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EFFECT OF BRANCHED-CHAIN AMINO ACID SUPPLEMENTATION ON RECOVERY OF PERFORMANCE AND MUSCLE DAMAGE, & AUTOPHAGY AND HEAT SHOCK PROTEIN RESPONSE

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Effect of Branched-Chain Amino Acid Supplementation on Recovery of Performance and Markers of Muscle Damage, & Autophagy and Heat Shock Protein Response

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ABSTRACT

Purpose: To investigate: 1) if branched-chain amino acid supplementation (BCAA) enhances recovery from acute, eccentric resistance exercise as measured by indirect markers of muscle damage, and 2) the impact of acute, eccentric resistance exercise on markers of autophagy and heat shock protein during recovery. **Methods:** Twenty resistance-trained males were randomly assigned to eight days of BCAA or PLCB and adhered to a diet consisting of 1.2 g/kg/d protein. On day five, all subjects completed 10x8 eccentric squats at 70% one repetition maximum (1RM), followed by 5x20 split jumps. Plasma creatine kinase (CK), erythrocyte glutathione, vertical jump (VJ), maximal

voluntary isometric contraction (MVIC), jump squat (JS), perceived soreness were measured as indirect markers of muscle damage. Autophagy (LC3-I, LC3-II, p62) and heat shock protein (HSP70) responses were assessed via peripheral blood mononuclear cells. Variables were measured immediately before, immediately post (IPE), as well as 1, 2, 4, 24, 48, and 72 hours (hr) post-exercise. **Results:** Plasma CK levels were significantly lower for the BCAA group at 48 hr post-exercise; however, no significant group-by-time effect was detected. Erythrocyte glutathione (GSSG/tGSH) was significantly elevated 1, 2, and 4 hr in both BCAA and PLCB groups compared to pre-exercise; however, no significant group-by-time effect was detected at any time-point. The BCAA group reported significantly less soreness compared to PLCB at 48 and 72 hr post-exercise. MVIC was significantly higher for the BCAA group at 24 hr post-exercise, but no significant group-by-time effect was observed. No significant difference between groups was detected for VJ or JS. No differences between groups were detected for protein expression of LC3-II, LC3 II/ I, p62, or HSP70 at any time-point. When combining groups, LC3-II decreased significantly 2 and 4 hr post-exercise. p62 decreased significantly IPE, 2, and 4 hr post-exercise and significantly increased 24 hr post-exercise. HSP70 significantly increased 48 and 72 hr post-exercise. **Conclusions:** BCAA may mitigate soreness following muscle damaging exercise; however, when consumed with a diet adequate in protein, the attenuation of performance decrements or corresponding CK levels are modest. These data support previous work suggesting that the heat shock response exerts regulatory control over autophagy.

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SYMBOLS / ABBREVIATIONS

>: greater than

≤: less than or equal to

<: less than

±: plus or minus

~: approximately

°C: degrees Celsius

µg: microgram

µl: microliter

ml: milliliter

µmol: micromole

1RM: one repetition maximum

AA: amino acid

ANOVA: analysis of variance

Atg: autophagy-related

ATP: adenosine triphosphate

BCAA: branched-chain amino acids

BF%: body fat percentage

BM: body mass

CK: creatine kinase

cm: centimeters

DOMS: delayed-onset muscle soreness

EAA: essential amino acid

ELISA: enzyme-linked immunosorbent assay

est: estimated

FOX: forkhead box

g: gram

g/kg/d: grams per kilogram per day

GSSG: oxidized glutathione

GSSG:tGSH: ratio of oxidized glutathione to total glutathione

H_2O: water

hr: hours

HSP: heat shock protein

IPE: immediately post-exercise

kDa: kilodalton

kg: kilogram

LC3: microtubule-associated protein light chain 3

M: molar

m/min: meters per minute

mg: milligram

Mg: myoglobin

Mm: millimeter

mM: millimolar

MPA: metaphosphoric acid

mTOR: mammalian target of rapamycin

MVIC: maximal voluntary isometric contraction

n= number of participants

NaCl: sodium chloride

nm: nanometer

Nm: newton-meter

p62: sequestosome 1

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PLCB: placebo

RDA: recommended daily allowance

RNS: reactive nitrogen species

ROS: reactive oxygen species

SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE: standard error

Tris: tris (hydroxymethyl) aminomethane

WPH: whey protein hydrolysate

CHAPTER 1

Introduction

Resistance-based exercise represents a commonly-practiced training mode for recreational athletes and accounts for a substantial portion of many athletes' training programs. The inclusion of resistance training has not only been shown to improve physical performance, but has been demonstrated to increase bone mineral density (Petersen, Hastings, & Gottschall, 2015; Strope et al., 2015), enhance metabolic rate (Dolezal, Potteiger, Jacobsen, & Benedict, 2000; Melby, Scholl, Edwards, & Bullough, 1993; Osterberg & Melby, 2000; Williamson & Kirwan, 1997), and improve muscular strength (Schoenfeld, Peterson, Ogborn, Contreras, & Sonmez, 2015). To maximize the stimuli of resistance training, it is recommended that individuals emphasize concentric, isometric, and eccentric muscle actions during their strength training sessions (ACSM, 2014; American College of Sports, 2009; Roig et al., 2009). Because of the distinct mechanical characteristics of eccentric muscle actions, it has been proposed that the incorporation of this training modality can lead to additional improvements of strength and performance, especially in well-trained individuals (Roig et al., 2009). Extensive reviews on eccentric exercise training have been published (Hyldahl & Hubal, 2014; Rasch, 1974).

Etiology of Muscle Damage and Eccentric Exercise

Skeletal muscle tissue is composed of both contractile and non-contractile (i.e. structural) proteins, both which may undergo damage during exercise. Specifically, eccentric muscle contractions or "lengthening contractions" have been shown to cause significant amounts of damage to both the contractile and non-contractile components of

skeletal tissue (Armstrong, Warren, & Warren, 1991; Hyldahl & Hubal, 2014). Though the exact mechanisms behind eccentric-induced skeletal muscle damage have yet to be fully elucidated, it is proposed that the instigation of this damage is a multi-factorial phenomenon (Armstrong et al., 1991; Hyldahl & Hubal, 2014).

Muscle damage is commonly defined as exercise-induced muscle cell and extracellular matrix injury that may ultimately impair normal function and increase the perception of soreness (Hyldahl et al., 2015). Research examining the etiology of muscle damage encompasses two principal explanations, mechanical strain and metabolic strain, which may occur in a biphasic arrangement during eccentric exercise training (Armstrong, 1984; Armstrong et al., 1991; Hyldahl & Hubal, 2014; Hyldahl et al., 2015; Warren, Ingalls, Lowe, & Armstrong, 2002).

Mechanical Strain

Early research on skeletal muscle damage demonstrates that muscle damage occurs via direct micro-tears of skeletal muscle fibers (contractile tissue) and other connective tissues or non-contractile tissues (Hough, 1900). Follow-up histological and biochemical evidence in both human and animal models confirms these findings, particularly when the exercise protocol consists of eccentric, or lengthening, muscle contractions (McHugh, 2003). Several investigations by Friden and colleagues (Friden, 1984; Friden & Lieber, 1998, 2001; Friden, Lieber, & Thornell, 1991; Friden, Seger, Sjostrom, & Ekblom, 1983; Friden, Sfakianos, & Hargens, 1986, 1989; Friden, Sfakianos, Hargens, & Akeson, 1988; Friden, Sjostrom, & Ekblom, 1983) showed evidence that eccentric muscle contractions evoke severe disarray of myofibrillar protein organization, altered z-line streaming, damaged t-tubule organization, and loss of skeletal

muscle membrane or sarcolemma integrity (Stupka, Tarnopolsky, Yardley, & Phillips, 2001). While the majority of studies examining the mechanical strain hypothesis have utilized the direct method of muscle biopsies, the use of less invasive and/or indirect techniques, including magnetic resonance imaging (MRI), quantification of muscle enzyme levels (e.g. creatine kinase, lactate dehydrogenase) and markers of oxidative stress (e.g. superoxide dismutase, reduced and oxidized glutathione, catalase) released into the blood have been utilized to determine the extent of mechanical skeletal muscle damage. One hypothesis that has been put forth with regard to eccentric muscle damage is the popping sarcomere hypothesis (Morgan & Proske, 2004, 2006) which suggests that the uneven stretching of sarcomeres during mechanical strain results in the muscle being stretched beyond the optimum force-producing length. The stretched and stressed sarcomeres become gradually weaker, reaching what is referred to as the "yield point" (Morgan & Proske, 2004, 2006). At the yield point, there is little or no overlap of the contractile myofilaments, and therefore, shearing of the myofibrils and sarcomere occurs throughout the skeletal muscle fibers as contraction continues. Though this may not occur in all sarcomeres, non-uniform lengthening and shearing of myofibrils exposes the t-tubules and disrupts intracellular calcium channel homeostasis, furthering cellular damage (Morgan & Proske, 2004, 2006). Following a bout of eccentric exercise training, the majority of the strained sarcomeres re-interdigitate and normal contractile function is restored; however, other sarcomeres may become permanently disrupted, at both the contractile (e.g. actin and myosin) and non-contractile (e.g. desmin, titin) levels of skeletal muscle (Friden & Lieber, 2001). Damage to individual sarcomeres may also lead to complications with excitation-contraction coupling, impairing the ability of the skeletal

muscle to produce force, as well as cause leakage of various intracellular proteins and enzymes (e.g. creatine kinase) into the extracellular space and circulation. Subsequently, a rise in blood concentrations of these proteins and enzymes may indicate an increase in permeability of the sarcolemmal membrane of the muscle cell in response to muscular insult (Kim & Lee, 2015).

Metabolic Strain

While less associated with delayed-onset muscle soreness following eccentric exercise, metabolic strain has been linked to damage of skeletal muscle. One component of the metabolic strain hypothesis of muscle damage implies that muscle damage results from environments in which adenosine-triphosphate (ATP) demand exceeds ATP production, often occurring during prolonged endurance-based exercise (Kuipers, 1994). Due to ATP-dependent calcium channel pumps, reduced ATP concentrations in the muscle cell may lead to excessively high calcium levels resulting in apoptosis or cell death. In regard to skeletal muscle damage resulting from resistance-based exercise, damage to the sarcoplasmic reticulum prevents proper re-sequestration of calcium, leading to elevations in intracellular calcium concentrations. Research utilizing rodent skeletal muscle fibers demonstrates that increased intracellular concentrations of calcium following muscle damage activates phospholipase A2, ubiquitin-proteasome and calpain-mediated proteases that assist in the degradation of the sarcolemma, as well as contractile proteins (Zhang et al., 2012; Zhang, Yeung, Allen, Qin, & Yeung, 2008). Furthermore, elevated resting intracellular calcium levels enhance cross-bridge formation, thus reducing the relaxation phase of contraction. This reduction in the relaxation phase of

muscle contraction has been associated with a reduced range of motion of the associated joint and increased muscle stiffness following damaging exercise (Kuipers, 1994).

While the ensuing events underlying muscle damage from lengthening contractions are not completely understood, it is likely that the mechanical stress that occurs during the eccentric exercise and the metabolic homeostatic imbalance during the recovery period contribute to the many symptoms associated with eccentric muscle damage. Common symptoms include decreased muscle function (muscle force production and power production), increased perceived muscle soreness, decreased range of motion and increased blood concentrations of skeletal muscle proteins and enzymes (Janssen et al., 1989; Komulainen et al., 2000; Van der Meulen, Kuipers, & Drukker, 1991).

Eccentric Exercise: For Better or For Worse

Engaging in unaccustomed lengthening contractions may result in reduced muscular function and increased recovery time. A premature return to strenuous activity after eccentric exercise without adequate recovery may increase the risk of musculoskeletal injury, and may hinder performance during subsequent training sessions and/or competition (Cheung, Hume, & Maxwell, 2003). Therefore, facilitating the restoration of muscle function may be a primary objective for individuals engaging in eccentric resistance exercise. A variety of treatments and modalities that may help alleviate symptoms associated with eccentric exercise damage have been suggested. These treatments include the administration of nonsteroidal anti-inflammatory drugs (Schoenfeld, 2012), tissue compression and massage (Shin & Sung, 2015) , cryotherapy (Ferreira-Junior et al., 2015), as well as a number of nutritional interventions (Beelen et

al., 2008; Buckley et al., 2010; Koopman, Saris, Wagenmakers, & van Loon, 2007; Tipton, 2015). However, the efficacy of most treatment strategies across a range of populations, including trained, untrained, and older adults, remains equivocal.

