, although it may simply serve as a buffer, should plasma Cr levels drop appreciably, such that cytosolic Cr levels may remain elevated during high contractile activity.

Several different *in vitro* studies have provided some insight into the regulation of creatine transport. Acute regulation (within hours) of CrT activity may be directly influenced by fluctuations in plasma creatine concentrations. This may involve alterations in the flux of sodium across the sarcolemma (Odoom, 1996). Similarly, there may be factors that might directly stimulate or inhibit the CrT protein (Nash, 1994). Chronic adaptations (days to weeks) may involve altering the number of CrT proteins available at the membrane, or by altering the number of CrT proteins expressed by the cell (Guerrero-Ontiveros, 1998). It seems that creatine uptake is actually inhibited with prolonged exposure to high plasma creatine levels, which may be due to decreased activity of CrT. Loike et al. (1986) found that cultured myocytes exposed to very high extracellular Cr concentrations (1-5 mM) displayed a decrement in Cr uptake, which was due to a loss in CrT activity, not a decreased affinity of Cr for the transporter. This was corroborated by findings that cells incubated in a sodium-free solution with the same Cr concentration did not demonstrate reduced Cr uptake. Boehm et al. (2003) reported that Cr supplementation in intact perfused rat heart leads to a decrease in the V_{max} of Cr

transport in the heart as well as a decrease in membrane CrT content, while supplementation with β-guanidinopropionic acid (β-GPA), a creatine analog and competitive inhibitor of CrT, resulted in a greater V_{max} and an increase in membrane CrT content (2003). This suggests that creatine uptake is actually dependent on intracellular creatine concentrations, and not extracellular creatine concentrations. It appears that elevated plasma creatine levels promote an initial rise in creatine uptake and resultant intracellular creatine concentration, which may in and of itself begin to inhibit uptake by negative feedback.

This down-regulation of Cr uptake with chronic elevated plasma Cr levels may be due to an inhibitory protein. Loike et al. (1986) demonstrated that cultured cells exposed to cycloheximide, a protein synthesis inhibitor, lost the down-regulation of Cr uptake. Another similar study found that the presence of cycloheximide allowed for a 2.4 fold increase in intracellular creatine (Oodom, 1996). This suggests that high intracellular concentrations may induce the expression of a protein that may either directly inhibit the CrT protein, or somehow reduce the number of transporter proteins available at the membrane. However, no such protein or mechanism has been identified up to this point. Another possible regulatory mechanism is an interaction of CrT with plasma membrane glycoproteins. It has been shown that the activity of a similar family of plasma membrane-spanning transporters, the monocarboxylate transporters, is dependent on the interaction with membrane bound regulatory glycoproteins (Halestrap, 1999). However, there is no current data to support any such interaction with CrT.

Hormones may also have an effect on CrT activity. Various catecholamines, amylin, insulin, and insulin growth factor I all stimulate the membrane-bound Na^+/K^+-

ATPase pump, which effectively increases the sodium gradient across the membrane (Clausen, 1998). This may enhance the electrochemical gradient that drives Cr transport against its concentration gradient. Insulin is further implicated by the finding that ingestion of Cr with high doses of carbohydrate promotes improved Cr uptake (Steenge, 1998). This is likely to be mediated by the carbohydrate-induced stimulation of insulin release.

Another possible regulatory mechanism for CrT activity is the protein's post-transcriptional modification state. Wang et al. (2002) demonstrated that the Tyr-416 residue of the putatative transporter protein is subject to phosphorylation, and furthermore, that Cr supplementation results in a decrease in the degree of CrT phosphorylation. Another group has previously ruled out cAMP dependent protein kinase (protein kinase A) as a modifier of the phosphorylation state of CrT, as treatment with compounds known to stimulate protein kinase A had no effect on Cr uptake in transfected cells (Nash, 1994). As a transmembrane protein, CrT may also be subject to glycosylation or lipoylation. It has been shown that the CrT isoform found in the plasma membrane is likely to be weakly glycosylated and that this glycosylation may serve as a label to ensure that that particular isoform is located at the plasma membrane (Tran, 2000). The control of these modification processes may exert a strong influence on both the activity of the CrT protein, and on the number of CrT proteins available for transport.

Lastly, it is possible that alterations in expression of the gene that codes for CrT might exert some control on the amount and activity of the CrT protein. The CrT gene is found on human chromosome Xq28, and is identified as the Cr transporter 1 (CrT1) gene (Iyer, 1996). Not surprisingly, the Xq28 locus has been linked to a number of

neuromuscular diseases, suggesting that mutations within this gene and the ensuing alterations of the wild-type protein may be to blame for these disorders (Consalez, 1998). Nearly identical CrT mRNA transcripts have been identified in human skeletal muscle, lung, brain, spleen, kidney, pancreas, testis, ovary, small intestine, thymus, placenta, and colon, and their relative quantities roughly coincide with their protein expression in each respective tissue (Nash, 1994). It is likely that the transcription levels of the CrT1 gene within each tissue are affected by the local creatine concentrations, which vary according to the quantity of CrT protein present in the tissue. Preliminary studies have validated the use of real-time polymerase chain reaction (RT-PCR) as a method for determining transcription levels of the CrT1 gene (Murphy, 2003). However, there is little if any data describing the direct effects that exogenous creatine supplementation may have on the transcription of the CrT1 gene and expression of the CrT protein in humans.

CHAPTER THREE

Methods and Materials

Methods

Participants

Nineteen healthy males between the ages of 18 and 23 served as participants in this study. Participants had at least one year of resistance training experience and had participated in resistance training protocols at least three times a week for the preceding six months, including at least one lower body exercise bout per week. Participants were required to perform a leg press 1-RM of at least 2.5 times their body weight (Wathen, 1993), followed by at least 10 repetitions at 70% of their 1-RM during a familiarization session to be considered trained to participate in the study. Participants were not allowed to participate in this study if they had any metabolic disorder, including known electrolyte abnormalities, heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism; a history of hypertension, hepatorenal, musculoskeletal, autoimmune, or neurologic disease; if they were taking thyroid, anti-hyperlipidemic, hypoglycemic, anti-hypertensive, anti-inflammatory, or androgenic medications; and, if they had taken ergogenic levels of creatine within six months prior to the start of the study and/or creatine naïve ideally. Participants were recruited from the male population at Baylor University and from surrounding Waco. Participants meeting eligibility criteria were informed of the requirements of the study and signed informed consent statements in compliance with the Human Subjects Guidelines of Baylor University and the American College of Sports Medicine. Participants were required to follow a specific resistance

training protocol for the duration of the study. Compliance to training regimen was confirmed by oral communication and weekly activity logs.

Study Site

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

Independent and Dependent Variables

The independent variables were the creatine supplement and the placebo that was used for the control groups. Dependent variables included leg strength, intracellular and extracellular fluid volume, fat-free mass, urinary creatine and creatinine, serum creatine and creatine, intramuscular free creatine, total creatine, and phosphocreatine, intramuscular CrT mRNA and protein expression, and whole body creatine retention.

Entry and Familiarization Session

Individuals expressing interest in the study were interviewed by phone to determine if they appeared to qualify to participate in the study. Those believed to meet eligibility criteria were then invited to attend an entry/familiarization session. After reporting to the lab, potential participants completed a medical history questionnaire, underwent a general physical examination, and then performed a 1-RM leg press and 70% 1-RM leg press for repetitions. The potential participants were required to leg press at least 2.5 times their body weight, as well as at least 10 repetitions at 70% of their 1-RM to confirm their training status. The 70% repetition set was performed five minutes

after completing the 1-RM assessment, as described below. Participants meeting entry criteria underwent a baseline body composition analysis by DEXA, as described below. The participants were then familiarized to the study protocol by way of a verbal and written explanation outlining the study design and were then given an appointment time to perform baseline/pre-supplementation assessments. Participants underwent their first testing session no longer than two weeks following their familiarization.

Strength Assessment

In order to determine possible effects of the supplement on muscular strength, participants performed four one-repetition maximum (1-RM) tests on the leg press sled: 1) at baseline (familiarization); 2) on Day 8 after the one week loading phase; 3) on Day 36 after the four week maintenance phase; and 4) on Day 64 after the four week washout phase. Participants warmed up by completing two sets of 8 to 10 repetitions at approximately 50% of the estimated 1-RM, with 2 minutes rest in between all sets. The participant then completed 3 to 5 repetitions at approximately 75% of the estimated 1-RM. The weight was then increased conservatively, and the participant attempted to lift the weight for one repetition. If the lift was successful, the participant rested for an additional 2 minutes before attempting the next weight increment. This procedure was continued until the participant failed to complete the lift. The 1-RM was recorded as the maximum weight that the participant was able to lift for one repetition. For the 70% 1-RM evaluation during the familiarization session, participants were allowed to rest five minutes following the determination of their 1-RM. The weight on the leg sled was adjusted to 70% of their 1-RM, and they were instructed to complete as many repetitions as possible.

Dietary Analysis

Participants were required to record their dietary intake for 24 hours prior to each of the five testing sessions where muscle samples were obtained. The participants' diets were not standardized and participants were instructed not to change their dietary habits during the course of the study. The 24-hour dietary recalls were evaluated with the ESHA Food Processor dietary assessment software program (ESHA Nutritional Research, Salem OR) to determine the average daily caloric, protein, carbohydrate, and fat intakes for the duration of the study.

Anthropometric and Body Composition Testing Procedures

Total body mass (kg) was determined on a Detecto digital scale (Webb City, MO). Total body water (total, intracellular, and extracellular) was determined with bioelectrical impedance (BIA) while percent body fat, fat mass, and fat-free mass, were determined using dual-energy x-ray absorptiometer (DEXA; Hologic Discovery, Bedford MA). Radiation exposure from DEXA for the whole body scan was approximately 1.5mR per scan. 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 low energy, high frequency current (500 micro-amps at a frequency of 50 kHz) transmitted through the body. The participants underwent total body mass and BIA measurements at each of the five testing sessions when muscle samples were obtained. A DEXA scan was performed during each of these testing sessions as well, except for the first testing session, since a baseline DEXA scan was taken during the familiarization session.

Supplementation Protocol

Participants were randomly assigned by age and body weight to orally ingest in a double blind manner packets containing a powdered dextrose placebo (AST Sports Science; Golden, CO) or micronized creatine monohydrate (AST Sports Science; Golden, CO). After baseline testing procedures and lean body mass determination via DEXA, participants ingested creatine or the placebo at a relative daily dose of 0.3 g/kg lean body mass (≈ 17-20 g/day) for one week 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 4 week maintenance phase. All groups ceased supplementation on Day 36 following the 4 week maintenance phase.

In order to standardize supplement intake throughout the study, participants were 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. Additionally, the participants were instructed to consume the supplement with water only, and ideally on an empty stomach in order to eliminate possibly confounding effects of insulin. Dextrose and creatine powders were comprised of similar mesh size, texture, taste, and appearance and were prepared for distribution by an objective third-party company (AST Sport Science, Colorado Springs, CO). Supplement packets for each participant were individually weighed on an analytical balance (Denver Instrument, Golden, CO) to the nearest 1% of the prescribed dose. During the loading phase, the participants consumed four packets a day, each containing an equal constituent dose of their prescribed daily dose. During the maintenance phase, the participants consumed one packet a day, which contained the whole daily dose. Participants were provided with

bundles of dose packets one week at a time, and were required to present empty packets each week to receive the next week's supplement. Midway through the maintenance phase (Day 22), the participants' daily doses were adjusted according to that day's DEXA body composition analysis. Compliance to the supplementation protocol was monitored by the return of empty supplement packets at the end of each week. In addition, the participants' compliance was verified by weekly verbal communication.