Protein-Based Nutritional Interventions

Recently, investigations have been done on the potential impact of nutritional strategies for aiding recovery from exercise-induced muscle damage as a result of resistance training, and in particular, the performance of eccentric muscle contractions. Specifically, the role of protein supplementation in attenuating muscle damage or increasing the restoration of damaged proteins and formation of new proteins to replace the damaged cellular components has garnered much scientific attention (Buckley et al., 2010; Farup et al., 2014; Rahbek, Farup, de Paoli, & Vissing, 2015; Yang et al., 2012). However, these investigations have not provided compelling results (Etheridge, Philp, & Watt, 2008; Levenhagen et al., 2002; Pasiakos, Lieberman, & McLellan, 2014).

While the recommended daily allowance (RDA) of protein is 0.8 grams per kilogram (g/kg) of body mass, the recommendation for individuals who engage in regular resistance training is 1.2 - 2.4 g/kg of body mass (Marini, 2015; Pencharz, Elango, & Wolfe, 2016). Previous reports indicate that 80-85% of professional athletes consume protein supplements, including amino acids (AA), based upon the presumption that these supplements enhance performance, skeletal muscle mass and strength, and recovery (Lun, Erdman, Fung, & Reimer, 2012; Maughan, Depiesse, Geyer, & International Association of Athletics, 2007). Branched-chain amino acids (BCAA) are the most popular of the AA supplements, especially among individuals who resistance train. The BCAA (leucine, isoleucine, and valine) are three essential amino acids (EAA) that cannot

be synthesized by the human body and must be consumed in the diet (Blomstrand & Saltin, 2001).

Branched-chain amino acids are primarily metabolized in skeletal muscle, whereas other essential AA are metabolized in the liver (Blomstrand & Saltin, 2001). The BCAA play a multitude of roles within the human body, serving as metabolic precursors for various substrates including tricarboxylic acid (TCA) cycle intermediates, constitutive elements of the structural and contractile proteins, signaling molecules for cellular pathways such as the mTOR (mammalian target of rapamycin) pathway, enhancing mitochondrial biogenesis, and serving as reactive oxygen species (ROS) scavenging constituents (Glynn et al., 2015; Hatazawa et al., 2014). On the basis of the many roles BCAA play, the possibility that BCAA supplementation may counteract the deleterious effects of exercise-induced muscle damage has been addressed. Previous research demonstrates that AA, specifically BCAA, stimulate metabolic pathways responsible for cell growth, improve exercise performance, as well as speed recovery following tissue damaging exercise (Coombes & McNaughton, 2000; Howatson et al., 2012; Shimomura, Murakami, Nakai, Nagasaki, & Harris, 2004).

Branched-Chain Amino Acids: Cellular Signaling

The ingestion of essential AA elicits a cascade of cellular events that underlie the adaptive responses to exercise training, particularly those accrued by resistance-based exercise (Atherton & Smith, 2012; Hulmi, Lockwood, & Stout, 2010; Kumar et al., 2012; B. E. Phillips, Hill, & Atherton, 2012). Of the EAA, the BCAA have demonstrated to exert the most potent effect on muscle protein balance, by increasing protein synthesis and precluding protein degradation (Anthony et al., 2000; Deldicque, Theisen, &

Francaux, 2005; Du, Shen, Zhu, & Ford, 2007; Herningtyas et al., 2008; Karlsson et al., 2004; Louard, Barrett, & Gelfand, 1990; Sadiq, Hazlerigg, & Lomax, 2007). A positive muscle protein balance allows for the accretion of myofibrillar proteins, and thus muscle cell size and strength, when combined with suitable resistance exercise protocols (Churchward-Venne, Murphy, Longland, & Phillips, 2013). An acute bout of resistance exercise leads to changes in protein turnover immediately following exercise, and up to 24 – 48 hours (hr) post-exercise (Biolo, Maggi, Williams, Tipton, & Wolfe, 1995; Dreyer et al., 2006; S. M. Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997) whereby both protein synthesis and degradation are increased. However, without the provision of AA, the net muscle protein balance is negative (Biolo et al., 1995; S. M. Phillips et al., 1997; Tipton & Wolfe, 1998). The intake of exogenous AA rich in BCAA in the proximal hr surrounding exercise, both prior to and following an acute bout, enhances protein synthesis to a degree that yields a positive net balance permitting cell growth (Churchward-Venne et al., 2013; Louis et al., 2003; Tipton & Wolfe, 1998). These effects are mediated by one central regulatory protein, the mTOR complex (Deldicque et al., 2005; Hawley, Hargreaves, Joyner, & Zierath, 2014; Marcotte, West, & Baar, 2015). The mTOR complex is sensitive to the energy state of the cell, as well as environmental stimuli, including mechanical stress, to control protein turnover and cell growth (Deldicque et al., 2005). Branched-chain amino acids elicit a profound effect on the mTOR signaling pathway by activating this protein-complex and using a mechanism distinct from mechanical load- and growth factor-induced stimuli (Jefferson & Kimball, 2003; Laplante & Sabatini, 2012; Marcotte et al., 2015). Once activated, mTOR exists as two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)

(Goodman et al., 2011; Hawley et al., 2014; Marcotte et al., 2015). Of the two complexes, mTORC1 is associated with a multitude of responses resulting in protein synthesis and cell growth, including mRNA translation and ribosomal biogenesis (Hawley et al., 2014; Laplante & Sabatini, 2012).

The protein synthetic effect of BCAA, most notably from the BCAA leucine, is multifaceted. In addition to down-regulating autophagy (Deldicque et al., 2005; Kanazawa et al., 2004; Sugawara, Ito, Nishizawa, & Nagasawa, 2009) and the ubiquitin-proteasome proteolysis pathway (Herningtyas et al., 2008; Sadiq et al., 2007), these AA must be present for other mTORC1 signaling sources (i.e. mechanical load, growth factors) to effectively result in increased protein synthesis, as they serve as substrates to be incorporated into new proteins (Blommaart, Luiken, Blommaart, van Woerkom, & Meijer, 1995; Crozier, Kimball, Emmert, Anthony, & Jefferson, 2005; Stipanuk, 2007; Tipton & Wolfe, 1998). Additionally, and uniquely, BCAA themselves are capable of up-regulating the mTORC1 signaling pathway (Du et al., 2007; Hara et al., 1998; Jefferson & Kimball, 2003; Norton & Layman, 2006). Though the molecular mechanisms by which AA activate mTORC1 have yet to be completely clarified, it is known that AA act through a growth factor- and loading-independent pathway (Kim & Guan, 2011; Laplante & Sabatini, 2012; Marcotte et al., 2015). Amino acids are capable of activating mTORC1 in an intrinsic manner, whereby upstream regulators including the PI3K – PKB/AKT pathway are not required (Deldicque et al., 2005; Kim & Guan, 2011; Marcotte et al., 2015; Meijer, Lorin, Blommaart, & Codogno, 2014), nor is mTORC1 acted upon directly (i.e. phosphorylation) (Kim & Guan, 2011; Meijer et al., 2014; van Sluijters, Dubbelhuis, Blommaart, & Meijer, 2000). Recent evidence suggests AA, particularly leucine, regulate

activity by co-localizing mTORC1 with its activator ras homolog enriched in brain (Rheb), a small GTPase located on the membrane of the lysosome (Bar-Peled & Sabatini, 2014; Efeyan et al., 2013; Marcotte et al., 2015; Sancak et al., 2010). In AA deprived cells, mTORC1 is diffuse throughout the cell; however, with the presentation of AA, the protein is translocated to the surface of the lysosome to bind with the Ragulator complex where it can then interact with Rheb (Bar-Peled & Sabatini, 2014; Efeyan et al., 2013; Marcotte et al., 2015; Sancak et al., 2008). This translocation is mediated by the Rag family of G-proteins (Rag A, B, C, D) where the presence of AA result in the activation of this complex which binds to raptor, the regulatory protein of mTORC1, and recruits mTORC1 to the lysosome through its interaction with the Ragulator on the lysosomal membrane (Watson & Baar, 2014). Interestingly, this process appears to be regulated by intralysosomal AA that accumulate within the vacuole, rather than AA in the cytosol (Bar-Peled & Sabatini, 2014; Meijer et al., 2014). The two most well-defined downstream targets of mTORC1 are ribosomal protein S6 kinase (S6K) and the eukaryote translation initiation factor 4E binding protein (4E-BP1), both of which are involved in translational control (Hawley et al., 2014; Kim & Guan, 2011; Ruvinsky & Meyuhas, 2006). mTORC1 regulates 4E-BP1 by hyper-phosphorylating the complex resulting in a dissociation from the translation initiation cap binding factor eIF4E which is then free to bind to eIF4G to form eIF4F, the initiation complex required for protein translation (Coffey & Hawley, 2007; Meijer et al., 2014; Richter & Sonenberg, 2005). mTORC1 phosphorylation of S6K results in an activation of ribosomal protein S6 and inhibition of elongation factors which enhances transcription and translation capacity of

mRNAs, and thus protein synthesis (Chauvin et al., 2014; Hawley et al., 2014; Ruvinsky & Meyuhas, 2006).

It has been observed that ingestion of BCAA in combination with resistance exercise, particularly prior to or within the 3 – 4 hr following exercise, results in an enhanced upregulation of the mTORC1 signaling cascade and protein synthesis (Apro & Blomstrand, 2010; Blomstrand & Saltin, 2001; Hawley, Tipton, & Millard-Stafford, 2006; Meijer et al., 2014). Briefly, a few well-designed crossover studies will be presented. Firstly, Karlsson et al. (Karlsson et al., 2004) investigated the effect of resistance training with and without the ingestion of BCAA. A BCAA supplement was administered as 100 mg/kg of body mass pre, during, and post-exercise at 15, 30, 60, and 90 minutes (min), resulted in a 3.5-fold increase in p70S6K phosphorylation at Ser^{424}/Thr^{421} (Serine and Threonine) and significantly greater S6 phosphorylation at $Ser^{235/236}$ 1 – 3 hr following exercise in comparison to the control trial. Similarly, Apro and Blomstrand reported the same administration of BCAA prior, during, and immediately, 15 and 45 min post-exercise led to a 30-fold increase in p70S6K phosphorylation at Thr^{389}, whereas the control trial remained unchanged 1 hr following exercise. S6 phosphorylation at $Ser^{235/236}$ was also more pronounced in the BCAA trial, though this difference did not reach statistical significance. Further, of the BCAA, leucine appears to be a primary effector of the protein synthetic response (Burd et al., 2011; Marcotte et al., 2015; Moberg et al., 2014; Tipton, Borsheim, Wolf, Sanford, & Wolfe, 2003). Following a bout of resistance exercise, subjects were asked to consume an EAA supplement with or without leucine (45 mg/kg body mass). Subjects followed a similar consumption schedule as the previously mentioned studies (pre-, during, and 15,

30, 60, and 90 min post-exercise). Phosphorylation of mTORC1 at SER^{2448} increased 120% 1 hr post-exercise with the leucine-containing solution versus 46% with the leucine-absent solution. Similarly, the leucine-containing solution resulted in a 59-fold increase in p70S6K phosphorylation at Thr^{389} while the leucine-absent solution only demonstrated an 8-fold increase. Lastly, a study by Moore et al. reported that subjects who consumed either 40 grams (g) or 20 g of egg protein (containing 1.7 g and 3.36 g of leucine, respectively) immediately following resistance exercise displayed similar responses in mixed muscle fractional synthetic rate 4 hr post-exercise (Moore et al., 2009). These data suggest that BCAA enhanced the protein synthetic response to resistance exercise and implicated leucine as the greatest driver of the mTORC1 signaling pathway and subsequent protein synthesis. From a supplementation perspective, leucine content, rather than overall protein content, appears to represent the limiting factor in stimulating the translational machinery underlying the adaptive responses to resistance-based exercise, namely skeletal muscle remodeling and hypertrophy (Campbell, 2013). Current evidence suggests that when a leucine intake of .02 – .045 g/kg/d is ingested, rates of muscle protein synthesis are significantly enhanced (Campbell, 2013).

Limitations in the Literature

Prophylactic interventions, including supplementation with BCAA, have been projected to attenuate the negative effects associated with resistance-induced muscle damage. To date, only 11 studies have examined BCAA supplements in humans on markers of recovery and muscular damage, one of which examined leucine exclusively. Assessments of muscle damage tissue (e.g. sarcolemma damage) utilized within these studies include muscle proteins (i.e. creatine kinase, myoglobin, lactate dehydrogenase),

while recovery was commonly assessed utilizing a visual analog scale for the assessment of delayed onset muscle soreness (DOMS) and recovery of muscle function (i.e. force, strength, jump height). A full literature review is included in Chapter 2.