Training Protocol

The participants followed a periodized 4-day per week resistance-training program split into two upper body and two lower body workouts per week, for a total of nine weeks. Prior to each workout, the participants performed a series of standardized stretches. For a given exercise, the participants performed 3 sets of 10 repetitions with as much weight as they could lift per set (typically 70 – 80% of 1RM). If they could lift fewer than 10 repetitions at a given weight, they were instructed to reduce the weight accordingly, and if they could lift more than 10 repetitions at a given weight, they were instructed to increase the weight accordingly. The participants were instructed to rest no longer than 3 minutes between exercises and no longer than 2 minutes between sets. During the familiarization session, the participants gave verbal confirmation that they were aware of how to properly perform each exercise.

The participants' upper body resistance-training program consisted of eight groups of exercises, and from each group they were instructed to choose only one exercise to perform for that workout. The upper body exercise groups were: 1) bench press, incline press, decline press, machine press, dumbbell bench press, or dumbbell incline press; 2) chest flies or cable crossovers; 3) wide-grip lat pulldown or close-grip lat

pulldown; 4) seated row, bent-over row, or dumbbell bent-over row; 5) shoulder press, dumbbell shoulder press, or machine shoulder press; 6) shoulder shrugs; 7) dumbbell curl, preacher curl, dumbbell preacher curl, or barbell curl; and 8) triceps pressdown, rope pressdown, reverse-grip pressdown, dumbbell triceps extension, or lying triceps extension. The participant's lower body resistance-training program consisted of seven groups of exercises, and from each group they were instructed to choose only one exercise to perform for that workout. The lower body exercise groups were: 1) leg press, back squats, smith machine squats, or machine squats; 2) leg extensions; 3) back extension, Russian deadlift, or good mornings; 4) step-ups, lunges, or split squats; 5) seated leg curls or lying leg curls; 6) standing heel raises or seated heel raises; and 8) abdominal crunches. As the sole exception, the abdominal crunches were performed in sets of twenty five repetitions instead of ten repetitions. For both upper and lower body workouts, the participants were only required to perform the same exercise from a particular group for the current workout, but were allowed to select a different exercise from each group during a subsequent workout at their discretion. All training took place at the Student Life Center (SLC) at Baylor University or at an area gym, and was documented by the participants in training logs to verify compliance and to monitor progress.

Urine Sampling

In order to determine the effects of the supplements on urinary creatine and creatinine levels and whole body creatine retention, participants were instructed to collect a total of nine 24-hour urine samples throughout the course of the study. During the familiarization session, each participant was provided with two 3 L urine collection

containers. The participants were instructed to begin each 24-hour urine collection at 8 am the day before their scheduled testing appointment, and to bring their collected urine in to the lab the next morning when they reported for testing. They were asked to record the number of times they urinated each day as well as total fluid intake during the period on a urine log. The participants collected 24-hour urine samples on Day 0 prior to the one week loading phase, and then also on Days 3 and 7 (samples 1-3) of the loading phase. Participants then collected 24-hour urine samples on Days 14, 21, 28, and 35 during the four week maintenance phase (samples 4-7), and also on Days 49 and 63 (samples 8, 9) of the four week washout period. Urine samples were refrigerated upon receipt during the participant's appointment, and 1 mL aliquots were frozen at -80° C for later analysis.

Muscle Biopsies and Venous Blood Sampling

Percutaneous muscle biopsies (50-70 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 subsequent four biopsies, attempts were made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy scar, depth markings on the needle, and a successive incision that was made approximately 0.5 cm to the former from medial to lateral. After removal, adipose tissue (if present) was trimmed from the muscle tissue and the sample was immediately flash-frozen in liquid nitrogen and subsequently stored at -80°C for later analysis. A total of five muscle samples were collected: 1) prior to the first dose of supplement (Day 1); 2) on Day 8, after the one week loading (which also served as the pre-maintenance phase biopsy); 3) on Day 22, after the first two weeks of

the maintenance phase; 4) on Day 36, after the four week maintenance phase; and 5) on Day 64, after the four week washout period following the four week maintenance phase.

Venous blood samples were collected from the antecubital vein into two 7.5 mL SST™ collection tubes and one 4 mL Plus EDTA™ tube using a standard Vacutainer® apparatus for serum and whole blood analysis, respectively. Participants were required to fast for eight hours prior to donating blood. Blood samples taken in the serum tubes were centrifuged for 15 minutes, and then the serum was removed and frozen at -20°C for later analysis. Whole blood samples were refrigerated at 4˚C until processed, within six hours of collection. A total of six blood samples were obtained: prior to the first dose of supplement (Day 1), on Day 4, on Day 8, after the one week loading phase (which also served as the pre-maintenance phase blood draw), on Day 22, after the first two weeks the maintenance phase, on Day 36, after the completion of the four week maintenance phase, and on Day 64, after the four week washout period following the four week maintenance phase.

Creatine Analysis

Urine, blood, and muscle tissue samples were analyzed for free creatine using the diacetyl/α-napthtol reaction. Additionally, muscle was analyzed for total creatine and phosphocreatine using the same method. Unmodified urine and serum samples were immediately ready for analysis, while muscle tissue samples required additional preparation. Muscle tissue samples (2-8 mg) were dried overnight in a ThermoSavant Speed Vac (Waltham, MA), then powdered at room temperature with mortar and pestle. Powdered muscle was weighed (0.5-3.0 mg) and then extracted in a 0.5 M perchloric acid/1 mM EDTA solution on ice for 15 minutes. The samples were then spun in a 4˚C

centrifuge at 15,000 rpm for 5 minutes, and the supernatant was neutralized with 2.1 M potassium bicarbonate/0.3 M MOPS solution, at which the supernatant was prepared for analysis. Total creatine of samples was determined by reaction with 6.94 mM α-napthtol and a 1:2500 dilution of diacetyl. The reaction was incubated for 15 minutes at room temperature in the dark, and color formation was detected at 520 nm with microplate reader (Wallac Victor-1420; Perkin-Elmer Life Sciences, Boston, MA). The samples were run against a standard curve of known creatine concentrations. Free creatine was determined by combining homogenate with 0.4 N hydrochloric acid and heating at 65˚C for 10 minutes. The homogenate was then neutralized with 2.0 N sodium hydroxide, and subsequently subjected to the diacetyl/α-napthtol reaction as previously described. Phosphocreatine concentration was calculated as free creatine concentration subtracted from total creatine concentration. For statistical analysis, total Cr content was expressed as the difference in concentration (delta) from baseline.

Urinary and Serum Creatinine Determination

Urine and serum levels of creatinine were determined photometrically by a DADE Dimension RXL clinical chemistry analyzer (Dade-Behring, Inc., Newark, DE). Unmodified samples were immediately ready for analysis.

Creatine Transporter mRNA Transcription

Muscle tissue samples were analyzed for creatine transporter mRNA transcription levels by quantitative real-time polymerase chain reaction (RT-PCR). Muscle tissue samples were weighed, then homogenized with Tri Reagent (Sigma, St. Louis, MO) and total RNA was isolated by isopropanol/ethanol extraction. Total RNA was then used to

generate a cDNA library using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), which was subsequently used as the template in the RT-PCR reaction. Primers (Integrated DNA Technologies, Coralville IA) were designed separately according to the human creatine transporter gene sequence and the constitutively active human β-actin gene sequence as published by the National Center for Biotechnology Information. Each sample was then run on an iCycler iQ RT-PCR system (Bio-Rad, Hercules CA), with both creatine transporter or β-actin primers in separate reactions, to determine relative transcription levels of the creatine transporter gene, which is expressed as the ratio of creatine transporter transcription levels to β-actin transcription levels.

Creatine Transporter Protein Expression

Muscle tissue samples were analyzed for creatine transporter expression by enzyme-linked immunosorbant assay (ELISA). Protein samples were obtained by isopropanol/ethanol extraction from the same homogenate used to extract total RNA. Total protein concentration of extracts was determined by the Bradford total protein assay (Sigma Chemical Co., St. Louis MO). Protein extracts were normalized to 20 µg total protein for analysis. The ELISA plate was prepared by incubating 120 µL of a 4µg/mL solution of the primary anti-CRT capture antibody (Alpha Diagnostics, San Antonio, TX) overnight at room temperature. The plate was then washed three times with Tris-buffered saline (TBS). Normalized protein extracts were loaded onto the ELISA plate and allowed to incubate at room temperature overnight. After incubation, the plate was washed three times with TBS. The washed plate was then loaded with 200 µL of a 10 µg/mL solution of anti-human IgG-horseradish peroxidase conjugate detection antibody (ICN Biomedical, Costa Mesa, CA) for 2 hours. The plate was once again washed three times

with TBS, and then exposed to a 3,3',5,5'-tetramethylbenzidine (TMB) solution for color development for 30 minutes. Once adequate color development was obtained, the reaction was stopped with the addition of 0.5 N hydrochloric acid. The absorbance was measured at 450 nm with a microplate reader (Wallac Victor-1420; Perkin-Elmer Life Sciences, Boston, MA). CrT protein content was expressed as the difference in absorbance units (delta) from baseline.

Reported Side Effects from Supplements

Participants were encouraged to report whether or not they tolerated the supplement, as well to report any medical problems or symptoms they encountered throughout the duration of the study.

Blood Chemistry Safety Markers

Serum samples were used to run clinical chemistry profiles (triglycerides, cholesterol, high-density lipoprotein, low-density lipoprotein, gamma-glutamyl transferase, lactic dehydrogenase, uric acid, glucose, blood urea nitrogen, BUN/creatinine ratio, calcium, total protein, albumin, total bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, and total creatine kinase) photometrically by way of a DADE Dimension RXL clinical chemistry analyzer (Dade-Behring, Inc., Newark, DE). Whole blood samples were used to run whole blood cell counts including hemoglobin, hematocrit, red blood cell counts, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, red cell distribution width, and white blood cell counts including neutrophils, lymphocytes, monocytes, eosinophils, and baosphils by

way of an Abbott Cell Dyn 3500 hematology analyzer (Abbott Laboratories, Chicago, IL). Prior to use each system was calibrated with standard quality assurance protocols.

Compensation and Incentives

Each participant was paid $200 upon completion of the study, including submission of all forms and logs, as well as completion of all testing sessions and sample collections. The participants were also provided with the opportunity of receiving free Cr supplements, as well as free consultation and medical monitoring by the staff of the ESNL. The individuals conducting this study had no financial interest or any other vested interest in the outcome of the study.

Statistical Analysis

Data was analyzed using group x time repeated univariate measures analysis of variance (ANOVA) with SPSS for Windows Version 11.5 software (*SPSS Inc., Chicago, IL*). Comparisons of demographics and supplement intake were determined using an independent Student's t-test. For the analyses of urine, blood, muscle, body composition, and strength, respective 2 x 9, 2 x 6, 2 x 5, 2 x 5, and 2 x 4 repeated measures ANOVAs were utilized. Data were considered significantly different when the probability of error was 0.05 or less. Significant differences among groups were identified by a Least Significant Differences (LSD) post hoc test. When necessary, Cohen's *d* effect calculations were performed to determine the size and significance between groups independent of group size.

CHAPTER FOUR

Results

Participant Demographics

Thirty-two healthy males were familiarized and signed informed consent statements in compliance with Baylor University's Institutional review board. Nine elected to abstain from the study following their recruitment. Four elected to withdraw from the study prior to completion. Two participants withdrew regarding concerns about undergoing additional muscle biopsies. One participant withdrew due to contraction of mononucleosis during the study, and one participant withdrew due to a condition of gastrointestinal stress that may have been contracted while traveling abroad. Nineteen (Cr = 9, Pl = 10) completed the study. Table 1 depicts the mean age, height, and body weight at familiarization for each group. Statistical analysis revealed that there was no significant difference between groups in age ($p = 0.76$) or weight ($p = 0.47$), but there was a significant difference between groups in height ($p = 0.03$).