Branched-Chain Amino Acid Supplementation: Recovery from Intense Exercise; Primary Study

Indirect Biochemical Markers: Creatine Kinase

Blood (plasma or serum) concentrations of myofiber proteins or enzymes are often used as indirect markers of muscle damage, including creatine kinase (CK), myoglobin (Mg), troponin, and lactate dehydrogenase. Creatine kinase is the most commonly measured biochemical marker for skeletal muscle damage. It should be noted that the concentration of the myofiber proteins in the circulation is negligible in comparison to that of the total amount in the muscle. However, research studies have reliably demonstrated significant increases in CK in the blood following a bout of muscle damaging exercise (Baird, Graham, Baker, & Bickerstaff, 2012). The permeability of the sarcolemma is mediated by arachidonic acid metabolites whereby the structural breakdown of the membrane is indicated by the efflux of myofiber proteins into circulation, specifically, CK (Baird et al., 2012).

The greatest maximal force loss is generally seen immediately after an eccentric exercise bout, whereas CK levels demonstrate a biphasic response, peaking at 24-48 hr and then again at 96-120 hr post-exercise (Newham, Jones, & Edwards, 1986). Furthermore, the response of CK to lengthening contractions is highly individualized, insofar as high inter-individual variability has been reliably documented (Newham et al., 1986). A difference in peak values for individuals has been shown to differ as much as

200 - 350% in response to the same eccentric exercise protocol (Nosaka & Clarkson, 1996).

Previous reports indicate that BCAA supplementation may decrease blood concentrations of the intramuscular enzymes CK and lactate dehydrogenase following prolonged, endurance exercise (Coombes & McNaughton, 2000). Two studies have examined the impact of BCAA supplementation on biochemical markers of muscle damage (e.g. CK) in response to resistance exercise (Aad et al., 2015; Jackman, Witard, Jeukendrup, & Tipton, 2010; Sharp & Pearson, 2010). Jackman *et al*. evaluated the effects of BCAA supplementation (3.5 g of leucine, 2.1 g of isoleucine, and 1.7 g of valine; divided in 4 daily doses) on recovery of CK following eccentric muscle damaging exercise. Results indicated that serum CK was significantly increased after exercise and remained elevated for 48 hr, however, BCAA supplementation did not attenuate CK responses. However, Sharp and Pearson (Sharp & Pearson, 2010) report conflicting results, as individuals who supplemented with BCAA (1.8 g of leucine, 0.75 g of isoleucine, and 0.75 g of valine) 3 weeks before exercise and 1 week during exercise exhibited reduced CK levels 2 and 4 hr post-exercise after completing a bout of total body resistance training workout (high-intensity, overreaching workout).

Indirect Biochemical Markers: Oxidative Stress

Oxidative stress represents an imbalance between free radical production and antioxidant defense. Free radicals include both ROS and reactive nitrogen species (RNS). Reactive oxygen species include oxygen-centered radicals (i.e. superoxide and hydroxyl radicals) and non-radical oxygen derivatives (i.e. hydrogen peroxide) whereas RNS include nitrogen-centered species such as nitric oxide. Both ROS and RNS demonstrate

exceptional chemical reactivity resulting in oxidation of numerous biomolecules (Kerksick & Zuhl, 2015).

An antioxidant is defined as a substance that has the potential to significantly delay or prevent oxidation by helping form less active radicals or by quenching the radical chain reactions in macromolecules such as proteins (i.e. amino acids) (Barber & Harris, 1994). Reactive oxygen species actions are commonly opposed by a complex network of antioxidant molecules including glutathione peroxidase, superoxide dismutase, catalase, as well as reduced glutathione. The quantification of oxidative stress commonly occurs via direct free radical detection or indirectly by measuring markers reflecting ROS/NOS-induced damage in proteins, lipids, DNA, and carbohydrates and/or antioxidants' concentration in several tissues (Jenkins, 1993).

Work by Paschalis and Nikolaidis was among the first to demonstrate that eccentric exercise increases systemic oxidative stress (Nikolaidis et al., 2007; Paschalis et al., 2007). Human studies indicate that muscle-damaging exercise elevates concentrations of ROS markers in blood by 9-122% for as long as 96 hr post-exercise. Concerning antioxidants' responses, exercise-induced muscle damage has previously been linked with elevated oxidized glutathione (GSSG) 2 hr post-exercise (Goldfarb, Bloomer, & McKenzie, 2005).

Though findings from research are not homogenous regarding oxidative stress responses following muscle-damaging exercise, it appears damaging exercise is capable of altering the concentrations of numerous protein, lipid, and DNA oxidation markers in both the muscle and blood. You et al. (2005) compared markers of oxidative stress in the blood and muscle tissue following muscle-damaging exercise and found no significant

differences between concentrations within the blood, compared to muscle tissue (You et al., 2005). However, less is known regarding how antioxidants, such as glutathione, respond to muscle-damaging exercise due to the variety of muscle-damaging exercise protocols, intensities, as well as training status and sex of participants used in previous studies. Thus far results are contradictory, reporting both early (~2 hr post-exercise) and more prolonged peaks in glutathione activity (~72 hr post-exercise) (Gambelunghe, Rossi, Micheletti, Mariucci, & Rufini, 2001). To our knowledge, no research has examined the oxidative stress response following eccentric exercise in humans supplementing with BCAA.

Markers of Physical Performance and Delayed Onset Muscle Soreness

Following a damaging bout of exercise, the extent of muscle damage is not immediately evident. Research examining recovery often includes "delayed onset of muscle soreness" or DOMS analysis (i.e. perception of soreness), as well as recovery of physical performance (e.g. vertical jump) at a multitude of post-exercise time points (e.g. 2, 4, 8, 24, 48, 72, 96 hr). Delayed onset muscle soreness often peaks 24-72 hr post-exercise, but may last up to 5-7 days following strenuous, muscle damaging physical activity (i.e. ultramarathon) (Cheung et al., 2003). Delayed onset muscle soreness is highly associated with decreased joint range of motion, decreased force production, and decreases in muscular strength and power, with accounts of decrements up to 72 hr post-exercise (Cheung et al., 2003). Human performance variables, such as vertical jump, maximal voluntary contraction, and the 100 meter dash, as well the visual analog scale for DOMS are commonly used in research studies to monitor muscle damage and recovery after exercise (Chapman et al. 2006; Vincent et al. 1997; Paschalis et al. 2005b).

Concerning the effect of BCAA supplements on DOMS and markers of performance, investigations have produced inconsistent results (Coombes & McNaughton, 2000; Howatson et al., 2012; Jackman et al., 2010; Shimomura et al., 2010).

Branched-Chain Amino Acids, Autophagy and Resistance Exercise; Secondary Study

Autophagy

The human body possesses several cellular housekeeping systems responsible for maintaining the proteome. Autophagy is an evolutionary-conserved intracellular recycling system present in all eukaryotic cells, from yeast to humans, and is involved in the clearance of dysfunctional and damaged cytosolic components (Levine & Klionsky, 2004; Todde, Veenhuis, & van der Klei, 2009). Three main types autophagy are commonly described, namely macroautophagy, microautophagy, and chaperone-mediated autophagy, though research on other cargo-specific autophagy pathways such as mitophagy, reticulophagy, and ribophagy are gaining popularity (Reggiori, Komatsu, Finley, & Simonsen, 2012). Macroautophagy, hereafter referred to as autophagy, is currently best-understood and is responsible for bulk degradation of damaged cellular material, including organelles, cytosolic proteins, and protein aggregates (Levine & Klionsky, 2004; Rubinsztein, Marino, & Kroemer, 2011). More specifically, autophagy involves the formation of double-membrane vesicles termed autophagosomes, which engulf damaged materials found within the cytoplasm, as well as entire organelles, and transport them to the lysosome for degradation (Levine & Klionsky, 2004). Normal autophagic function has been correlated with less incidence of degenerative diseases, including insulin resistance, Parkinson, Alzheimer's, cancer, and sarcopenia, and thus

carries broad and apparent implications on organismal function (Fan et al., 2016; Todde et al., 2009). Moreover, autophagy appears to play a major role in the adaptive processes to exercise (He, Bassik, et al., 2012; He, Sumpter, & Levine, 2012). Though the first description of exercise-induced autophagy dates back to 1984, the relationship among autophagy, exercise and metabolic regulation has been largely unexplored and its role in the maintenance of muscle mass remains controversial (Fan et al., 2016).

Microtubule-associated protein 1A/1B-light chain 3 (LC3) immunoblotting is a commonly used biochemical technique to assess autophagic function, in that LC3-II is the only protein marker that is reliably associated with completed autophagosomes and the LC3-II/LC3-I ratio is recognized as a valuable marker of autophagosome content in tissues (Klionsky et al., 2016). The adaptor protein p62 (sequestosome 1) which is involved in substrate delivery to the autophagosome, is also commonly used as a marker of autophagy activity, as its degradation represents degradation of the autophagosome at the lysosome (Klionsky et al., 2012). Various autophagy-related proteins (Atgs) associated with sequestering cytosolic components and formation of the autophagosome have been used as measures of autophagic activity (Klionsky et al., 2012; Klionsky et al., 2016).

Autophagy and Exercise

The study of autophagic function and exercise is currently in its infancy and results regarding the acute and chronic responses to exercise are equivocal (Schwalm et al., 2015; Jamart et al., 2012; Moller et al., 2015). Work by Grumati and colleagues demonstrates that voluntary physical exercise to fatigue in rodents activated autophagy in skeletal muscle as indicated by the enhanced conversion of LC3-I to LC3-II

(Microtubule-associated protein 1-light chain [cytosolic]; LC3II-phosphophatidyleethanolamine conjugate), as well as the increased presence of autophagosomes (Grumati & Bonaldo, 2012; Grumati et al., 2011; Vainshtein, Grumati, Sandri, & Bonaldo, 2014). Research by Luo and colleagues shows that chronic resistance training for nine weeks resulted in increased muscle mass accretion, improved muscular strength, as well as reduced the LC3-II/LC3-I ratio, reduced p62 protein levels, and increased levels of autophagy regulatory proteins, including Beclin 1, Atg5/12, Atg7, and the lysosomal enzyme cathepsin (Luo et al., 2013). Moreover, the upstream targets including total AMPK (adenosine monophosphate-activated protein kinase), phosphorylated AMPK, and downstream target FOXO3 (forkhead transcription factor) were significantly upregulated following a chronic resistance training protocol. These data are also supported in the recent works by Fry et al., who demonstrate that levels of autophagy are not significantly different in younger and older individuals following an acute bout of resistance exercise (Fry et al., 2013). To date, no research has examined autophagy following an acute bout of eccentric-based resistance exercise or in individuals supplementing with BCAA.

Heat Shock Proteins and Exercise

Heat shock proteins (HSP) are a family of chaperones involved in the repair, specifically refolding, of misfolded and damaged cellular proteins and have been shown to possess an essential role in cellular response to stressors such as exercise, particularly that of the recovery phase (Dokladny, Myers, & Moseley, 2015). Exercise elicits several stressors that activate the HSP response, including heat, oxidative, and metabolic stress as well as inflammation (de Moura, Lollo, Morato, Carneiro, & Amaya-Farfan, 2013; Fittipaldi,

Dimauro, Mercatelli, & Caporossi, 2014; Lollo, Moura, Morato, & Amaya-Farfan, 2013; Mikkelsen et al., 2013; Peart, Kirk, Madden, Siegler, & Vince, 2013). Acute exercise has been demonstrated to stimulate the expressions of certain HSP in a variety of cells and tissues including muscle, heart, liver, brain, leukocytes, and plasma. The HSP response to exercise has been documented in both animals and humans (de Moura et al., 2013; Fehrenbach & Niess, 1999; Fittipaldi et al., 2014).

The extent of the HSP response is dependent on type, intensity, and duration of exercise, environmental conditions, and cell type. Training status and sex also have an impact on the HSP response (Kiang, 2004). Large individual variability of HSP expression has been reported and the particular HSP being examined have to be considered when assessing heat shock response (Kiang, 2004). Repeated exercise bouts, regular training, and high ambient temperature induce an adaptation of the HSP response in muscles and immune cells (Kiang, 2004). Dokladny et al. (2015) reported that exercise and training interfere with HSP in a complex way, which includes alterations in HSP content and function through acute exercise and sophisticated regulation through regular training. To date, the extent to which HSP responds to an acute, damaging bout of eccentric resistance training has yet to be fully elucidated (Dokladny et al., 2015).