Table 1

Participant Demographics

Variable	*Cr Group (±SD)*	*Pl Group (±SD)*	*Group p-level*
Age (yr)	20.1 (1.6)	20.3 (1.1)	0.76
Height (cm)	181.26 (6.41)	174.43 (5.79)	*0.03
Weight (kg)	83.4 (19.4)	77.6 (15.0)	0.47

Note: This data represents the average demographics of all participants that completed the study. Data are expressed as means ± SD. Significant group interactions contain an asterisk.

Familiarization and Strength Assessment

All participants performed a strength assessment during the familiarization session to demonstrate minimum strength requirements for entry into the study. Table 2 represents the average strength assessment parameters for both groups of participants who completed the study. Statistical analysis revealed that there were no significant differences between groups in body weight multiples of leg press 1-RM ($p = 0.66$) or number of repetitions at 70% of their respective 1-RM ($p = 0.65$).

Table 2

Baseline Strength Assessment

Variable	*Cr Group (±SD)*	*Pl Group (±SD)*	*Group p-level*
Body Weight Multiples of Leg Press 1-RM	4.20 (1.00)	4.46 (1.43)	0.66
# of Repetitions at 70% 1-RM	20.6 (5.3)	22.0 (7.9)	0.65

Note: This data represents the average strength assessment parameters of all participants that completed the study. Data are expressed as means ± SD.

Dietary Intake

Participant dietary intakes during the study were determined by self-reported 24 hour food records completed prior to each of the five testing sessions which involved muscle biopsies (Day 1, Day 8, Day 22, Day 36, and Day 64) and were analyzed by the Food Processor nutritional software (ESHA Research, Salem, OR). Table 3 represents the average daily dietary intakes for both groups over the course of the study. Statistical analysis revealed that there was a significant decrease in protein intake ($p = 0.03$) for both groups, but there was not a significant difference between groups in caloric intake ($p = 0.45$) or protein intake ($p = 0.61$) over the course of the study.

Table 3

Dietary Intake

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
Calories (kcal/kg)	Day 1	34.8 (11.5)	36.7 (21.5)	-	-	-
	Day 8	26.8 (10.0	28.7 (11.6)	-	-	-
	Day 22	25.2 (7.2)	28.3 (10.3)	-	-	-
	Day 36	20.3 (8.2)	27.1 (9.0)	-	-	-
	Day 64	19.7 (12.1)	35.0 (13.7)	0.07	0.10	0.45
Protein (g/kg)	Day 1	1.39 (0.67)	1.69 (0.93)	-	-	-
	Day 8	1.26 (0.29)	1.13 (0.52)	-	-	-
	Day 22	1.16 (0.37)	1.15 (0.38)	-	-	-
	Day 36	1.00 (0.24)	1.12 (0.31)	-	-	-
	Day 64	0.92 (0.56)	1.10 (0.43)	*0.03	0.56	0.61

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
Carbohydrate (g/kg)	Day 1	3.92 (1.78)	3.78 (2.15)	-	-	-
	Day 8	2.49 (1.46)	3.41 (1.94)	-	-	-
	Day 22	2.53 (0.60)	3.25 (1.45)	-	-	-
	Day 36	2.27 (1.02)	3.10 (1.60)	-	-	-
	Day 64	2.19 (1.49	3.86 (1.75)	0.28	0.12	0.63
Dietary Fat (g/kg)	Day 1	1.52 (0.76)	1.62 (1.30)	-	-	-
	Day 8	1.31 (0.59)	1.22 (0.48)	-	-	-
	Day 22	1.18 (0.51)	1.18 (0.40)	-	-	-
	Day 36	0.81 (0.51)	1.14 (0.35)	-	-	-
	Day 64	0.70 (0.63)	1.76 (0.75)	0.08	0.08	0.26

Note: This data represents the average dietary intake of all participants that completed the study. Data are expressed as means ± SD. Significant time effects contain an asterisk.

Supplement Administration

The participants were assigned supplement doses as determined by their fat-free body mass assessments on Day 1, Day 8, and Day 22. Table 4 represents the average supplemental intakes of both groups over the course of the study. Statistical analysis reveals that there were no significant differences in loading dose ($p = 0.32$) or maintenance dose 2 ($p = 0.21$) between groups, but that there was a significant difference in maintenance dose 1 ($p = 0.05$) between groups. However, it should be noted that these intakes reasonably approached the paradigm creatine doses of ~20 g/d for a loading period and ~5 g/d for a maintenance period. Furthermore, these doses were not absolute, but relative to each participant's fat-free body mass; thus an apparent difference in doses

between groups is reflective of variance in fat-free body mass and not indicative of dissimilar dosing.

It was an assumption of this study that each participant completely ingested the entire amount of supplement that was given to them. Each participant presented empty supplement packets and gave oral confirmation that they had consumed the supplement prior to receiving the subsequent week's supplement. There was no indication of any participant failing to consume a supplement dose. In this manner, compliance to the supplement protocol was assumed to be 100%. Furthermore, there were no adverse side effects reported by any of the participants during the study that may have been related to ingestion of the supplement.

Table 4

Supplement Administration

Variable	Cr Group (± SD)	Pl Group (± SD)	Group p-level
Loading Dose (g/d)	18.80 (2.04)	17.84 (2.08)	0.32
Maintenance Dose 1 (g/d)	4.75 (0.48)	4.33 (0.37)	*0.05
Maintenance Dose 2 (g/d)	4.78 (0.49)	4.48 (0.53)	0.21

Note: This data represents the average daily (grams/day) supplement intake of all participants that completed the study. Data are expressed as means ± SD.

Training Protocol and Training Volume

Each participant followed a periodized 4-day per week resistance-training program including two upper body and two lower body workouts per week. It was an assumption of this study that each participant regularly completed the training protocol for the duration of the study. The participants were asked to record their weights lifted during each session in a training log, and gave verbal confirmation of completion of their

training at each testing appointment. Each participant turned in their completed training logs at the end of the study. There was no verbal or recorded indication of any participant failing to complete any of their training. In this manner, compliance to the resistance-training protocol was assumed to be 100%.

Training volume was determined by multiplying weight lifted by number of repetitions for each exercise throughout the duration of the study. Table 5 represents the average training volumes of both groups for upper body exercises, lower body exercises, and overall training over the course of the study. Statistical analysis revealed that there was no significant difference between groups in any measure of training volume.

Table 5

Training Volume

Variable	Cr Group (± SD)	Pl Group (± SD)	Group p-level
Upper Body Training Volume (kg)	149840 (74036)	209303 (42530)	0.08
Lower Body Training Volume (kg)	222382 (63875)	269551 (117927)	0.32
Total Training Volume (kg)	372223 (118398)	478855 (139309)	0.12

Note: This data represents the average weight lifted by all participants that completed the study. Data are expressed as means ± SD.

Body Composition and Strength Measures

Table 6 represents the average body composition and strength measures of both groups over the course of the study. Statistical analysis revealed significant increments in total body mass ($p = 0.03$), lean body mass ($p = 0.01$), intracellular fluid ($p = 0.01$), and total body water ($p = 0.004$) in the Cr group. Figures 1 and 2 depict the changes in total body mass and lean body mass, respectively, for the duration of the study. Hypothesis H_7 is accepted as Cr supplementation did significantly augment total body mass and fat free

mass. There were no significant differences in body fat percentage ($p = 0.13$), extracellular fluid ($p = 0.12$), relative total body water ($p = 0.07$), extracellular: intracellular ratio ($p = 0.19$), or extracellular:total ratio ($p = 0.21$). Hypothesis H_6 is accepted, as Cr supplementation did not affect relative total body water or overall fluid distribution. There was a significant main effect for leg press 1-RM to increase with time ($p < 0.001$), however there was no significant group x time interaction ($p = 0.17$). The changes in leg press 1-RM are shown in Figure 3. The overall strength gain as determined by change in leg press 1-RM over the course of the study was 33.01 ± 23.29 kg. When split into groups, the strength gain for the Cr group was 41.16 ± 26.90 kg, while the strength gain for the Pl group was 25.68 ± 17.77 kg. Hypothesis H_5 is rejected as no significant increase in leg press strength was found to be attributed to Cr supplementation.

Table 6

Body Composition and Strength Measures

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
Total Body Mass (kg)	Day 1	83.5 (19.8)	77.9 (14.7)	-	-	-
	Day 8	84.4 (19.5)	77.6 (14.8)	-	-	-
	Day 22	85.0 (19.5)	78.3 (14.6)	-	-	-
	Day 36	84.8 (18.9)	77.5 (14.6)	-	-	-
	Day 64	84.6 (19.4)	77.8 (15.0)	0.10	0.41	*0.03
Lean Body Mass (kg)	Day 1	62.7 (6.8)	59.3 (7.0)	-	-	-

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
	Day 8	63.3 (6.4)	59.1 (6.8)	-	-	-
	Day 22	63.8 (6.5)	59.7 (7.1)	-	-	-
	Day 36	63.9 (6.3)	58.9 (6.9)	-	-	-
	Day 64	63.5 (6.2)	58.8 (6.8)	0.33	0.18	*0.01
Body Fat %	Day 1	17.6 (8.8)	17.0 (9.1)	-	-	-
	Day 8	17.7 (8.9)	17.2 (9.4)	-	-	-
	Day 22	17.5 (8.7)	17.1 (8.9)	-	-	-
	Day 36	17.6 (8.5)	17.5 (9.3)	-	-	-
	Day 64	17.6 (8.4)	17.4 (9.1)	0.64	0.93	0.13
Extracellular Fluid (L)	Day 1	18.3 (2.1)	17.9 (2.5)	-	-	-
	Day 8	18.9 (2.3)	17.6 (2.6)	-	-	-
	Day 22	18.4 (2.3)	17.9 (3.0)	-	-	-
	Day 36	18.9 (2.6)	17.4 (2.5)	-	-	-
	Day 64	18.5 (1.9)	18.2 (2.3)	0.49	0.69	0.12
Intracellular Fluid (L)	Day 1	25.0 (3.0)	24.9 (3.2)	-	-	-
	Day 8	26.2 (3.2)	24.4 (2.7)	-	-	-
	Day 22	26.4 (3.2)	24.5 (3.1)	-	-	-
	Day 36	26.7 (3.2	24.4 (3.0)	-	-	-
	Day 64	26.0 (3.0)	25.0 (3.3)	0.10	0.44	*0.01
Total Fluid (L)	Day 1	43.3 (3.9)	42.8 (5.4)	-	-	-
	Day 8	45.2 (3.9)	42.1 (5.2)	-	-	-
	Day 22	44.8 (3.7)	42.4 (6.0)	-	-	-
	Day 36	45.6 (4.1)	41.8 (5.3)	-	-	-
	Day 64	44.5 (3.2)	43.2 (5.3)	0.21	0.47	*0.004

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
Relative Total Body Water (L/kg)	Day 1	0.534 (0.078)	0.550 (0.04)	-	-	-
	Day 8	0.550 (0.081)	0.542 (0.042)	-	-	-
	Day 22	0.543 (0.080)	0.543 (0.034)	-	-	-
	Day 36	0.551 (0.077)	0.540 (0.044)	-	-	-
	Day 64	0.542 (0.083)	0.545 (0.037)	0.73	0.99	0.07
Extracellular : Intracellular	Day 1	0.739 (0.120)	0.721 (0.069)	-	-	-
	Day 8	0.733 (0.138)	0.721 (0.058)	-	-	-
	Day 22	0.709 (0.148)	0.728 (0.051)	-	-	-
	Day 36	0.685 (0.195)	0.716 (0.054)	-	-	-
	Day 64	0.719 (0.132)	0.730 (0.057)	0.10	0.80	0.19
Extracellular : Total	Day 1	0.423 (0.036)	0.418 (0.023)	-	-	-
	Day 8	0.420 (0.040)	0.419 (0.017)	-	-	-

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
	Day 22	0.412 (0.044)	0.421 (0.020)	-	-	-
	Day 36	0.415 (0.043)	0.417 (0.018)	-	-	-
	Day 64	0.416 (0.039)	0.422 (0.019)	0.09	0.76	0.21
Leg Press 1 RM (kg)	Day 1	342.9 (84.0)	347.7 (134.1)	-	-	-
	Day 8	375.3 (105.5)	359.1 (152.1)	-	-	-
	Day 36	379.8 (91.2)	366.1 (139.5)	-	-	-
	Day 64	384.1 (101.9)	373.4 (146.0)	*<0.001	0.88	0.17

Note: This data represents the average body composition and strength parameters of all participants that completed the study. Data are expressed as means ± SD.

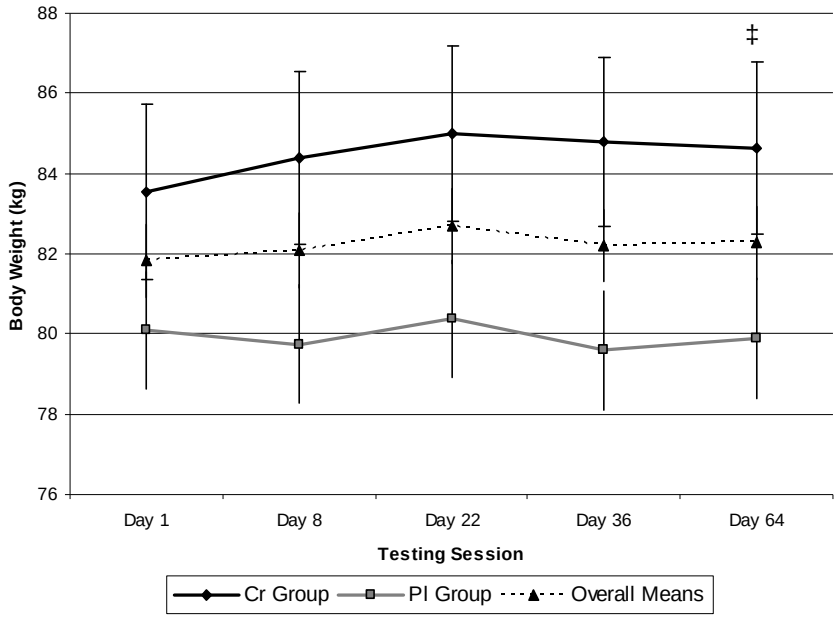

Figure 1. Changes in body weight (kg) over the course of the study. A significant increase was observed in the Cr group ‡($p = 0.03$).

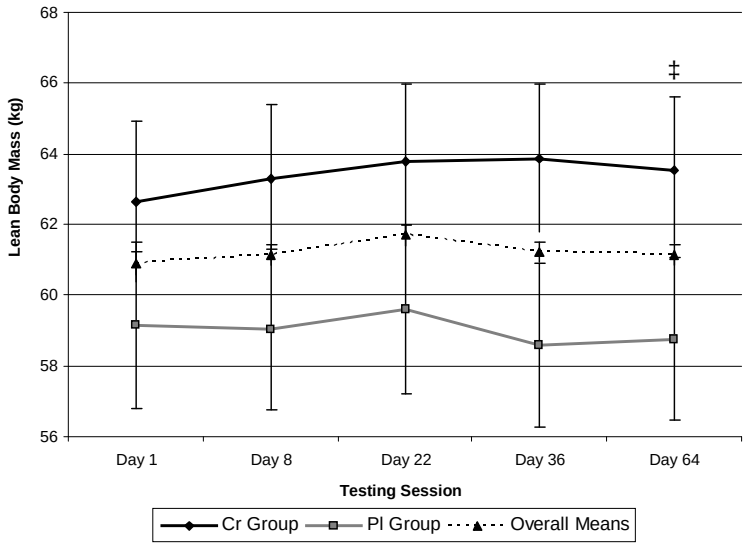

Figure 2. Changes in lean body mass (kg) over the course of the study. A significant increase was observed in the Cr group ‡ ($p = 0.01$).

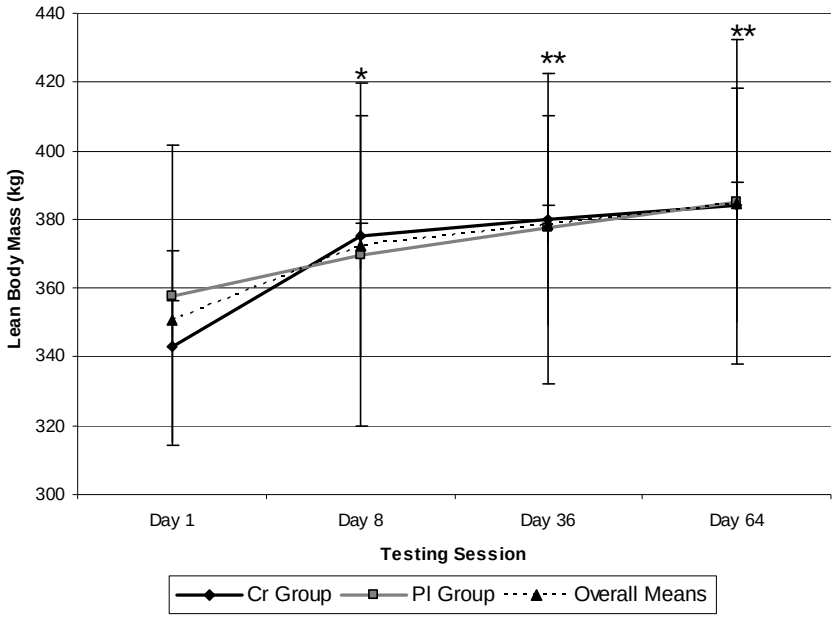

Figure 3. Changes in leg press 1-RM (kg) over the course of the study. A significant time effect for both groups was observed at day 8 *($p = 0.001$), day 36 **($p < 0.001$), and day 64 **($p < 0.001$).

White Blood Cell Values

Table 7 represents the average white blood cell values of both groups over the course of the study. Statistical analysis revealed no significant interactions for white blood cell count ($p = 0.93$), neutrophils ($p = 0.92$), lymphocytes ($p = 0.35$), monocytes ($p = 0.19$), eosinophils ($p = 0.16$), or basophils ($p = 0.29$). Thus, it appears that Cr supplementation did not alter white blood cell values.

Table 7

White Blood Cell Values

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
WBC (K/µL)	Day 1	5.55 (1.20)	5.47 (1.68)	-	-	-
	Day 4	4.66 (0.75)	4.68 (1.28)	-	-	-
	Day 8	5.22 (1.48)	5.81 (1.60)	-	-	-
	Day 22	6.29 (2.32)	5.34 (1.95)	-	-	-
	Day 36	5.71 (1.34)	5.50 (2.61)	-	-	-
	Day 64	6.67 (1.05)	5.63 (2.15)	0.16	0.82	0.93
Neutrophils (K/µL)	Day 1	3.25 (1.40)	3.15 (1.43)	-	-	-
	Day 4	2.49 (0.91)	2.99 (1.12)	-	-	-
	Day 8	2.94 (1.47)	3.49 (1.39)	-	-	-
	Day 22	3.95 (2.25)	3.18 (1.64)	-	-	-
	Day 36	3.28 (1.33)	3.19 (2.20)	-	-	-
	Day 64	3.27 (1.02)	3.47 (1.76)	0.24	0.67	0.92
Lymphocytes (K/µL)	Day 1	1.68 (0.38)	1.68 (0.34)	-	-	-
	Day 4	1.53 (0.36)	0.98 (0.38)	-	-	-
	Day 8	1.66 (0.33)	1.67 (0.34)	-	-	-
	Day 22	1.63 (0.39)	1.56 (0.27)	-	-	-
	Day 36	1.74 (0.28)	1.64 (0.41)	-	-	-
	Day 64	1.68 (0.23)	1.53 (0.45)	0.10	0.66	0.35

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
Monocytes (K/µL)	Day 1	0.457 (0.116)	0.454 (0.118)	-	-	-
	Day 4	0.445 (0.089)	0.481 (0.179)	-	-	-
	Day 8	0.419 (0.112)	0.456 (0.152)	-	-	-
	Day 22	0.519 (0.169)	0.438 (0.262)	-	-	-
	Day 36	0.501 (0.083)	0.466 (0.174)	-	-	-
	Day 64	0.485 (0.131)	0.474 (0.184)	0.47	.017	0.19
Eosinophils (K/µL)	Day 1	0.107 (0.049)	0.118 (0.074)	-	-	-
	Day 4	0.126 (0.072)	0.145 (0.098)	-	-	-
	Day 8	0.130 (0.041)	0.124 (0.089)	-	-	-
	Day 22	0.126 (0.044)	0.102 (0.042)	-	-	-
	Day 36	0.129 (0.055)	0.125 (0.057)	-	-	-
	Day 64	0.173 (0.126)	0.105 (0.036)	0.91	0.86	0.16
Basophils (K/µL)	Day 1	0.050 (0.022)	0.062 (0.022)	-	-	-
	Day 4	0.070 (0.031)	0.077 (0.031)	-	-	-
	Day 8	0.059 (0.022)	0.074 (0.034)	-	-	-
	Day 22	0.060 (0.019)	0.064 (0.018)	-	-	-
	Day 36	0.050 (0.015)	0.076 (0.046)	-	-	-
	Day 64	0.057 (0.021)	0.053 (0.019)	0.35	0.99	0.29

Note: This data represents the average white blood cell parameters of all participants that completed the study. Data are expressed as means ± SD. Significant time effects contain an asterisk.

Red Blood Cell Values

Table 8 represents the average red blood cell values of both groups over the course of the study. Statistical analysis revealed no significant interactions for red blood cell count ($p = 0.23$), hemoglobin ($p = 0.38$), hematocrit ($p = 0.30$), mean cell volume ($p = 0.51$), mean cell hematocrit ($p = 0.35$), or mean cell hematocrit concentration ($p = 0.81$). Main effects for time were observed for hematocrit ($p = 0.001$), mean cell volume ($p = 0.003$), mean cell hematocrit ($p = 0.002$), and mean cell hematocrit concentration ($p < 0.001$); however, these values remained within normal clinical ranges, and no group x time interactions were found for these variables. Thus, it appears that Cr supplementation had no effect on red blood cell values.