Recently, researchers have examined the HSP response following eccentric exercise in skeletal muscle in individuals who consumed non-steroidal anti-inflammatory drugs (NSAIDS) (Mikkelsen et al., 2013). Following exercise, Mikkelsen et al. report that, compared to pre-exercise values, the mRNA expression of HSP70 and HSP27 was increased; specifically, HSP70 increased 26-fold 5 hr post-eccentric exercise and remained significantly elevated from baseline levels 24 hr after exercise (3.4-fold)

(Mikkelsen et al., 2013). Immunohistochemical staining for HSP70 revealed increased staining in some samples, but no significant differences were found between control and eccentrically damaged samples at any time point. Moreover, de Moura et al. reported larger increases in HSP70 protein expression in the soleus, gastrocnemius and lung of the whey protein hydrolysate-fed (WPH) rats, compared to whey-protein or casein-fed rats following a stress bout of endurance exercise (22 m/min for 30 min) (de Moura et al., 2013). Interestingly, HSP70 expression in the sedentary rats was very low, independent of the diet or tissue type. Markers of oxidative stress were lower in the group that consumed WPH. de Moura et al. concluded that the consumption of WPH enhances HSP70 expression; however, these findings have yet to be replicated in humans (de Moura et al., 2013).

Interestingly, the HSP response appears to exert regulatory control of autophagic activity (Dokladny et al., 2015). With regard to exercise, this cooperative relationship exhibits itself in a phasic manner in that autophagy is primarily upregulated in the initial degradation phase of exercise and the heat shock response is largely activated in the building phase following exercise (Dokladny et al., 2015). Current evidence suggests that, HSP70 in particular, acts as a intracellular control mechanism responsible for shifting the cell from the degradation phase, as denoted by elevated autophagic activity, to the repair and protein synthetic phase in response to exercise (Dokladny et al., 2015). To date, no studies have examined the HSP response in individuals undergoing eccentric exercise, nor individuals supplementing with BCAA.

Study Objectives

The objectives of this study were: 1) determine the impact of BCAA supplementation on recovery of muscular performance, indirect biochemical markers of muscle damage, and perceived soreness ratings following muscle-damaging exercise in resistance trained males; 2) examine the impact of acute, damaging resistance exercise on markers of autophagy (microtubule-associated protein 1 Light Chain 3 (LC3); sequestosome 1 (SQSTM1/p62)) and heat shock protein 70 (HSP70) during recovery in resistance trained males.

Specifically, we investigated:

Objective 1- To determine if supplementation with BCAA favorably impacts the ability of the body to recover from muscle damaging eccentric exercise, as measured by indirect markers of muscle damage (creatine kinase, glutathione) and recovery of markers of muscular performance (vertical jump, maximum voluntary isometric contraction, and jump squat).

Objective 2- To determine if supplementation with BCAA influences rating of perceived soreness following an acute bout of damaging eccentric resistance exercise.

Objective 3 - To determine if acute muscle-damaging resistance exercise affects the autophagy (LC3-I, LC3-II, p62) response acutely and at multiple time points over 72 hr post-exercise.

Objective 4- To determine if there is an impact of BCAA supplementation on markers of autophagy (LC3-I, LC3-II, p62) in individuals who perform a muscle-damaging bout of resistance exercise.

Objective 5- To determine if muscle-damaging resistance exercise affects heat shock protein 70 (HSP70) response acutely and at multiple time points over 72 hr post-exercise.

Objective 6- To determine whether there is any difference in autophagy and HSP 70 responses between groups supplementing with BCAA and placebo.

Objective 7- To determine the relationship between autophagy and HSP70 responses in individuals who perform a muscle-damaging bout of resistance exercise.

Limitations

1: We were unable to measure protein expression of LC3-I and LC3-II, p62, and HSP70 in skeletal muscle. Markers of autophagy and HSP were measured in peripheral blood mononuclear cells (PBMC) and may not represent regulation of these systems in skeletal muscle.

2: Results from previous research have established that a single bout of unfamiliar eccentric exercise causes symptoms of muscle damage such as strength loss, pain and muscle tenderness. However, it has been demonstrated that a repeated bout of the same or similar eccentrically based exercise protocol results in markedly reduced symptoms of damage, as compared to the previous bout (McHugh, 2003; McHugh & Tetro, 2003). Due to the potential protective nature of this "repeated bout effect" phenomenon, we were unable to use a cross-over design.

3: When studying a dietary supplement, dietary control is important. We did not possess the necessary resources (e.g. kitchen, funds) to prepare food for the participants in this study. Rather, a registered dietician provided specific dietary guidelines for

participants, including weighing food and consulting dietary analysis software to calculate intake of macronutrients.

4: We measured only LC3-I, LC3-II, and p62 proteins as markers of autophagy and measured only HSP70 protein as a marker of the heat shock system.

5: We estimated the steady state autophagy protein expression at each time point, as we were unable to administer chloroquine to participants.

Assumptions

This study was conducted based on the following assumptions:

1: All subjects understood the exercise and dietary restrictions of the study, and reliably adhered to these guidelines honestly. Subjects reported their dietary food intake honestly and accurately.

2: Subjects put forth a maximal effort during vertical jump, maximal voluntary isometric contraction, and jump squat testing.

3: The creatine kinase measured in the blood are of skeletal muscle origin.

4: Subjects accurately reported their previous and current resistance training status.

5: Subjects consistently consumed their randomized beverage twice per day, morning and evening for all of the study days.

Hypotheses

This study tested the following hypotheses:

1: BCAA supplementation will reduce (or blunt) the increase in plasma CK levels post muscle-damaging exercise compared to the placebo group (PLCB).

Howatson et al. demonstrated that individuals supplementing with BCAA resulted in significantly lower CK levels post muscle damaging exercise (Howatson et al., 2012). Specifically, they demonstrated significantly lower CK levels in the BCAA group compared to placebo. Both BCAA and placebo groups peaked at 24 h post-exercise (312 $IU.L^{-1}$ and 398 $IU.L^{-1}$, respectively) (Howatson et al., 2012).

2: There will be no significant difference in glutathione responses between individuals who supplement with BCAA versus PLCB post muscle-damaging exercise.

To date, there has been no research examining the effect of BCAA supplementation alone on glutathione responses following muscle-damaging exercise.

3: BCAA supplementation will reduce the decline in force production during maximal voluntary isometric contraction (MVIC) post muscle-damaging exercise compared to PLCB.

Previous works by Greer et al. and Howatson et al. (Greer, Woodard, White, Arguello, & Haymes, 2007; Howatson et al., 2012) have reported decreased decrements in force production, as measured by MVIC, in individuals who supplemented with BCAA.

4: There will be no significant difference in peak power outcomes, as measured by 40% 1RM jump squat, between individuals who supplement with BCAA versus PLCB post muscle-damaging exercise.

To date, there is no research examining the effect of BCAA supplementation on jump squat power production following muscle-damaging exercise.

5: There will be no significant difference in vertical jump performances between individuals who supplement with BCAA versus PLCB post muscle-damaging exercise.

To date, two studies (Areces et al., 2014; Howatson et al., 2012) have been done examining the effect of BCAA supplementation on vertical jump performance following a damaging bout of exercise. Both Areces et al. and Howatson et al. report no impact of BCAA supplementation on recovery of vertical jump performance in individuals supplementing with 5 and 20 g of BCAA, respectively.

6: BCAA supplementation will reduce the perception of soreness (DOMS) post muscle-damaging exercise compared to the PLCB group.

Previous work by Jackman et al. (Jackman et al., 2010) demonstrated significant reductions in perceived muscle soreness 72 hr post muscle-damaging exercise in individuals who supplemented with 29.2 g/d of BCAA. Work by Shimomura et al. showed significant reductions in perceived soreness at 24 and 48 hr post muscle-damaging exercise in individuals who supplemented with BCAA (Shimomura et al., 2010).

7: There will be no significant difference in: a.) LC3-II/β-actin, b.) LC3-II/I ratio, and c.) p62/β-actin protein expressions in PBMC between individuals who supplement with BCAA versus PLCB post muscle-damaging exercise.

It has been reported that nine weeks of resistance exercise training increased autophagy and reduce apoptosis in skeletal muscles of aged rats. In particular, reduced p62 protein levels, increased levels of Beclin1, Atg5-Atg12, and reduced LC3-II/LC3-I ratio have been documented. However, this has yet to be documented in human PBMC.

8: There will be no significant difference in HSP70 protein responses in PBMC between individuals who supplement with BCAA versus PLCB post muscle-damaging exercise.

de Moura et al. reported larger increase in HSP70 protein expression in the soleus, gastrocnemius and lung of whey protein hydrolysate-fed (WPH) rats, compared to whey-protein or casein-fed rats following a stress bout of endurance exercise (22 m/min for 30 min) (de Moura et al., 2013). However, there has been no research examining the impact of BCAA supplementation on HSP responses in human PBMC following resistance exercise.

Significance of Study

This study is expected to produce unique and important knowledge in the fields of sports nutrition and exercise physiology. In addition to elucidating whether BCAA supplementation plays a role in recovery from high-intensity, eccentric resistance exercise and prevention of/recovery from skeletal muscle damage, these results will be some of the first to provide insight on how resistance exercise affect markers of autophagy and HSP70, and if BCAA supplementation influences these systems.

Definitions

Autophagy: highly conserved intracellular catabolic pathway that recycles damaged organelles and proteins in order to protect the cell.

Branched chain amino acids: leucine, isoleucine, valine; essential amino acids.

Creatine kinase: indirect marker of muscle damage.

Delayed-onset muscle soreness: DOMS; feeling of muscular pain 24 -96 hours post novel or strenuous exercise.

Eccentric exercise: lengthening muscular contraction; has been demonstrated to induce significant amounts of muscle damage.

Glutathione: antioxidant capable of preventing or reducing damage caused by reactive oxygen species such as free radicals. The relationship between oxidized and reduced glutathione is often used as a measure of oxidative stress.

Heat shock protein: a family of proteins that are produced by cells in response to exposure to stressful conditions.

Jump squat: ballistic exercise requiring participants to squat down to approximately 100 degrees of knee flexion and then jump vertically as powerfully as possible.

Maximal voluntary isometric contraction: standardized method for measurement of force production.

Microtubule-associated protein 1A/1B light chain (LC3): a soluble protein with a molecular mass of approximately 17 kDa that is distributed ubiquitously in mammalian tissues (e.g. peripheral blood mononuclear cells). During autophagy, autophagosomes engulf cytoplasmic components, including cytosolic proteins and organelles. Concomitantly, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II).

mTOR: mammalian target of rapamycin; a serine/threonine protein kinase that regulates cell growth and proliferation, cell survival, protein synthesis, and autophagy.

Muscle damage: physical disruption of muscle structures involved in force transmission and production.

Peripheral blood mononuclear cells: includes lymphocytes, monocytes, and dendritic cells; extracted from whole blood for analysis of autophagy and heat shock activity

Sequestosome 1: the ubiquitin-binding protein p62, it is an autophagosome cargo protein that targets other proteins that bind to it for selective autophagy.

Vertical jump: the maximal height an athlete can achieve, in inches, completed as a double-leg, countermovement jump. The jump is measured was measured by a Vertec, which is designed specifically to measure vertical jump height.

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CHAPTER 2

This chapter presents a review article, entitled "Branched-Chain Amino Acid Supplementation and Recovery: An Evidence-Based Review of Both Endurance and Resistance-Based Research" which will be submitted for publication to the *International Journal of Sports Nutrition and Exercise Metabolism*. It is authored by Trisha VanDusseldorp, Kurt Escobar, Chad Kerksick, Roger Vaughan, Karol Dokladny, Len Kravitz, and Christine Mermier. The manuscript follows the formatting guidelines of the journal. References, tables, and figures are provided at the end of manuscript.