Table 8

Red Blood Cell Values

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
RBC Count (K/µL)	Day 1	5.13 (0.33)	5.05 (0.38)	-	-	-
	Day 4	5.20 (0.48)	4.87 (0.31)	-	-	-
	Day 8	5.13 (0.44)	5.13 (0.11)	-	-	-
	Day 22	5.34 (0.40)	5.23 (0.34)	-	-	-
	Day 36	5.06 (0.47)	5.14 (0.26)	-	-	-
	Day 64	5.24 (0.43)	5.29 (0.20)	0.07	0.70	0.23
Hemoglobin (g/dL)	Day 1	15.7 (0.9)	15.4 (0.9)	-	-	-
	Day 4	15.5 (1.1)	14.9 (0.8)	-	-	-

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
	Day 8	15.4 (1.0)	15.5 (0.6)	-	-	-
	Day 22	15.3 (0.9)	15.3 (0.9)	-	-	-
	Day 36	15.2 (0.9)	15.4 (0.6)	-	-	-
	Day 64	15.8 (1.2)	15.7 (0.4)	0.73	0.97	0.38
Hematocrit (%)	Day 1	45.0 (2.7)	44.8 (2.4)	-	-	-
	Day 4	46.7 (3.9)	43.9 (3.0)	-	-	-
	Day 8	46.1 (3.8)	45.6 (1.2)	-	-	-
	Day 22	49.0 (3.2)	47.9 (2.4)	-	-	-
	Day 36	46.6 (3.6)	47.0 (1.4)	-	-	-
	Day 64	48.0 (3.8)	48.3 (2.4)	*0.001	0.94	0.30
MCV (fL)	Day 1	88.6 (3.1)	88.9 (4.4)	-	-	-
	Day 4	89.8 (2.1)	90.4 (3.4)	-	-	-
	Day 8	89.9 (2.1)	88.9 (3.7)	-	-	-
	Day 22	91.8 (2.9)	91.6 (2.9)	-	-	-
	Day 36	92.2 (3.2)	91.5 (3.0)	-	-	-
	Day 64	91.7 (2.8)	90.8 (3.0)	*0.003	0.34	0.51
MCH (pg)	Day 1	30.6 (1.2)	30.5 (1.1)	-	-	-
	Day 4	30.0 (1.6)	30.8 (1.3)	-	-	-
	Day 8	30.1 (1.4)	30.1 (1.4)	-	-	-
	Day 22	28.7 (1.2)	29.2 (1.6)	-	-	-
	Day 36	30.1 (1.4)	29.9 (1.1)	-	-	-
	Day 64	30.2 (1.0)	29.8 (1.0)	*0.02	0.52	0.35

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
MCHC (g/dL)	Day 1	34.6 (0.8)	34.3 (1.0)	-	-	-
	Day 4	33.3 (1.4)	34.0 (1.1)	-	-	-
	Day 8	33.5 (1.2)	33.9 (1.3)	-	-	-
	Day 22	31.2 (0.4)	31.9 (0.9)	-	-	-
	Day 36	32.7 (0.9)	32.7 (0.6)	-	-	-
	Day 64	33.9 (0.2)	32.8 (0.5)	*<0.001	0.89	0.81

Note: This data represents the average red blood cell parameters of all participants that completed the study. Data are expressed as means ± SD. Significant time effects contain an asterisk.

Serum Chemistry Values

Table 9 represents the average clinical chemistry values of both groups over the course of the study. Statistical analysis revealed no significant interactions for triglycerides ($p = 0.73$), total cholesterol ($p = 0.21$), HDL ($p = 0.46$), LDL ($p = 0.16$), GGT ($p = 0.78$), LDH ($p = 0.51$), uric acid ($p = 0.21$), BUN ($p = 0.27$), glucose ($p = 0.56$), BUN/creatinine ratio ($p = 0.75$), calcium ($p = 0.17$), total protein ($p = 0.12$), total bilirubin ($p = 0.41$), ALP ($p = 0.17$), AST ($p = 0.41$), ALT ($p = 0.54$), or total creatine kinase ($p = 0.25$). There was a significant increment in serum albumin in the Pl group ($p = 0.05$). However, these values remained within normal clinical ranges. Thus, it appears that Cr supplementation had no adverse affect on serum chemistry safety markers.

Table 9

Serum Chemistry Values

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
Triglycerides (mg/dL)	Day 1	117.7 (77.0)	143.5 (79.8)	-	-	-
	Day 4	105.0 (42.9)	79.3 (44.1)	-	-	-
	Day 8	125.8 (121.9)	108.8 (76.3)	-	-	-
	Day 22	104.7 (65.0)	103.1 (68.6)	-	-	-
	Day 36	96.0 (46.9)	125.0 (87.7)	-	-	-
	Day 64	97.1 (56.2)	99.0 (64.0)	0.31	0.76	0.73
Total Cholesterol (mg/dL)	Day 1	168.6 (19.3)	163.1 (36.6)	-	-	-
	Day 4	170.0 (35.4)	147.1 (15.2)	-	-	-
	Day 8	161.9 (18.1)	157.9 (39.3)	-	-	-
	Day 22	172.7 (27.6)	182.7 (35.5)	-	-	-
	Day 36	159.4 (23.0)	157.0 (32.3)	-	-	-
	Day 64	168.6 (20.7)	179.1 (36.1)	0.40	0.51	0.21
HDL (mg/dL)	Day 1	53.2 (13.3)	44.7 (6.7)	-	-	-
	Day 4	51.9 (12.7)	45.1 (5.0)	-	-	-
	Day 8	55.6 (11.1)	46.1 (6.6)	-	-	-
	Day 22	57.9 (14.2)	51.0 (8.6)	-	-	-
	Day 36	56.6 (15.1)	44.1 (5.7)	-	-	-
	Day 64	58.8 (16.0)	51.3 (7.2)	*0.02	0.12	0.46
LDL (mg/dL)	Day 1	99.0 (21.4)	102.7 (27.5)	-	-	-

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
	Day 4	103.5 (27.6)	91.0 (15.8)	-	-	-
	Day 8	93.7 (21.2)	99.1 (29.0)	-	-	-
	Day 22	100.1 (25.5)	116.7 (30.6)	-	-	-
	Day 36	91.1 (19.5)	98.7 (26.5)	-	-	-
	Day 64	98.0 (19.3)	115.0 (29.6)	0.60	0.89	0.16
GGT (U/L)	Day 1	36.7 (7.9)	29.8 (4.1)	-	-	-
	Day 4	37.8 (12.5)	28.5 (3.4)	-	-	-
	Day 8	38.2 (16.4)	29.1 (4.6)	-	-	-
	Day 22	43.0 (23.1)	31.3 (3.7)	-	-	-
	Day 36	41.1 (21.6)	29.2 (4.3)	-	-	-
	Day 64	36.3 (19.0)	30.7 (5.1)	0.70	0.14	0.78
LDH (U/L)	Day 1	143.2 (31.5)	130.9 (27.4)	-	-	-
	Day 4	140.1 (39.6)	138.8 (43.6)	-	-	-
	Day 8	172.2 (76.8)	165.8 (70.2)	-	-	-
	Day 22	141.4 (37.8)	134.1 (23.1)	-	-	-
	Day 36	141.3 (27.7)	130.8 (29.9)	-	-	-
	Day 64	144.9 (27.2)	147.1 (34.7)	0.98	0.72	0.51
Uric Acid	Day 1	6.4 (0.8)	6.9 (1.5)	-	-	-
	Day 4	5.8 (1.3)	6.3 (1.0)	-	-	-
	Day 8	5.3 (0.9)	7.0 (1.7)	-	-	-
	Day 22	6.6 (1.6)	7.0 (0.7)	-	-	-
	Day 36	6.5 (1.6)	6.6 (1.5)	-	-	-
	Day 64	6.7 (1.2)	6.6 (1.4)	*0.04	0.39	0.21
BUN (mg/dL)	Day 1	15.8 (3.5)	17.1 (5.9)	-	-	-

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
	Day 4	14.1 (4.4)	14.5 (3.9)	-	-	-
	Day 8	15.4 (3.3)	13.3 (3.7)	-	-	-
	Day 22	15.8 (3.7)	14.4 (4.2)	-	-	-
	Day 36	16.2 (5.3)	15.2 (4.5)	-	-	-
	Day 64	16.7 (4.1)	15.6 (3.4)	0.52	0.79	0.27
Glucose (mg/dL)	Day 1	93.3 (5.9)	150.5 (143.5)	-	-	-
	Day 4	91.8 (11.8)	174.8 (153.0)	-	-	-
	Day 8	97.2 (14.8)	156.5 (93.7)	-	-	-
	Day 22	98.0 (9.6)	115.1 (121.6)	-	-	-
	Day 36	104.9 (15.9)	151.4 (183.5)	-	-	-
	Day 64	101.3 (11.0)	129.7 (155.7)	0.78	0.11	0.56
BUN : Creatinine	Day 1	13.6 (2.8)	15.2 (4.6)	-	-	-
	Day 4	11.5 (2.4)	14.5 (3.3)	-	-	-
	Day 8	12.5 (3.0)	12.5 (4.6)	-	-	-
	Day 22	12.5 (3.9)	12.6 (3.7)	-	-	-
	Day 36	12.3 (3.5)	13.4 (3.3)	-	-	-
	Day 64	13.7 (4.3)	13.4 (3.1)	0.89	0.23	0.75
Calcium	Day 1	10.0 (0.5)	9.8 (0.7)	-	-	-
	Day 4	9.8 (1.2)	9.5 (1.1)	-	-	-
	Day 8	9.9 (0.6)	9.8 (0.6)	-	-	-
	Day 22	10.1 (0.8)	10.2 (0.8)	-	-	-
	Day 36	9.9 (0.5)	9.9 (0.7)	-	-	-
	Day 64	10.0 (0.5)	10.1 (0.5)	*0.05	0.38	0.17
Total Protein	Day 1	7.8 (0.5)	7.3 (0.9)	-	-	-

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
	Day 4	7.6 (1.1)	6.9 (1.0)	-	-	-
	Day 8	7.6 (0.7)	7.2 (0.7)	-	-	-
	Day 22	7.8 (0.8)	7.9 (1.3)	-	-	-
	Day 36	7.7 (0.6)	7.5 (0.7)	-	-	-
	Day 64	7.8 (0.6)	7.8 (0.6)	*0.05	0.26	0.12
Albumin	Day 1	4.9 (0.3)	4.7 (0.5)	-	-	-
	Day 4	4.8 (0.7)	4.4 (0.6)	-	-	-
	Day 8	4.8 (0.3)	4.6 (0.5)	-	-	-
	Day 22	4.9 (0.4)	5.0 (0.8)	-	-	-
	Day 36	4.5 (1.1)	4.7 (0.5)	-	-	-
	Day 64	4.8 (0.4)	5.0 (0.3)	0.29	0.80	*0.05
Total Bilirubin	Day 1	0.37 (0.14)	0.61 (0.50)	-	-	-
	Day 4	0.40 (0.20)	0.68 (0.35)	-	-	-
	Day 8	0.41 (0.17)	0.64 (0.42)	-	-	-
	Day 22	0.49 (0.24)	0.72 (0.41)	-	-	-
	Day 36	0.40 (0.31)	0.77 (0.79)	-	-	-
	Day 64	0.40 (0.21)	0.81 (0.73)	0.16	0.09	0.41
ALP (U/L)	Day 1	77.4 (19.0)	62.0 (15.1)	-	-	-
	Day 4	77.5 (25.6)	61.6 (16.4)	-	-	-
	Day 8	80.7 (23.1)	65.8 (14.0)	-	-	-
	Day 22	80.0 (26.1)	74.3 (20.9)	-	-	-
	Day 36	82.1 (24.6)	72.4 (18.5)	-	-	-
	Day 64	84.8 (23.2)	80.8 (17.4)	*0.001	0.30	0.17
AST (U/L)	Day 1	54.7 (85.6)	27.0 (6.4)	-	-	-

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
	Day 4	31.4 (14.4)	28.8 (8.9)	-	-	-
	Day 8	38.4 (28.8)	28.9 (9.0)	-	-	-
	Day 22	30.2 (9.2)	25.8 (406)	-	-	-
	Day 36	27.3 (7.8)	22.8 (8.1)	-	-	-
	Day 64	25.4 (7.5)	24.1 (8.1)	0.24	0.28	0.41
ALT (U/L)	Day 1	26.6 (18.8)	19.4 (2.7)	-	-	-
	Day 4	23.0 (9.8)	19.4 (3.7)	-	-	-
	Day 8	24.8 (12.0)	19.2 (2.2)	-	-	-
	Day 22	23.2 (5.4)	19.0 (2.4)	-	-	-
	Day 36	21.3 (3.4)	17.9 (2.1)	-	-	-
	Day 64	23.2 (5.0)	21.0 (2.8)	0.47	0.17	0.54
Total Creatine Kinase	Day 1	533 (716)	132 (86)	-	-	-
	Day 4	538 (594)	95 (37)	-	-	-
	Day 8	674 (1014)	107 (62)	-	-	-
	Day 22	379 (241)	105 (29)	-	-	-
	Day 36	370 (209)	106 (53)	-	-	-
	Day 64	311 (287)	136 (140)	0.22	*0.01	0.25

Note: This data represents the average clinical chemistry parameters of all participants that completed the study. Data are expressed as means ± SD.

Urine Values

Table 10 represents the average urine creatine and creatinine values of both groups over the course of the study. Statistical analysis revealed no significant interaction for urine creatinine ($p = 0.15$). However, a significant group x time

interaction for urine creatine ($p = 0.01$) to be increased in the Cr group was found. Therefore, hypothesis H_{1A} is accepted, while hypothesis H_{1B} must be rejected. Figure 4 depicts the changes in urine creatine over the course of the study.

Table 10

Urine Values

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
Urine Creatine (µM)	Day 1	249 (73)	473 (719)	-	-	-
	Day 4	27833 (5933)	209 (132)	-	-	-
	Day 8	23716 (11168)	197 (107)	-	-	-
	Day 15	4521 (2302)	152 (64)	-	-	-
	Day 22	5529 (3410)	202 (118)	-	-	-
	Day 29	4751 (4722)	238 (160)	-	-	-
	Day 36	8214 (6524)	225 (141)	-	-	-
	Day 50	648 (607)	213 (95)	-	-	-
	Day 64	531 (567)	260 (112)	*0.01	*0.002	*0.01
Urine Creatinine (mg/dL)	Day 1	135 (62)	118 (75)	-	-	-
	Day 4	157 (62)	89 (68)	-	-	-
	Day 8	150 (129)	96 (68)	-	-	-
	Day 15	338 (218)	83 (33)	-	-	-
	Day 22	135 (41)	94 (55)	-	-	-

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
	Day 29	180 (74)	148 (115)	-	-	-
	Day 36	164 (54)	104 (66)	-	-	-
	Day 50	193 (65)	118 (63)	-	-	-
	Day 64	161 (69)	144 (76)	0.19	.34	0.15

Note: This data represents the average urinary creatine parameters of all participants that completed the study. Data are expressed as means ± SD. Significant time and/or time x group interactions contain an asterisk.

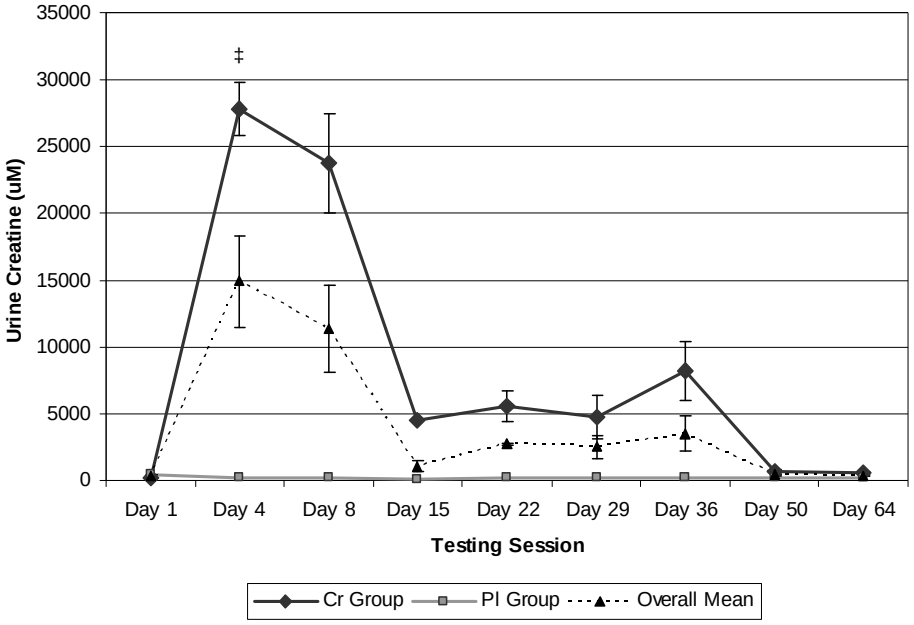

Figure 4. Changes in urine creatine over the course of the study. A significant increase was observed in the Cr group ‡(p = 0.01).

Serum Values

Table 11 represents the average change (delta) in serum creatine and creatinine values of both groups over the course of the study. Statistical analysis revealed no significant interaction for serum creatinine (p = 0.14), but a significant group x time interaction for increased serum creatine in the Cr group was found (p = 0.003).

Therefore, hypothesis H_{1C} is accepted, while hypothesis H_{1D} must be rejected. Figure 5 depicts the changes in serum creatine over the course of the study.

Table 11

Serum Values

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
Δ Creatine (μM)	Day 1	0	0	-	-	-
	Day 4	54.7 (126.6)	-36.1 (47.9)	-	-	-
	Day 8	204.1 (193.4)	-11.0 (76.6)	-	-	-
	Day 22	-20.0 (71.7)	-77.8 (91.3)	-	-	-
	Day 36	23.2 (81.1)	-44.7 (87.8)	-	-	-
	Day 64	-13.4 (86.2)	-28.4 (122.0)	*0.05	0.09	*0.003
Δ Creatinine (mg/dL)	Day 1	0	0	-	-	-
	Day 4	1.24 (0.28)	1.01 (0.22)	-	-	-
	Day 8	1.26 (0.18)	1.10 (0.13)	-	-	-
	Day 22	1.29 (0.20)	1.16 (0.22)	-	-	-
	Day 36	1.32 (0.15)	1.04 (0.31)	-	-	-
	Day 64	1.23 (0.11)	1.17 (0.12)	0.54	0.18	0.14

Note: This data represents the average serum creatine parameters of all participants that completed the study. Data are expressed as means ± SD. Significant time and/or time x group interactions contain an asterisk.

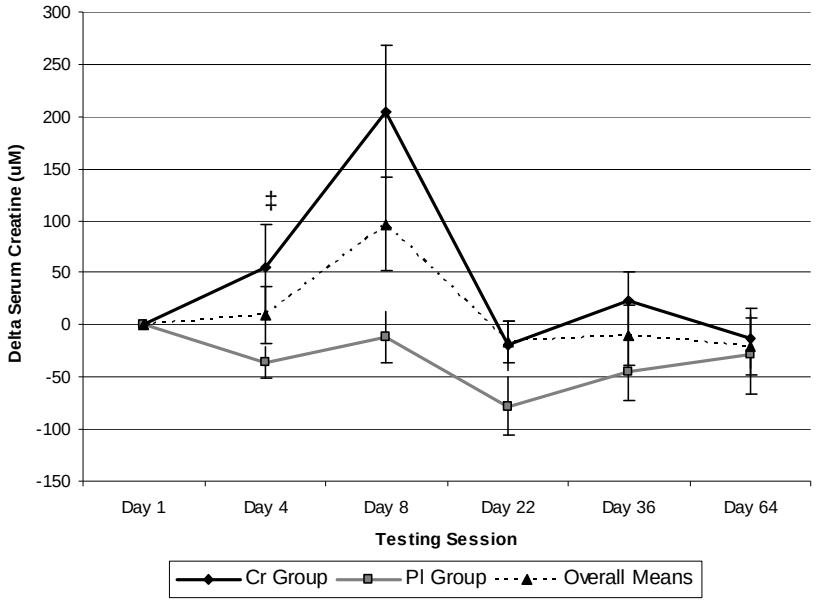

Figure 5. Changes in serum creatine (ΔμM) over the course of the study. A significant increase was observed in the Cr group ‡($p = 0.003$).

Skeletal Muscle Measures

Table 12 represents the average skeletal muscle measures of both groups over the course of the study. Statistical analysis revealed no significant interaction for total muscle Cr ($p = 0.85$), total muscle Cr % from baseline ($p = 0.99$), CrT mRNA content ($p = 0.78$), CrtT mRNA expression as % from baseline ($p = 0.93$), CrT protein content ($p = 0.36$), or CrT protein content as % from baseline ($p = 0.60$). Main effects for group were found in CrT mRNA content ($p = 0.004$) and CrT protein content ($p < 0.001$). Independent t-tests reveal that the baseline means at Day 1 for each of these variables were different between groups ($p = 0.02$ & $p = 0.01$, respectively). When expressed as percent deviation from baseline, the main effects for group are no longer present. Hypothesis H_2 must be rejected as intramuscular levels of Cr did not increase with Cr supplementation. Hypotheses H_3 and H_4 are accepted as there was no significant

difference in CrT mRNA or protein expression found, respectively, with Cr supplementation.

Table 12

Skeletal Muscle Measures

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
Total Muscle Cr (mmol/kg dry wt)	Day 1	67.1 (31.9)	95.7 (73.9)	-	-	-
	Day 8	72.4 (33.7)	72.4 (37.2)	-	-	-
	Day 22	101.8 (51.8)	79.2 (36.4)	-	-	-
	Day 36	84.5 (45.8)	85.9 (60.5)	-	-	-
	Day 64	71.1 (40.7)	81.2 (27.8)	0.90	0.73	0.77
Muscle Phospho Cr (mmol/kg dry wt)	Day 1	46.2 (19.7)	58.2 (47.4)			
	Day 8	50.7 (22.0)	45.0 (22.4)			
	Day 22	73.3 (46.8)	45.9 (21.3)			
	Day 36	57.5 (30.8)	51.8 (36.7)			
	Day 64	47.8 (23.2)	46.2 (18.8)	0.86	0.43	0.67
Muscle Free Cr (mmol/kg dry wt)	Day 1	20.9 (12.8)	37.5 (27.4)			
	Day 8	21.7 (13.3)	27.4 (15.2)			
	Day 22	28.6 (8.8)	33.2 (15.3)			
	Day 36	26.4 (15.6)	34.0 (24.1)			
	Day 64	23.3 (18.9)	35.0 (12.9)	0.92	0.10	0.92

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
CrT mRNA Content	Day 1	0.89 (0.11)	0.74 (0.08)	-	-	-
	Day 8	0.85 (0.06)	0.75 (0.05)	-	-	-
	Day 22	0.83 (0.09)	0.74 (0.06)	-	-	-
	Day 36	0.84 (0.10)	0.71 (0.07)	-	-	-
	Day 64	0.82 (0.12)	0.71 (0.07)	0.18	*0.004	0.78
CrT mRNA % from Baseline	Day 1	0	0	-	-	-
	Day 8	0.3 (10.9)	-2.3 (6.0)	-		-
	Day 22	2.6 (14.3)	-1.2 (6.1)	-		-
	Day 36	2.0 (16.8)	4.3 (4.3)	-		-
	Day 64	5.0 (19.1)	1.9 (10.5)	0.16	0.72	0.93
CrT Protein Content	Day 1	0.062 (0.01)	0.076 (0.02)	-	-	-
	Day 8	0.061 (0.01)	0.097 (0.07)	-	-	-
	Day 22	0.062 (0.01)	0.090 (0.06)	-	-	-
	Day 36	0.062 (0.01)	0.078 (0.04)	-	-	-
	Day 64	0.064 (0.01)	0.077 (0.05)	0.33	*<0.001	0.36
CrT Content % from Baseline	Day 1	0	0	-	-	-
	Day 8	-1.9 (16.7)	27.4 (57.4)	-	-	-
	Day 22	-0.6 (15.4)	22.8 (76.3)	-	-	-

(table continues)

Variable	Session	Cr Group (± SD)	Pl Group (± SD)	Time Effect p-level	Group Effect p-level	Group x Time p-level
	Day 36	-0.3 (12.6)	8.0 (51.5)	-	-	-
	Day 64	5.3 (16.6)	4.7 (49.2)	0.61	0.52	0.60

Note: This data represents the average muscle creatine and creatine transporter parameters of all participants that completed the study. Data are expressed as means ± SD.