CHAPTER 2

Branched-Chain Amino Acid Supplementation and Recovery: An Evidence-Based Review of Both Endurance and Resistance-Based Research

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Running Title: BCAA and eccentric exercise recovery

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ABSTRACT

The understanding of the influence of branched-chain amino acids (BCAA) supplementation on athletic performance and recovery following strenuous training is of interest to active and athletic populations. Athletes and recreationally active individuals frequently consume BCAA supplements in order to facilitate recovery; however, the efficacy of BCAA supplementation on recovery from acute, muscle-damaging endurance and resistance training remains unclear. In this context, the aims of this evidence-based review are fourfold: 1) provide a comprehensive analysis of the literature that investigates the hypothesis that BCAA supplementation enhances recovery of physical performance following strenuous endurance and resistance exercise; 2) provide a comprehensive analysis of the literature that investigates the hypothesis that BCAA supplementation decreases muscle damage as evaluated by the extracellular release of indirect biochemical markers into systemic circulation; 3) provide a comprehensive analysis of the literature that investigates the hypothesis that BCAA supplementation mitigates perceived soreness following intense exercise, and based on the results of this review; 4) provide general recommendations for the use of BCAA supplements as a means to facilitate recovery. Research articles (January 1975 - June 2016; English only) were retrieved from PubMed, Google Scholar, SPORTDiscus, and Web of Science databases using keywords that included BCAA or leucine alone or in combination with exercise, performance, running, cycling, resistance training, weight training, skeletal muscle, muscle damage, recovery, and markers of muscle damage. Included studies evaluated the effects of BCAA supplements alone (i.e. those with no other ingredients) on markers of athletic performance and muscle damage following a strenuous bout of endurance- or resistance-based exercise. Nine articles met the criteria, of which six examined resistance-based exercise and three examined endurance-based exercise. Of the nine studies, seven articles examined the effectiveness of BCAA supplementation on recovery of muscular performance, six examined recovery of indirect biochemical markers of muscle damage, and eight assessed perceived soreness ratings. BCAA may attenuate perception of soreness and indirect makers of muscle damage during recovery from muscle-damaging exercise; however, this attenuation may not speed recovery of physical performance.

INTRODUCTION

Athletes, both recreational and competitive, have long sought out nutritional supplementation strategies for a number of reasons centering on physical performance including: 1) to enhance athletic performance, such as strength, power, and/or speed; 2) to speed recovery and ameliorate delayed-onset muscle soreness (DOMS); 3) to mitigate skeletal muscle degradation; 4) to strengthen immune function in an attempt to prevent illness; and 5) to prevent inflammation (Blendon, Benson, Botta, & Weldon, 2013). To assess whether a nutritional method might actually benefit exercise performance and recovery, researchers have devoted substantial amounts of time to the study of nutrient needs of physically active individuals, and the usefulness of ergogenic supplements in athletic performance and recovery (Naderi, Earnest, Lowery, Wilson, & Willems, 2016; Titchenal, 1988). Recently, researchers have dedicated a great deal of interest to branched-chain amino acid supplements (BCAA), in an attempt to understand how and if BCAA influence specific biochemical processes that may in turn decrease decrements in athletic performance, and speed recovery following a strenuous bout of exercise (Coombes & McNaughton, 2000; da Luz, Nicastro, Zanchi, Chaves, & Lancha, 2011; Howatson et al., 2012; Jackman, Witard, Jeukendrup, & Tipton, 2010).

In 1975, Buse et al. garnered initial interest in BCAA when their lab demonstrated that BCAA supplementation, leucine in particular, stimulated protein synthesis and deterred protein degradation *in vitro* (Buse, Jursinic, & Reid, 1975). A little over 4 decades later, researchers have consistently demonstrated the ability of BCAA, principally leucine, to exert a profound effect on protein synthesis (Churchward-Venne et al., 2012; Moberg et al., 2014; Proud, 2007a, 2007b). The extensive literature supporting

the anabolic effect of BCAA (Anthony, Anthony, Kimball, Vary, & Jefferson, 2000; Blomstrand, Eliasson, Karlsson, & Kohnke, 2006; Churchward-Venne et al., 2014; Crozier, Kimball, Emmert, Anthony, & Jefferson, 2005; Karlsson et al., 2004; Moberg et al., 2016) has led to the widespread use and further study of BCAA supplements for the regulation of skeletal muscle protein turnover (Blomstrand et al., 2006), as well as to enhance post-exercise recovery (Howatson et al., 2012), and decrease central fatigue (Blomstrand, 2006).

While the efficacy of BCAA on skeletal muscle turnover is well-established, as this has been repeatedly demonstrated using a variety of methodologies (Anthony et al., 2000; Appuhamy, Knoebel, Nayananjalie, Escobar, & Hanigan, 2012; Blomstrand et al., 2006; Churchward-Venne et al., 2012; Wolfe, 2000), the commercial attention capitalizing on these findings has led to liberal interpretations of these data and claims of efficacy that are not yet conclusive. This includes the advertisement of BCAA supplements as efficacious dietary supplements to mitigate muscle damage and enhance recovery time. To date, the effects of BCAA supplementation on markers of muscular recovery, such as exercise performance, indirect biochemical markers of muscle damage, and perceived ratings of soreness, are inconclusive, though not always advertised as such.

Previous works studying the effects of BCAA on recovery from exercise using indirect markers of muscle damage, such as creatine kinase (CK), and delayed onset muscle soreness ratings (DOMS) have produced conflicting results (da Luz et al., 2011; Foure et al., 2015; Greer, White, Arguello, & Haymes, 2011; Greer, Woodard, White, Arguello, & Haymes, 2007; Howatson et al., 2012; Jackman et al., 2010; Shimomura et al., 2010). The inconsistent findings regarding the practicality of BCAA as a means to

enhance recovery are likely due to the use of variable exercise models, types of supplements (e.g., mixed amino acids, carbohydrate with protein, isolated amino acids), lack of dietary control, duration of supplementation, training status of participants, as well as dosage and timing of ingestion (da Luz et al., 2011; Foure et al., 2015; Greer, White, Arguello, & Haymes, 2011; Greer, Woodard, White, Arguello, & Haymes, 2007; Howatson et al., 2012; Jackman et al., 2010; Shimomura et al., 2010). Nonetheless, the use of protein supplements and individual BCAA supplements deserves special attention, as these essential amino acids have been shown to play a critical role in the homeostatic maintenance of biological systems essential for athletic performance and recovery, including protein synthesis and degradation and immune function (Bohe, Low, Wolfe, & Rennie, 2003; Rennie, 2001; Wolfe, 2000).

Currently, a systematic review of the evidence to support or refute the consumption of BCAA supplements as a means to enhance recovery from endurance- or resistance-based exercise does not exist. We feel this review is necessary as the commercial presentation of BCAA supplements as a means to enhance recovery is part of a lucrative industry as well as a popular practice amongst active and athletic communities. Therefore, the aim of this review was to: 1) provide a comprehensive analysis of the literature investigating the hypothesis that BCAA supplementation enhances recovery of physical performance following strenuous endurance and resistance exercise; 2) provide a comprehensive analysis of the literature investigating the hypothesis that BCAA supplementation decreases the release of indirect markers of muscle damage; 3) provide a comprehensive analysis of the literature investigating the hypothesis that BCAA supplementation mitigates perceived soreness following intense

exercise, and based on the results of this review; 4) provide general recommendations for the use of BCAA supplements in the context of recovery from an acute, intense bout of exercise.

METHODOLOGY

A literature search was conducted utilizing a restricted date range from January 1975 to June 2016. Only English language, peer-reviewed articles were retrieved from PubMed (PM), Google Scholar (GS), SportsDiscus (SD), and Web of Sciences (WoS) using keywords that included branched-chain amino acids or leucine, alone or in combination with exercise, performance, running, cycling, weight training, resistance training, skeletal muscle, muscle damage, recovery, markers of muscle damage, oxidative stress, creatine kinase, myoglobin, and lactate dehydrogenase. Articles relevant to the review were further screened for the following inclusion criteria: 1) peer-reviewed publications; 2) healthy human participants (trained or untrained; 18-50 years of age); 3) studies including BCAA supplementation-only condition with no other supplementation; 4) provided the BCAA dosage; 5) involved an intense bout of endurance or resistance-based exercise (e.g. marathon, eccentric resistance exercise); 6) included muscular performance outcome measures (e.g. time-trial, vertical jump, maximal voluntary contraction); and 5) included direct or indirect markers of muscle damage (e.g. creatine kinase, myoglobin). In addition, articles from the reference lists of papers reviewed were examined when applicable using the same inclusion criteria. Articles examining the BCAA leucine alone on markers of recovery from a bout of endurance or resistance exercise were also considered due to leucine's purported effects on muscle protein

synthesis and recovery (Anthony et al., 2000; Blomstrand, 2006; Moberg et al., 2016; Xu, Tan, Zhang, Gui, & Yang, 2014). Studies that examined BCAA supplements in combination with other amino acids, vitamins, carbohydrate, or antioxidants were excluded due to the difficulty in isolating the effects attributable to the BCAA supplements themselves. All papers were examined in detail and searched for confounding experimental design issues that could explain discrepant outcomes. The full search strategy and the search terms used are portrayed in **Figure 1**.

RESULTS

Our search identified 33 articles **(Figure 1)** of which nine examined BCAA supplementation or leucine alone (8 BCAA; 1 leucine) and their ability to decrease muscle damage and soreness, and improve recovery of muscle function following endurance or resistance exercise in humans. A total of three endurance-based studies and six resistance-based studies were utilized. Studies reviewed used protocols for initiating muscle damage that ranged from eccentric squatting to prolonged, intense cycling. Performance outcome measures consisted primarily of force production assessment and jump height. Creatine kinase (CK) and myoglobin (Mg) were frequently used as indirect biochemical markers of muscle protein damage. Perceived soreness ratings were assessed using a variety of forms of the visual analog scale (VAS).

Insert Figure 1 about here

BCAA SUPPLEMENTATION AND RECOVERY

Following single or multiple bouts of high-intensity endurance or resistance exercise, the recovery of isometric (Foure et al., 2015) and dynamic (Howatson et al., 2012) muscle function parameters, such as maximal voluntary isometric contractions (Greer et al., 2011; Howatson et al., 2012), sprinting (Twist & Eston, 2005), and jumping (Areces et al., 2014), may take several days to return to baseline values, especially in instances where the exercise task is novel to the individual. The use of BCAA supplements has been hypothesized to decrease the amount of time needed for skeletal tissue remodeling and to decrease exercise-induced protein breakdown, potentially permitting faster recovery of physical performance markers (Blomstrand et al., 2006; Moberg et al., 2016). In order to examine these hypotheses, researchers have investigated whether BCAA or leucine supplementation alone improve subsequent muscle function and physical performance up to 96 hours (hr) after the initial bout of exercise.

Following unaccustomed resistance or endurance exercise, muscle soreness, often referred to as delayed onset muscle soreness (DOMS), develops gradually and lasts for several days. DOMS is a commonly-associated symptom of muscle damage, especially prevalent following activities involving eccentric muscle contractions (Paddon-Jones, Muthalib, & Jenkins, 2000). It has been suggested that BCAA supplements may decrease ratings of soreness following a damaging bout of exercise. In order to examine this hypothesis, researchers have investigated whether BCAA supplementation decreases ratings of perceived soreness up to 96 hr after the initial bout of exercise.

Furthermore, following novel eccentric exercise or high-intensity, prolonged endurance-type exercise, it is not uncommon for blood-borne markers of muscle damage,

such as CK and Mg, to become elevated from 10-fold to as much as 300-fold (Baird, Graham, Baker, & Bickerstaff, 2012; Clarkson, Byrnes, Gillisson, & Harper, 1987; Hyldahl & Hubal, 2014; Rawson, Conti, & Miles, 2007). The use of BCAA supplements may decrease skeletal muscle damage, potentially decreasing the extracellular release of indirect biochemical markers of muscle damage, as less damage may be induced due to BCAA's purported effects on protein turnover in skeletal muscle (Escobar, Frank, Suryawan, Nguyen, & Davis, 2007; Proud, 2007a; Tipton, Borsheim, Wolf, Sanford, & Wolfe, 2003). In order to examine this hypothesis, researchers have investigated whether BCAA supplementation decreases the response of indirect biochemical markers of muscle damage up to 96 hr after the initial bout of exercise.

The arrangement of the findings for this review is separated into protocols using endurance-based or resistance-based exercise. Studies examining recovery of performance markers are addressed first, followed by indirect biochemical markers of muscle damage, and lastly perceived soreness ratings.