CHAPTER FIVE

Discussion

The purpose of this study was to conduct a preliminary examination of the effects of a typical creatine monohydrate supplementation regimen on the activity of the creatine transporter at the transcriptional and translational levels in resistance-trained males. In order to properly evaluate these attributes, it was deemed necessary to attempt to normalize the amount of creatine exposed to the skeletal muscle of each participant. It was similarly desirable to approximate a standard creatine dosing regimen in parallel. This was achieved by prescribing doses of Cr unique to each participant that were determined by their respective fat-free body masses. In this manner, the creatine group consumed on average 18.8 g/d for a loading phase of one week, followed by an average of 4.77 g/d for a maintenance phase of four weeks. These doses closely approach the paradigm doses of 20 g/d and 5 g/d for loading and maintenance phases, respectively (Harris, 1992; Greenhaff, 1994; Volek, 1996; Preen, 2003).

Significant Changes in Body Composition and Strength

Previous studies have indicated that similar consumption of creatine coupled with resistance exercise significantly improves body composition and fat free mass, as well as muscular strength (Greenhaff, 1994; Rawson, 2003). As expected, we found a significant group x time interaction for total body weight and lean body mass, both of which were significantly elevated in the Cr group. We did not find a group x time interaction for percent body fat ($p = 0.13$); however, statistical analysis revealed a low to moderate

effect size percent body fat decrease in the Cr group (d = 0.33). There was a significant increase in intracellular fluid and total body water in the Cr group, while there was no group x time interaction for extracellular water, nor the extracellular:intracellular and extracellular:total ratios. Furthermore, when total body water is expressed relative to body weight, there is no longer a group x time interaction present (p = 0.07). These findings coincide with a previous study (Powers, 2003), which found that similar Cr loading and maintenance increased total body water without altering overall fluid distribution.

As shown in figure 3, there was a significant increase over time in muscular strength for both groups, as measured by leg press 1-RM. This strength increase in both groups was expected as a consequence of the periodized resistance training program (Rawson, 2003). The mean strength gain for the Cr group was 41.2 ± 26.9 kg, while the mean strength gain for the Pl group was 25.7 ± 17.7 kg, and so the difference in strength gains between groups was 15.5 kg. However, Cr supplementation failed to confer an additional strength increase beyond the Pl group (p = 0.11). Statistical analysis revealed a moderate effect-size increase for leg press 1-RM in the Cr group (d = 0.51). This suggests that additional participants might have provided adequate statistical power to reveal a significantly greater strength gain in the Cr group, which would coincide with previous research that demonstrates additional strength increments from Cr supplementation (Earnest, 1995; Volek, 1996; Rawson, 2003).

The participants were instructed to maintain their normal mixed diets for the duration of the study, and there were no group x time interactions for daily calorie, carbohydrate, protein, and fat intakes. It is therefore unlikely that dietary intake exerted

any influence on these strength measures. There were also no differences between groups for upper body training volume, lower body training volume, or total training volume, which suggests that the training stimulus between groups was not different. As mentioned previously, all participants submitted completed training logs at the end of the study, and compliance to the resistance training protocol as measured by these training logs and verbal communication approached one hundred percent. The training logs included self-reported weights lifted, which were subsequently used to calculate training volume for the duration of the study. It is feasible that some participants did not comply fully with the resistance training protocol, despite indicating otherwise, or incorrectly reported weights lifted, which would have introduced inaccuracies in the calculation of training volumes.

It is likely that the study did not contain enough participants to discern differences in strength between groups. If the sample size was indeed too small, the statistical power of the study becomes inadequate, and the likelihood of making a type II error increases. Statistical analysis revealed a moderate effect size for leg press 1-RM. As such, the probability of detecting a meaningful effect and making a correct rejection of hypothesis H_5 is less than optimal. It is evident that it would have been beneficial to include more participants in this study. We did not run any explicit power or effect calculations prior to the study to determine the adequate sample size to achieve acceptable statistical power. Based on our laboratory's previous experience with studies involving muscle biopsy collections and three groups, 10 participants in each group would yield moderate statistical power (power = 0.84) and effect size (d = 1.35). Therefore, using a two group design it was determined that a group size of n = 12 would be reasonable to achieve

noteworthy results and yet still be feasible from a participant recruitment standpoint. However, this study fell short of that number, with nine participants comprising the creatine group and ten participants comprising the placebo group; although our reduced sample size was not for a lack of participant recruitment. A post hoc power calculation reveals that in order to detect a statistically significant difference in strength gain of 15.5 kg with a standard deviation of 23.3 kg and a power equal to 0.8, 34 participants per group would have been necessary.

Significant Changes in Urine and Serum Creatine

In a study that provided a loading dosage of 0.1 g Cr/kg lean body mass/d, as determined by skinfold measurement, 46% of ingested Cr was excreted in the urine after the first 24 hours of supplementation, with no requisite changes in creatinine excretion (Burke et al., 2001). Our participants ingested approximately three times that amount of Cr during the loading phase, and after three days of supplementation, urine Cr concentrations had increased over 1000-fold from baseline levels, and remained similarly elevated after one week of supplementation. As a result, statistical analysis revealed a significant group x time effect for urinary Cr to be elevated. On the contrary, there was no significant increase in urinary creatinine. These findings are similar to recent studies which showed increases in urinary Cr but not in urinary creatinine (Powers, 2003; Syrotuik, 2004). We did not record urine volume during the study, and so we are unable to express by mass what amount of ingested Cr was being excreted at this point.

In tandem, serum Cr concentrations were significantly elevated from baseline at three days and at one week of supplementation, as well as at the end of the maintenance phase of the Cr supplementation. Statistical analysis revealed a significant group x time

interaction for serum Cr to be elevated in the Cr group. Conversely, there was no significant group x time interaction for serum creatinine. These findings provide an indication that the exogenous Cr supplementation effectively increased Cr concentration within the circulation, augmenting the Cr pool that was available to the skeletal muscle for uptake, which coincides with recent studies (Schedel, 1999; Robinson, 2000).

Non-significant Changes in Intramuscular Creatine

Unlike numerous other studies (Harris, 1992; Greenhaff, 1994; Casey, 1996; Hultman, 1996; Snow, 1998; Burke, 2003; Watt, 2004), we did not find that Cr supplementation significantly increased free Cr ($p = 0.92$), phosphocreatine ($p=0.67$), or total Cr ($p = 0.77$) . Our supplement protocol was quite similar the protocols of these comparable studies, and supplementation effectively elevated serum concentrations of Cr, which suggests the possibility that our result is erroneous. It appears that the intramuscular Cr data contain substantial measurement errors. For the total Cr measurements, the coefficients of variation at individual time points range from 34-77%, while the coefficients of variation for PCr range from 41-72%, and the coefficients of variation for free Cr range from 37-82%. These measurements do not compare favorably with recent studies, which report coefficients of variation for the same measurements in the range of 3-8% (Snow, 1998; Watt, 2004). This indicates a high degree of variability in the assays performed, and as such, it is dubious at best to draw conclusions from these data.

In the present study we had only 0.5-3.0 mg of powdered muscle available per sample for metabolite extraction and subsequent Cr analysis, with many samples falling towards the lower end of that range, while others used as much as 5-10 mg of powdered

muscle for their extractions (Burke, 2003). The raw data outcome of the intramuscular Cr assay provides the concentration of Cr found in the extraction buffer. Because different masses of powdered muscle tissue were used for each sample, the Cr concentrations of the extraction buffer were divided by the powdered muscle mass used in order to normalize Cr concentration per unit of muscle weight. The powdered muscle mass acts as the denominator in a fraction that determines measured intramuscular Cr concentrations, and since the masses used in our study were considerably smaller, there is by virtue of the mathematics a lesser degree of precision between the measured value and actual value. This artifact from the calculation may have introduced measurement error, thereby confounding the results of the assay, and possibly masking any statistical trends.

Additionally, in preparing our wet tissue samples for Cr analysis, we dried them under a SpeedVac overnight. The temperature of this apparatus is not controlled, and although the vacuum likely reduced the environment to less than room temperature, the samples did not remain frozen during the drying process, and they were exposed to the open atmosphere. It is possible that a substantial amount of the Cr contained within the tissue samples was degraded, or that the sample was otherwise contaminated. Previous studies have lyophilized their muscle samples prior to metabolite extraction and analysis (Burke, 2003; Watt, 2004). We did not have the capabilities to freeze dry the tissue samples, and so we chose a method that approximated the process, perhaps inadequately so. In hindsight, it may have been ideal to directly powder the tissue samples under liquid nitrogen, which would have maintained the integrity of the metabolic state of the tissue at the point of the biopsy, or to use frozen tissue and express the Cr values relative to muscle wet weight. We were admittedly naïve in the process of extracting Cr from

skeletal muscle tissue for analysis, and it would have served us well to familiarize ourselves with the proper techniques prior to utilizing our samples by comparing the results from the dried and wet muscle techniques. As a result, we must conclude that substantial measurement error may have obscured any true differences in intramuscular Cr content.

Non-significant Changes in CrT mRNA and Protein

We failed to find any group x time interaction for CrT mRNA transcription levels. Statistical analysis did reveal a significant main effect for group where CrT mRNA transcription levels were elevated in the Cr group. However, an independent t-test reveals that the baseline means for this variable were different ($p = 0.02$). Because these participants were drawn from a relatively homogenous population, and placed randomly into groups, we have no reason to believe that an actual difference in baseline values exists. When expressed as percent deviation from baseline, the group effect is no longer present ($p = 0.72$), and there is still no group x time interaction ($p = 0.93$). It is likely that this difference in baseline means is due to interassay variations. The mRNA data was generated from two separate RT-PCR reactions, one of which contained a full complement of samples from the Cr group, and the other of which contained a full complement of samples from the Pl group. In retrospect, it would have been ideal to include an internal standard in both reactions, in order to appropriately relate the results of the assay across groups, or alternatively, to have included an equal number of samples from each group in the RT-PCR reactions in order to reduce interassay variability.

There is a sparse amount of existing data regarding the possible or expected outcomes of exogenous Cr supplementation on the transcription levels of the CrT gene in

humans. As such, we hypothesized that no differences would be found and we have no reason to reject that hypothesis.

Murphy et al. demonstrated that there is no difference in basal CrT mRNA transcription levels between healthy young males and healthy young females, but did not expose their participants to any type of Cr supplementation (Murphy, 2003).

Tarnopolsky's group conducted a pair of studies which in tandem mimic the aims of our study, the first of which addresses CrT mRNA content following a loading period of Cr supplementation (Tarnopolsky et al, 2003). Thirteen young moderately active men and fourteen young moderately active women consumed Cr at a dose of 4 x 5 g/d for five days, followed by a maintenance dose of 5 g/d for 3-4 days. The participants consumed the supplement with milk, juice, or similar carbohydrate-containing beverage. Muscle biopsies were collected pre- and post- supplementation, and RT-PCR was performed using 18S ribosomal RNA as an internal control. Although Cr supplementation increased intramuscular Cr content in a similar manner in both the males and females, no differences in CrT gene transcription were identified.

Murphy et al. recently validated the use of β-actin as a housekeeping gene to study gene expression in human skeletal muscle, particularly in response to nutritional supplements and/or exercise interventions that might impact skeletal muscle metabolism (Murphy, 2002). This provides for a gene that is constitutively and equally expressed among all individuals regardless of supplementation or intervention. We employed β-actin as an internal control in our RT-PCR reactions to normalize for any possible induction of the CrT gene during the study. It is feasible that using β-actin and not 18S RNA as the internal control may be more ideal for the purposes of measuring CrT gene

transcription in human skeletal muscle. CrT gene transcription levels were, therefore, expressed as a ratio of CrT mRNA transcript amount to β-actin mRNA amount. Despite the differences in internal control, neither ours nor the Tarnopolsky study found any induction of the CrT gene following a loading phase of Cr supplementation. Furthermore, our study was carried out beyond a loading phase, and similarly no induction of the CrT gene was identified during the maintenance or washout phases.

Watt et. al similarly utilized β-actin as an internal control in RT-PCR while measuring CrT mRNA content (Watt et. al, 2004). In this study, seven vegetarians and seven non-vegetarians consumed either 0.4 g/kg body weight Cr or placebo for five days in a crossover design. Muscle biopsies were collected pre- and post- supplement, as well as at 1 day following the beginning of the supplementation. Vegetarianism did not affect CrT gene transcription, but when data from both groups were pooled, a significant treatment by time interaction was found where CrT transcription levels were significantly elevated at 1 day, but returned to baseline at 5 days.

Certain genes found to be inducible in human skeletal muscle respond relatively quickly to a stimulus, often within two days or less, after which the transcription of the gene returns to basal levels (Willoughby, 2002; Yang, 2006; Louis, 2007). In our study, we took a baseline muscle tissue sample prior to administering the supplement, and then did not collect another muscle tissue sample until after a full week of Cr supplementation. It may be possible that the Cr supplementation indeed altered transcription of the CrT gene within the first few days of supplementation, but the induction of the CrT gene had waned and the CrT mRNA transcript level had returned to basal levels by the time the second tissue sample was collected one week later. In this manner, it is possible that the

timing of our tissue sampling was inadequate to detect any alterations in gene transcription, which are generally transient and responsive to acute changes, but may not be apparent with chronic exposure to a stimulus. Even if a chronic stimulus of an inducible gene resulted in a varied expression of the target gene, the transcription activity required to maintain the varied expression would likely be no different than in a non-perturbed state, as the turnover of the target protein would presumably occur at the same rate. In light of recent findings, it would certainly be of interest to more precisely evaluate CrT gene transcription at shorter intervals during the loading phase of Cr supplementation.

Herein we report one of the first attempts at identifying alterations in CrT protein expression levels in humans given an exogenous Cr supplement throughout a standard supplementation protocol. With an outcome analogous to our CrT mRNA expression data, we failed to find any group x time effect for CrT protein content. Statistical analysis revealed a significant main effect for group, where CrT protein expression was elevated in the Pl group ($p < 0.001$). However, an independent t-test shows that the baseline means for CrT expression levels are different ($p = 0.01$). When expressed as percent deviation from baseline, this group effect is lost ($p = 0.52$), and there is still no group x time interaction ($p = 0.60$). Similar to the CrT mRNA data, the CrT protein expression data were generated from two separate assays, where the first assay included all samples from the Cr group, while the second assay included all samples form the Pl group. As such, it is likely that this group effect is a consequence of interassay variability, and not a true statistical difference.

Murphy et al. demonstrated that total Cr content and CrT protein expression levels, as measured by Western blot, do not differ between healthy young males and females (Murphy, 2003). However, this was a descriptive study and did not involve a treatment or Cr supplementation.

Another study from Tarnopolsky's group evaluated CrT protein content in a manner similar to ours (Tarnopolsky, 2003). Nineteen moderately active young men participated in eight weeks of supervised resistance training and consumed 10 g Cr + 75 g dextrose for 6 d/wk over the eight week period. Muscle biopsies were collected pre- and post- supplementation. Neither supplementation nor resistance training altered CrT protein content, although supplementation did significantly increase total intramuscular Cr concentration.

Boehm (Boehm et al., 2003) measured CrT kinetics and content in perfused rat hearts of animals fed a diet for six weeks containing either 3% Cr, 1.5% β-guanidinoproprionic acid (a Cr analog that competitively binds CrT), or placebo. They noted that Cr supplementation reduced transport activity of CrT by ~27%, as well as plasma membrane CrT content by ~35%, although the total CrT pool remained unchanged. Furthermore, the β-guanidinoproprionic acid effectively reduced intramuscular Cr content by >80%, despite the transport activity of CrT being enhanced by ~62%, and plasma membrane CrT content increased fivefold. Again, the total CrT pool remained unchanged. This suggests that in rat cardiomyocytes, intramuscular Cr content is determined by the content and activity of the plasma membrane CrT isoform.

These experiments utilized Western blots to determine CrT content, and thus differentiated between plasma membrane and mitochondrial isoforms due to their varying

molecular weights of 72 kDa and 52 kDa, respectively. In our experiment, the ELISA analysis did not allow for differentiation between CrT isoforms because the anti-CrT antibody cross-reacts with both membrane-bound and mitochondrial CrT antigen. Boehm noted that the content of the plasma membrane CrT isoform was much less (>10 times) than the total CrT content. In this respect, the ELISA technique was insensitive to possible alterations in plasma membrane CrT content, although our finding that there was no change in total CrT content with Cr supplementation is in agreement with Boehm's findings. Admittedly, it would have been ideal to utilize Western blotting to distinguish the CrT isoforms, but in the interest of time and its more robust assay throughput, we chose to utilize the ELISA technique. It is possible that our treatment in fact induced specific alterations in plasma membrane CrT content, but the ELISA was inadequate to detect such variations.

A similar experiment (Brault et al, 2003) either supplemented rats with a 1% Cr diet for 7 weeks or utilized 1% β-guanidinoproprionic acid to deplete intramuscular Cr content for 7 weeks, after which some rats were repleted with 3.5 weeks of 1% Cr supplementation. Intramuscular Cr content, uptake, and CrT protein expression in the soleus, red gastrocnemius, and white gastrocnemius muscles were measured. Creatine supplementation failed to elicit any changes in CrT protein expression as measured at 1, 3, 5, and 7 weeks of supplementation. Creatine uptake in the white gastrocnemius significantly decreased with supplementation.

β-GPA supplementation caused a significant accumulation of β-GPA and a concomitant reduction of intramuscular Cr to ~15% of basal levels in all fiber types. With subsequent Cr supplementation, intramuscular Cr was replenished at the same rate

in each fiber type, and returned to near-basal levels within 3.5 weeks. The Cr depletion by β-GPA corresponded to a 1.9-2.5 fold increase in CrT protein expression. However, subsequent repletion by 1% Cr supplementation did not alter or reduce the elevated expression levels of CrT. Creatine uptake rates significantly increased in the soleus and red gastrocnemius during the Cr depletion, but did not change in the white gastrocnemius. With subsequent Cr repletion, the creatine uptake rates decreased to near-basal levels within 3.5 weeks in the soleus and red gastrocnemius. These findings suggest that perturbations in intramuscular Cr levels affect Cr uptake rates into the sarcolemma, and that these effects are observed in more highly oxidative muscle fiber types as those found in the soleus and red gastrocnemius, but not in the less oxidative white gastrocnemius. Creatine uptake rates were determined by constant hindlimb perfusion of [^{14}C] Cr at concentrations well above the K_m of the transporter, and so extracellular Cr availability is not likely the cause of this divergent phenomenon.

Our tissue samples for CrT analysis were obtained from the vatus lateralis, which contains approximately 57% type II muscle fibers (Hakkinen, 2001). Brault's data suggests that this muscle may be more resistant to alterations in Cr uptake than a more oxidative muscle such as the soleus. However, we did not deplete and subsequently replete intramuscular Cr as Brault did. Boehm found that Cr supplementation reduced Cr transport by ~27%, but this was in perfused rat hearts, which are comprised of highly oxidative tissue. It is unclear how Cr uptake levels are affected by supplementation in less oxidative fiber types, and so the Cr metabolic status of the vastus lateralis may not be indicative of whole body muscle adaptive uptake of supplemented Cr.

Intuitively, it would seem logical that CrT protein content might shift with exposure to varying extracellular Cr concentrations. A deficiency of circulating Cr would require more import into the sarcolemma to maintain basal intramuscular Cr, and therefore an increase in the number of available transport proteins could effect that change. Similarly, an excess of circulating Cr would likely dictate decreased import into the sarcolemma, possibly by reducing the number of available transport proteins. Interestingly, Brault's study confirmed that CrT protein content rose with Cr depletion, but they did not observe the reverse outcome, as subsequent exposure to elevated extracellular Cr via 3.5 weeks of supplementation failed to reduce CrT protein content, although Cr uptake rates began to decrease in spite of this. As such, it appears that modulation of CrT protein content is not a primary mechanism by which Cr uptake increases in the face of reduced extracellular Cr.

In our study, significant increases in serum and urine Cr concentrations, particularly during the loading phase of supplementation, indicate that the extracellular Cr concentration was elevated by our dose of exogenous creatine. The supplement protocol did not attempt to deplete intramuscular Cr and so we did not expect to find an increase in CrT protein content, but perhaps a decrease in CrT protein content, which might mitigate Cr flux in the face of supraphysiological extracellular Cr levels. Nonetheless, we found no differences in total CrT protein content. As mentioned previously, our methods lacked the ability to differentiate between the mitochondrial and plasma membrane isoforms of CrT. It is apparent that the plasma membrane isoform is relatively less abundant, and it is possible that our study may have induced changes in expression of the plasma membrane isoform but not in the expression of the

mitochondrial isoform, which would be rendered undetectable by our methods. As such, we can only conclude that our supplementation failed to induce changes in the total CrT pool, but we cannot exclude the possibility that supplementation may have induced changes in the content of the plasma membrane isoform, which presumably may have influenced creatine kinetics and uptake in the face of exogenous creatine.

It would certainly be of interest to ascertain what influence CrT content and activity has on basal intramuscular Cr levels and the variable responsiveness to Cr supplementation. The existing data suggest that plasma membrane CrT content is reduced in the face of additional exogenous Cr, which corresponds with a decrease in Cr uptake. It is unclear if the reduced uptake is a function of fewer transporters available at the plasma membrane, or a saturable kinetic phenomenon of the transporters themselves, or perhaps a combination of the two. If the former indeed plays a role, then it is possible that variations in the regulation of plasma membrane CrT content, via the processes of gene transcription, expression, protein degradation, and sequestering, may account for individual differences in responsiveness to Cr supplementation. Unfortunately, the present study did not reveal any particular insight in this regard, and the question accordingly merits further investigation.

Conclusions

In summary, one week of Cr supplementation at 0.3 g/kg lean body mass followed by four weeks of Cr supplementation at 0.075 g/kg lean body mass did not alter any measured blood safety markers, and no adverse effects were reported by any of the participants, so we conclude that the supplement had no effect on overall health. The prescribed periodized resistance training protocol successfully promoted strength gains

across groups, although Cr did not have an additive effect on strength. There was a moderate effect size for strength increase, and this effect is likely due to the study being underpowered. As expected, the Cr supplement did elicit significant gains in total body mass, lean tissue mass, and total body water, without altering relative body water and overall fluid distribution. Supplementation significantly elevated serum Cr levels as well as Cr excretion in the urine, which indicates adaptive handling of exogenous creatine. Contrary to other studies involving Cr supplementation and resistance training, our Cr supplementation had no effect on muscular strength or intramuscular Cr levels. We believe that these discrepancies with the literature are largely a result of an inadequate number of participants to reveal the expected effect, and with respect to intramuscular Cr, of a substantial measurement error caused by inappropriate handling of tissue samples during analysis. By our measures, neither CrT mRNA transcription levels nor total CrT protein content were altered during the loading, maintenance, and washout phases of the Cr supplementation protocol. We suggest however, that Cr supplementation might yet influence the transcription and subsequent expression of the CrT gene, in such a manner that was undetectable by our sampling points or measurement techniques, but may yet be responsible for the adaptive Cr metabolism that occurs with supplementation.

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