Muscle Damaging Endurance Exercise and Markers of Physical Performance During Recovery

To date only two studies **(Table 1)** have examined BCAA supplementation on markers of physical performance during recovery following an intense bout of endurance-based exercise. Most recently, Areces et al. (Areces et al., 2014) studied the effectiveness of 7 days of 5 grams per day (g/d) of BCAA *(ratio: leucine 1.0: isoleucine 0.5: valine 0.5)* on recovery from a marathon in 46 amateur runners with a mean age of 41.4 years. Their experimental design included a randomized, double-blind, isocaloric and isovolumetric placebo controlled study, whereby the placebo consisted of a 1:1

cellulose:dextrose solution. Prior to and after the marathon, countermovement vertical jump max, leg maximal power output, leg muscle force, and leg muscle velocity were determined using a force platform, followed by the assessment of handgrip maximal force production in both the right and left hands. In comparison to the control group, supplementing with BCAA did not prevent post-marathon reductions in muscle power and force for both the legs and hands, as well as jump height and leg muscle velocity. These findings coincide with results from Greer et al. (Greer et al., 2007), who reported no differences 4 or 24 hr post-exercise for both leg-flexion and leg-extension torque following 90 minutes of cycling at 55% of VO_2 peak in 9 untrained men supplementing with 5 g of BCAA compared to an isocaloric CHO beverage or a non-caloric beverage. When compared with isocaloric carbohydrate and placebo trials, leg-flexion torque was higher 48 hr post-exercise in the BCAA supplement group.

Muscle Damaging Resistance-Based Exercise and Markers of Physical Performance During Recovery

Thus far, only three investigations **(Table 1)** have examined the effects of BCAA supplementation on recovery of markers of physical performance following a damaging bout of resistance exercise. Jackman et al. examined the role of BCAA supplementation during recovery from intense eccentric exercise in 24 non-resistance trained males. Participants were asked to complete an eccentric protocol consisting of 12 x 10 repetitions of supramaximal unilateral eccentric knee extension at 120% of concentric 1 repetition maximum (1RM). BCAA supplementation was administered 4 times per day as a 7.3 g bolus (*ratio: 3.5 leucine, 2.1 isoleucine, 1.7 valine g; 29.2 g/d total)* on the day of exercise and the following 2 days (i.e. 24, 48 hr). Maximal isometric strength of the

exercised leg was assessed 1, 8, 24, 48, and 72 hr post-exercise. A significant decrease in maximal isometric strength was observed in both the placebo and BCAA supplement groups following exercise, with no difference between groups. The degree of force loss was also unaffected by the BCAA supplementation at all measured time-points in comparison to the placebo group (Jackman et al., 2010). More recently, in an acute, double-blind placebo controlled study, Howatson et al. provided competitive national league rugby and football players 20 g of BCAA each day for 7 days before and 4 days after completing a damaging bout of exercise. Participants were asked to complete 100 consecutive drop jumps in order to induce muscle damage, followed by assessment of MVIC of the dominant knee extensors at 90 degrees of flexion and vertical jump 24, 48, 72, and 96 hr post-exercise. No significant difference at any time-point for any group was found for vertical jump performance, however, the decrement in force of MVIC was lower and subsequent recovery of force was greater in the BCAA supplement group. The MVIC force output was greater at all time points in the BCAA supplement group compared to controls following exercise (Howatson et al., 2012). Conversely, Foure et al. examined 26 college-aged men who consumed 7 total BCAA supplements consisting of 100 mg/kg in a *2:1:1 leucine, isoleucine, valine ratio*. Three doses were taken on the muscle-damaging day, while a single dose was consumed each day for the 4 days following. Maximal voluntary isometric contraction was unaffected by either exercise or supplementation (Foure et al., 2015).

To date, Kirby and colleagues (2012) are the only group to examine the effect of leucine supplementation alone on recovery of muscular function following an acute bout of damaging exercise (Kirby et al., 2012). Twenty-seven untrained, healthy college-aged

males were randomly assigned to one of three groups: leucine, placebo, or control. Both the leucine and placebo groups performed an eccentric exercise protocol consisting of 100 – 60 cm depth jumps followed by six sets of ten 3-second repetitions of eccentric-only leg press. The leucine group consumed a leucine supplement consisting of 250 mg/kg of body mass mixed with 3 g of non-caloric sweetener (Splenda®), while the PLCB group consumed non-caloric sweetener only. All subjects consumed their designated supplement 30 minutes prior to resistance exercise, immediately pre-exercise, immediately post-exercise, and immediately prior to the 24, 48, 72, and 96 hr time points. Recovery of muscular function was determined via maximal isometric squat and vertical jump analyses pre-exercise, as well as 24, 48, 72, and 96 hr post-exercise. Isometric squat peak force was measured as the highest force output achieved during the 3-sec isometric contraction. When expressed relative to pre-eccentric exercise values, the leucine group isometric peak force was significantly higher relative to placebo supplemented groups immediately post-exercise, 24, 48, 72, and 96 hr, however, no difference in jump height was found.

Insert Table 1 about here

Muscle Damaging Endurance Exercise and Indirect Biochemical Markers of Muscle Damage During Recovery

Damage to muscle tissue following intense exercise has been associated with a disrupted sarcolemma and damaged Z discs, permitting the efflux of skeletal muscle proteins and enzymes, including CK, lactate dehydrogenase (LDH), Mg, as well as

troponin, aldolase, and myosin heavy chain into the blood or urine (Baird et al., 2012; Kuipers, 1994). The blood concentrations of CK, LDH, and Mg are commonly used as indirect indicators of the extent of muscle damage that has occurred following intense exercise. Researchers have examined the effect of BCAA on these biochemical markers following strenuous endurance exercise intended to provoke muscle damage.

To date, only three studies **(Table 2)** have examined indirect markers of muscle damage following endurance exercise and BCAA supplementation. In addition to examining markers of performance, Areces et al. reported no differences in post-marathon urine Mg concentrations between placebo and BCAA groups (Areces et al., 2014). In their study of 9 untrained males, Greer et al. reported significant reductions in LDH 4 hr post-cycling, as well as significant reductions 4, 24, and 48 hr post-cycling in serum concentrations of CK (Greer et al., 2007). These findings coincide with the earlier work of Coombes and McNaughton, who indicated that supplementary BCAA significantly decreased serum concentrations of the intramuscular enzymes LDH and CK. In their investigation, Coombes and McNaughton reported that following 120 minutes of cycling at 70% VO_{2max}, LDH was significantly reduced from 2 hr post-exercise to 5 days post-exercise in the BCAA supplement cohort, as compared to the control group. A similar response was found for CK concentrations, as the BCAA group exhibited a significant reduction 4 hr to 5 days post-cycling exercise (Coombes & McNaughton, 2000).

Muscle Damaging Resistance-Based Exercise and Indirect Markers of Muscle Damage During Recovery

In both of their investigations, Howatson et al. and Foure et al. utilized CK as the primary index of muscle damage after muscle damaging exercise in their trained cohort of individuals (Foure et al., 2015; Howatson et al., 2012). Howatson et al. reported that plasma CK levels were significantly increased 24 hr following exercise, demonstrating that their drop jump protocol successfully induced muscle damage. Branched-chain amino acid supplementation resulted in significantly lower CK concentrations compared to the placebo. Alternatively, Foure et al. observed no reductions in CK in the BCAA supplemented group. Shimomura et al. assessed both CK and Mg in young untrained women supplemented with a 5.5 g BCAA mixture (100 mg/kg) 15 minutes prior to performing 7 sets of 20 repetitions of squats. A significant increase in serum Mg concentration 48 hr post-exercise was observed in the placebo trial, but no changes occurred for the BCAA group. A detectable increase was noted in CK for both groups; however, the change was only significant at 48 hr post in the placebo group, indicating that the intensity of the squat-exercise task used was low, and not significantly damaging (Shimomura et al., 2010). Similarly, the eccentric-based resistance exercise protocol used by Kirby et al. resulted in minimal changes in serum muscle damage markers (Kirby et al., 2012). Both the BCAA and placebo groups demonstrated significant increases in Mg and CK at 24 hr compared to baseline concentrations. However, there were no significant differences between groups for both CK and Mg responses (Kirby et al., 2012) **(Table 2)**.

Insert Table 2 about here

Muscle Damaging Endurance-Based or Resistance-Based Exercise and Perceived Soreness Rating Recovery

Two of the aforementioned endurance-based (Areces et al., 2014; Greer et al., 2007) and five of the resistance-based studies (Foure et al., 2015; Howatson et al., 2012; Jackman et al., 2010; Kirby et al., 2012; Shimomura et al., 2010) examined the effect of BCAA supplementation on perception of soreness following exercise **(Table 3)**. Significant reductions in soreness ranged from 24 to 72 hr post-exercise, in comparison to placebo groups (Greer et al., 2007; Howatson et al., 2012; Jackman et al., 2010; Shimomura et al., 2010). In their study of untrained males supplementing with BCAA, Greer and coworkers reported significant reductions in perceived soreness 24 hr post-cycling (90 min @ 55% VO_2peak) in comparison to both placebo and carbohydrate supplement groups (Greer et al., 2007). Both Shimomura et al. (Shimomura et al., 2010) and Howatson et al. (Howatson et al., 2012) indicated significant reductions in soreness both 24 and 48 hr post-exercise (body weight squats and drop jumps, respectively) when compared to placebo groups. In the investigation by Jackman et al. (Jackman et al., 2010), participants rated general muscle soreness, as well as soreness with the knee in flexed and extended positions. Participants supplementing with BCAA indicated significant lower knee flexed soreness 72 hr post-unilateral eccentric supramaximal knee extension; however, no differences between BCAA and placebo groups were found at any time point for knee extension perceived soreness ratings. Conversely, the work of both Areces et al. (Areces et al., 2014) in trained, amateur marathon runners and Foure et al. (Foure et al., 2015) in recreationally trained males, who were primarily endurance trained, observed no differences in muscle soreness between BCAA and control

conditions. Results from the leucine supplementation study by Kirby and colleagues demonstrated unexpected results, as the leucine supplementing group experienced significantly increased soreness compared to the placebo group at all post-exercise time points (Kirby et al., 2012).

Insert Table 3 about here

DISCUSSION

Recovery of Muscle Function, Indirect Biochemical Markers of Muscle Damage, and Soreness

Following our review, the potential of BCAA supplements to enhance subsequent measures of muscular function and mitigate indices of muscle damage in response to strenuous endurance or resistance exercise remains equivocal. While the physiological evidence for BCAA, leucine in particular, to enhance skeletal muscle anabolism is ample (Escobar et al., 2007; Proud, 2007a; Tipton et al., 2003), the hypothesis that protein synthesis augmentation with BCAA reduces indices of muscle damage and facilitates recovery of muscular performance are inconsistent in both trained and untrained human trials.

To date, only six investigations have examined the potential for BCAA supplements to enhance subsequent measures of muscular function in response to strenuous endurance or resistance exercise. All six studies included various force production assessments, often consisting of maximal voluntary isometric contraction (MVIC) of the quadriceps, while three included assessment of jump height ranging from

immediate post-exercise up to 96 hr post-exercise. Consistent findings from studies evaluating recovery of exercise performance variables following an acute, muscle damaging bout of exercise demonstrate that BCAA supplementation does not necessarily confer an enhancement of performance recovery (Areces et al., 2014; Foure et al., 2015; Greer et al., 2007; Jackman et al., 2010). More specifically, the positive effects on the maintenance of isometric muscle force production following muscle damaging eccentric resistance exercise and strenuous endurance exercise were only documented in three of the six studies (Greer et al., 2007; Howatson et al., 2012; Kirby et al., 2012). The applicability of these findings, however, may be minor as athletic movement rarely involves isometric activities. The capability to maintain force output during these type of contractions may not translate into performance of more complex movements, such as sprinting or jumping. This was demonstrated by the results of the reviewed literature, as no studies (Areces et al., 2014; Howatson et al., 2012; Kirby et al., 2012) showed a difference between placebo and BCAA groups when assessing dynamic muscle function (i.e. vertical jump height). Moreover, the training status of the participants used in these studies varied and included unaccustomed exercise protocols in some cases (Areces et al., 2014; Howatson et al., 2012; Kirby et al., 2012). This is common practice by researchers investigating recovery, as utilizing untrained individuals for muscle damage studies ensures substantial muscle injury. Three (Greer et al., 2007; Jackman et al., 2010; Kirby et al., 2012) of the six studies examining BCAA supplements utilized untrained males, with two (Greer et al., 2007; Kirby et al., 2012) demonstrating attenuated decreases in peak force production following supplementation. Of the three studies studying trained participants (Areces et al., 2014; Foure et al., 2015; Howatson et al., 2012), only

Howatson and colleagues (Howatson et al., 2012) reported that decrements in force production during MVIC of the knee extensors were lower and recovery of force was greater in response to BCAA supplementation (Howatson et al., 2012). It is important to note the timing and dose of BCAA varied markedly between studies, ranging 5 to 20 g/d for multiple days prior to and following exercise (i.e. 7 days prior to 4 days post) or exclusively the day of exercise. Interestingly, results of the two resistance-based studies by Kirby et al. and Howatson et al. both reported significant recovery of force in participants supplementing with high dosages of leucine. On the day of exercise, Kirby et al. asked subjects to consume two 250 mg/kg of leucine supplements (~20 grams of leucine each), as well as 1 dose immediately post-exercise, totaling 60 g on the day of exercise. Similarly, Howatson et al. asked subjects to consume a total of 60 g of BCAA on the day of exercise, totaling 30 g of leucine. The effect of high dosages of leucine on the day of exercise may contribute to the enhanced recovery in trained individuals and should be further examined.

Research investigating nutritional supplements as a means for recovery of muscle function often includes indirect markers of muscle injury and subjective measures of soreness. Interestingly, individuals supplementing with BCAA demonstrated attenuated increases or significantly lower values of indirect, blood-borne muscle damage indices and perception of soreness at multiple post-exercise time points. Both Greer et al. (Greer et al., 2007) and Coombes et al. (Coombes & McNaughton, 2000) reported significant reductions in LDH and CK post damaging endurance-type exercise, ranging 2 hr post-exercise to 5 days after exercise. Similar results were found in national league rugby and football players following 100 consecutive drop jumps (Howatson et al., 2012). In the

only investigation of women supplementing with BCAA alone to date, Shimomura et al. (Shimomura et al., 2010) reported no significant increase in CK and Mg concentrations. While an increase in CK was reported, peak serum concentrations of CK did not exceed 120 IU/L, demonstrating that the squat exercise protocol meant to induce muscle damage was not very strenuous for the untrained women. However, men supplementing with 250 mg/kg of leucine (~20 g) per day demonstrated no attenuation in plasma CK and Mg levels when compared to placebo (Kirby et al., 2012), albeit with a different protocol for muscle damage. In addition to the differences in indirect biochemical markers of muscle damage, Shimomura et al. (Shimomura et al., 2010), Howatson et al. (Howatson et al., 2012), and Greer et al .(Greer et al., 2007) all reported significantly lower perceived soreness ratings in subjects supplementing with BCAA 24 and 48 hr post-exercise, while Jackman et al. (Jackman et al., 2010) reported significant reductions at 72 hr post in knee flexed soreness. Overall, investigations including an acute bout of damaging endurance or resistance exercise demonstrate attenuated increases in indirect makers of muscle damage as well as incidences of decreased perception of soreness in subjects consuming approximately 5-20 g of BCAA per day. However, it is important to note, that the relationship between recovery of muscle function and these indirect markers of muscle damage (e.g. CK, perceived soreness) is poor. Similar findings have been reported in both acute and chronic studies investigating individuals ingesting other protein supplements (Cermak, Res, de Groot, Saris, & van Loon, 2012; Pasiakos, Lieberman, & McLellan, 2014).

Further, it may be suggested that the ergogenic effects associated with BCAA supplements and other protein supplements on physical performance following an acute

exercise bout may only be evident if participants are in negative nitrogen balance without the use of supplements (Tarnopolsky et al., 1992). Previous reports suggest individuals consuming a diet adequate in protein (1.2 -2.4 g/kg/d) promotes similar recovery rates as individuals using protein supplements while consuming adequate dietary protein (Pasiakos et al., 2014; Pasiakos et al., 2011; Pasiakos, McLellan, & Lieberman, 2015; Sousa, Teixeira, & Soares, 2014). Only Jackman et al. (Jackman et al., 2010) included strict dietary control and analysis within their study design; therefore, discrepancies in markers of recovery may be attributed to the adequacy of participants' dietary protein intake which may be determined by the intake of exogenous amino acid supplementation, such as BCAA, or lack thereof. With no dietary control or records, it is not imprudent to conclude the positive effects elicited from BCAA supplementation stems from the inherent properties of the amino acids found within these supplements rather than simply facilitating a positive protein balance through an adequacy of overall protein intake. Additional work with greater dietary control is needed to elucidate whether BCAA supplementation does, indeed, possess an ergogenic advantage with respect to recovery, or if BCAA supplementation is beneficial only in its ability to rectify protein inadequacy and its practice when consuming a diet adequate in protein is superfluous. This work would seem paramount given that such nuances and distinctions are typically not readily available to consumers, and individuals consuming a diet sufficient in protein may be interested in BCAA supplementation despite a lack of evidence as to its' efficacy.

General Study Limitations

While several of the investigations in this review implemented a double-blind, placebo-controlled experimental design (Areces et al., 2014; Foure et al., 2015;

Howatson et al., 2012; Kirby et al., 2012), the majority of the studies reviewed in this analysis used a between-group rather than a repeated-measure experimental design (Areces et al., 2014; Coombes & McNaughton, 2000; Howatson et al., 2012; Jackman et al., 2010; Kirby et al., 2012). This is common practice, especially in studies involving novel eccentric exercise, due to the purported repeated bout phenomenon, which suggests that repeating similar eccentric exercise results in less damage (e.g. lower CK efflux, decreased perception of soreness) (Clarkson et al., 1987; Ebbeling & Clarkson, 1989; Howatson, Van Someren, & Hortobagyi, 2007). While between-groups comparisons are often used in the convenience of time, future investigations should consider using a repeated-measure design by separating participant testing sessions by a minimum of 10 weeks to ameliorate the repeated bout effect (Clarkson et al., 1987). Additionally, controlling participant dietary intake is imperative for constructing meaningful assessments of nutritional supplements. The majority of the reviewed studies asked participants to maintain their regular diet, with no reports of macronutrient intake. Lastly, the muscle damaging protocols selected by the reviewed investigations varied considerably, from neuromuscular electrical stimulation to unilateral eccentric knee exercise at 120% 1RM, making comparisons difficult.

CONCLUSIONS

This analysis has reviewed the current evidence for the support of BCAA supplementation before and/or after strenuous, muscle-damaging endurance and resistance-based exercise to enhance recovery. Based on our interpretation of the

current peer-reviewed research, we conclude the following regarding BCAA supplementation and recovery:

- Both trained and untrained individuals acutely supplementing with 5-20 g/d of BCAA or 250 mg/kg of leucine from 7 days prior to exercise up to immediately prior to exercise, may exhibit increased recovery of isometric force production following muscle-damaging endurance or resistance exercise. However, evidence suggests that the recovery of isometric force does not translate to recovery of common athletic movements.
- Both trained and untrained individuals supplementing with 5-20 g/d of BCAA may experience decreased perception of soreness and mitigate increases in indirect biochemical markers of muscle damage following an acute bout of strenuous and resistance exercise.
- Additional research with BCAA supplementation is needed in individuals undergoing strict dietary control, specifically protein intake, in order to determine if BCAA supplements indeed enhance recovery rather than facilitating the adequacy of overall protein intake. We suggest examining BCAA supplementation and recovery in individuals consuming a diet consisting of 0.8 -1.0 g/kg/day of protein. Research is also needed to examine BCAA supplementation and recovery in individuals consuming a plant-based diet, as plant-based proteins are low in essential amino acids or are missing certain essential amino acids.
- More research is needed examining BCAA supplements and markers of recovery in women, perhaps using trained women who would most likely use BCAA supplements, as only one study has been completed in untrained women.

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Table 1. A summary of endurance and resistance-based investigations examining recovery of exercise performance.

Author and Participants	Dietary Control	Study Design	Exercise Mode	Supplementation	Timing	Force Production	Jump Height
Endurance Exercise: Markers of Muscle Function Recovery							
Greer et al. (2007) **UT males (n=9)**	Participants were encouraged to maintain the same dietary patterns for the 3 d before each trial	Acute; randomized single-blind, placebo controlled crossover	90 min of cycling at 55% VO_2peak	5 g/d 2.5 g x 2 doses 0.48: 1.22: 0.73 g Ile: Leu: Val	BCAA beverage consumed 5 min prior to exercise and at the 60-min mark	MVC KF: Sig. higher 48 hr post compared to PLCB/ CHO; No diff MCV KE	---
Areces et al. (2014) **T amateur marathon runners (n =46 M; 7 F)**	Maintain regular diet; no differences between PLCB and BCAA group in dietary intake	Acute; double-blind, placebo controlled, randomized	Marathon	5 g/d 0.5: 1.0: 0.5 Ile: Leu: Val	7 d prior	No sig diff between groups IP	No sig diff between groups IP
Resistance Exercise: Markers of Muscle Function Recovery							
Jackman et al. (2010) **UT males (n=24)**	Participants energy requirements were determined via 3-d diet record; Food was prepared and given to participants to ensure similar diets: 55% CHO, 1.5 g PRO/kg BM; remaining FAT	Acute; single-blind, placebo controlled	12 x 10 rep of unilateral eccentric KE @ 120% 1RM	7.3 g BCAA; 2.1: 3.5: 1.7 g Ile: Leu: Val	30 min before exercise, 1.5 hr post-exercise, between lunch and dinner, and before bed On 24 and 48 hr post-exercise, consumed between meals	No difference between groups	---
Howatson et al. (2012) **T competitive national league rugby and football players (n=12)**	No additional nutritional supplements; Maintain regular diet; Replicate diet for PLCB and BCAA	Acute; double-blind, placebo controlled	100 consecutive drop-jumps	20 g/d 1:2:1 Ile: Leu: Val Exercise trial: additional 20 g 1 hr pre- and immediately post-exercise	7 d loading phase 11 d total	MVC KE: decrement in force was lower and recovery of force was greatest in BCAA group	No group or interaction effects
Foure et al. 2016 **Rec trained males (n=26)**	Maintain regular diet; No high protein foods for breakfast on the d of exercise	Acute; double-blind, placebo controlled	NMES	100 mg/kg 1:2:1 Ile:Leu:Val:	7 total doses; 2 pre-exercise, 1 IP, 24, 48, 72, 96 hr post	No differences between groups	---
Kirby et al. (2012) **UT, healthy college-aged males**	Maintain regular diet;; no differences between PLCB and BCAA group in dietary intake	Acute; double-blind, placebo controlled	5 x 20 max drop jumps (60 cm) and 6 x 10 bilateral 3 s leg press @ 120% 1RM	250 mg/kg Leu	7 total doses; 2 pre-exercise, 1 IP, 24, 48, 72, 96 hr post	Leu attenuated drop in mean peak force across all post-exercise time points compared to PLCB	A significant decrease in the overall marginal mean for Leu from pre-exercise values; no interaction, no group effect

*d =day, min=minute, g=gram, MVC=maximal voluntary contraction, KF=knee flexion, sig= significant, PLCB=placebo, CHO=carbohydrate, KE=knee extension, M=male, F=female, T=trained, UT=untrained, BCAA= branched chain amino acid, IP= immediate post, mg=milligram, kg=kilogram, s=sec, NMES=neuromuscular electrical stimulation, Rec= recreationally, PRO= protein, 1RM = one repetition maximum, BM= body mass, Leu=leucine, Ile= isoleucine, Val=valine

Table 2. A summary of endurance and resistance-based investigations examining recovery of indirect biochemical markers of muscle damage.

Authors & Participants	Dietary Control	Study Design	Exercise Mode	Supplementation	Timing	LDH	CK	Mb
Endurance Exercise: Indirect Biochemical Markers of Muscle Damage								
Coombes et al (2000) **Males (n=16)**	Normal diet; All subjects were averaging a protein intake in excess of 1.6 g/d	Acute; PLCB controlled	120 min cycle @ 70% VO_2max	12 g/d 33.3% Ile, Leu, Val; add. 20 g was given before and after exercise test	Twice a d, with breakfast and dinner	Sig ∨ in BCAA group 2, 3, 4 h post-ex. As well as 1, 3, and 5 d post	Sig ∨ in BCAA group 4 hr post-ex. As well as 1, 3, and 5 d post	---
Greer et al. (2007) **UT males (n=9)**	Participants were encouraged to maintain the same dietary patterns for the 3 d before each trial	Acute; randomized single-blind, placebo controlled crossover	90 min of cycling at 55% VO_2peak	5 g/d 2.5 g x 2 doses 0.48: 1.22: 0.73 g Ile: Leu: Val	BCAA beverage consumed 5 min prior to exercise and at the 60-min mark	Sig ∨ in BCAA group 4 h post-ex	Sig ∨ in BCAA group 4, 24, 48 h post-ex.	---
Areces et al. (2014) **T amateur marathon runners (n =46 M; 7 F)**	Maintain regular diet; no differences between PLCB and BCAA group in dietary intake	Acute; double-blind, placebo controlled, randomized	Marathon	5 g/d 0.5: 1.0: 0.5 Ile: Leu: Val	7 d prior	---	---	No sig diff btwn groups
Resistance Exercise: Indirect Biochemical Markers of Muscle Damage								
Shimomura et al. (2010) **UT sedentary women (n=12)**	Maintain regular diet	Acute cross-over	BW squats 7 x 20 (total 140)	5.5 g BCAA mixture Ile:Leu:Val: 1:2:3:2:1 Participants consumed 100 mg BCAA/kg BW	Supp: prior to exercise	---	Sig. ∧ in placebo group 48 hr; No change in BCAA group	Sig. ∧ in placebo group only at 48 hrs post; No change in BCAA group
Howatson et al. (2012) **T competitive national league rugby and football players (n=12)**	No additional nutritional supplements; Maintain regular diet; Replicate diet for PLCB and BCAA	Acute; double-blind, placebo controlled	100 consecutive drop-jumps	20 g/d 1:2:1 Ile: Leu: Val Exercise trial: additional 20 g 1 hr pre- and immediately post-exercise	7 d loading phase 11 d total	---	Sig ∨ in BCAA group	---
Kirby et al. (2012) **UT, healthy college-aged males**	Maintain regular diet;; no differences between PLCB and BCAA group in dietary intake	Acute; double-blind, placebo controlled	5 x 20 max drop jumps (60 cm) and 6 x 10 bilateral 3 s leg press @ 120% 1RM	250 mg/kg Leu	7 total doses; 2 pre-exercise, 1 IP, 24, 48, 72, 96 hr post		No sig diff between groups	No sig diff between groups

*d =day, min=minute, g=gram, MVC=maximal voluntary contraction, KF=knee flexion, sig= significant, PLCB=placebo, CHO=carbohydrate, KE=knee extension, M=male, F=female, T=trained, UT=untrained, BCAA= branched chain amino acid, IP= immediate post, mg=milligram, kg=kilogram, s=sec, PRO= protein, 1RM = one repetition maximum, BM= body mass, Leu=leucine, Ile= isoleucine, Val=valine, Sig= significant, ^ = increased, ∨ = decreased

Table 3. A summary of endurance and resistance-based investigations examining recovery of perceived muscle soreness.

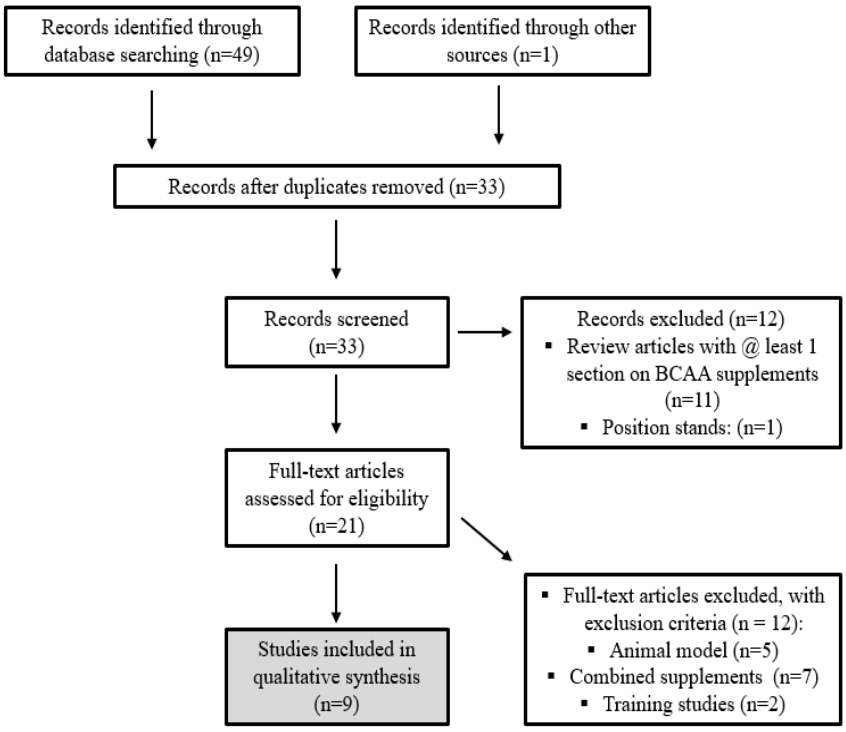
Authors & Participants	Dietary Control	Study Design	Exercise Mode	Supplementation	Timing	Perceived Muscle Soreness (VAS)
Endurance Exercise: Perceived Soreness						
Greer et al. (2007) **UT males (n=9)**	Participants were encouraged to maintain the same dietary patterns for the 3 d before each trial	Acute; Randomized single-blind, placebo controlled crossover	90 min of cycling at 55% VO_2peak	1 serving = 2.5 g consisting of 1.22 g Leu, 480 mg Ile, and 730 mg Val Total consumption over 2 time points = 200 kcal	BCAA beverage consumed 5 min prior to exercise and at the 60-min mark	Sig. reduced 24 hrs post in BCAA compared to PLAC /CHO
Areces et al. (2014) **T amateur marathon runners (n = 46; 7 F)**	Maintain regular diet; no differences between PLCB and BCAA group in dietary intake	Acute; double-blind, placebo controlled, randomized design; Supp 7 d	Marathon	5 g/d Ile:Leu:Val 0.5:1.0:0.5	7 d	No sig diff between groups
Resistance Exercise: Perceived Soreness						
Howatson et al. (2012) **T competitive national league rugby and football players (n=12)**	No additional nutritional supplements; Maintain regular diet; Replicate diet for PLCB and BCAA	Acute; double-blind, placebo controlled	100 consecutive drop-jumps	20 g/d 1:2:1 Ile: Leu: Val Exercise trial: additional 20 g 1 hr pre- and immediately post-exercise	7 d loading phase 11 d total	Sig reduced 24 and 48 hours post in BCAA compared to placebo
Jackman et al. (2010) **UT males (n=24)**	Participants energy requirements were determined via 3-d diet record; Food was prepared and given to participants to ensure similar diets: 55% CHO, 1.5 g PRO/kg BM; remaining FAT	Acute; single-blind, placebo controlled	12 x 10 rep of unilateral eccentric KE @ 120% 1RM	7.3 g BCAA; 2.1: 3.5: 1.7 g Ile: Leu: Val	30 min before exercise, 1.5 hr post-exercise, between lunch and dinner, and before bed On 24 and 48 hr post-exercise, consumed between meals	Sig reduced in BCAA group 72 h post in KF; no diff between groups for KE at any time point
Foure et al. 2016 **Rec trained males (n=26)**	Maintain regular diet; No high protein foods for breakfast on the d of exercise	Acute; double-blind, placebo controlled	NMES	100 mg/kg 1:2:1 Ile:Leu:Val:	7 total doses; 2 pre-exercise, 1 IP, 24, 48, 72, 96 hr post	No sig diff between groups
Kirby et al. (2012) **UT, healthy college-aged males**	Maintain regular diet,; no differences between PLCB and BCAA group in dietary intake	Acute; double-blind, placebo controlled	5 x 20 max drop jumps (60 cm) and 6 x 10 bilateral 3 s leg press @ 120% 1RM	250 mg/kg Leu	7 total doses; 2 pre-exercise, 1 IP, 24, 48, 72, 96 hr post	No sig diff between groups
Shimomura et al. (2010) **UT sedentary women (n=12)**	Maintain regular diet	Acute cross-over	BW squats 7 x 20 (total 140)	5.5 g BCAA mixture Ile:Leu:Val: 1:2:3:2:1 Participants consumed 100 mg BCAA/kg BW	Supp: prior to exercise	Sig lower than PLCB 24 and 48 hr post-exercise

*d =day, min=minute, g=gram, MVC=maximal voluntary contraction, KF=knee flexion, sig= significant, PLCB=placebo, CHO=carbohydrate, KE=knee extension, M=male, F=female, T=trained, UT=untrained, BCAA= branched chain amino acid, IP= immediate post, mg=milligram, kg=kilogram, s=sec, NMES=neuromuscular electrical stimulation, Rec= recreationally, PRO= protein, 1RM = one repetition maximum, BM= body mass, Leu=leucine, Ile= isoleucine, Val=valine

FIGURE LEGENDS

Figure 1. Flow diagram of study selection and articles included in this review.

Figure 1



CHAPTER 3

This chapter presents a research manuscript, entitled "Effect of Branched-Chain Amino Acid Supplementation on Markers of Muscle Damage and Recovery Following Acute Eccentric Resistance Exercise". This manuscript is authored by Trisha VanDusseldorp, Kurt Escobar, Kelly Johnson, James McCormick, Terence Moriarity, Matthew Stratton, Nathan Cole, Chad Kerksick, Roger Vaughan, Karol Dokladny, Len Kravitz, and Christine Mermier. The manuscript follows the formatting and style guidelines of the journal *Medicine and Science in Sports & Exercise*. References are provided at the end of the chapter. Figures are provided within the chapter.

Title: Effect of Branched-Chain Amino Acid Supplementation on Markers of Muscle Damage and Recovery Following Acute Eccentric Resistance Exercise

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Running Title: BCAA and eccentric exercise recovery

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Abstract

Purpose: The purpose of this study was to investigate the effect of branched-chain amino acid (BCAA) supplementation (0.22 g/kg body weight) on recovery from eccentric exercise. **Methods:** Twenty resistance-trained males were randomly assigned to a supplement (n=10) or placebo (PLCB) (n=10) group. Subjects consumed either BCAA or PLCB for eight days in a double-blind manner, with a four-day loading period prior to a muscle damaging exercise bout. During the eight-day protocol, subjects adhered to a diet consisting of 1.2 g/kg/d protein as recommended by a registered dietician. On day five, the damaging exercise protocol was performed which consisted of 10 sets of 8 repetitions of 4-second eccentric squats at 70% one repetition maximum (1RM). Immediately following, subjects completed 5 sets of 20 split squat jumps (10 each leg). Creatine kinase (CK), glutathione (GSSG/tGSH), vertical jump (VJ), maximal voluntary isometric contraction of the quadriceps (MVIC), jump squat (JS; 40% 1RM), and perceived muscle soreness were measured as indirect markers of muscle damage. Variables were measured immediately before the exercise protocol, as well as at 1, 2, 4, 24, 48, and 72 hours (hr) post-exercise. **Results:** Plasma CK concentrations were significantly elevated above baseline ($p<0.001$) in both BCAA and PLCB groups at 4, 24, 48, and 72 hr post-exercise. While no significant group-by-time effect was detected for plasma CK ($p=0.10$), plasma CK levels were significantly lower for the BCAA group at 48 hr post-exercise ($p=0.02$; BCAA: 799.2±197.6; PLCB: 1422.9±630.8 IU/L). Erythrocyte oxidized glutathione to total glutathione ratio (GSSG/tGSH) was significantly elevated ($p<0.05$) 1 hr (BCAA: 0.42±0.06; PLCB: 0.36±0.04), 2 hr (BCAA: 0.67±0.24; PLCB: 0.71±0.21), and 4 hr (BCAA: 0.36±0.21; PLCB: 0.42±0.12) in both BCAA and PLCB groups compared to pre-exercise; however, no significant group-by-time effect was detected at any time point. Perceived muscle soreness increased from baseline ($p<0.01$) in both groups at 1, 2, 4, 24, 48, and 72 hr, however the BCAA group reported significantly less soreness ($p<0.01$) at 48 hr (BCAA: 4.59±1.42; PLCB: 7.14±1.65 cm) and 72 hr post-exercise (BCAA: 1.38±1.83; PLCB: 3.90±1.52 cm). MVIC was significantly higher for the BCAA group at 24 hr post-exercise ($p=0.04$; BCAA: 270.8±68.8; PLCB: 210.9±38.5 Nm), but no significant group-by-time effect was observed ($p=0.18$). No significant difference between groups ($p>0.05$) was detected for VJ or JS. **Conclusions:** BCAA

supplementation (0.22 g/kg body weight) may mitigate muscle soreness following muscle damaging eccentric exercise. However, when consumed with a diet adequate in daily protein (1.2 g/kg/d), the attenuation of muscular performance decrements or corresponding plasma CK levels are modest.

Key Words: protein supplements, unaccustomed exercise, perceived soreness

INTRODUCTION

Skeletal muscle damage induced by resistance-based exercise is known to promote microdamage in muscle fibers which may lead to temporary increased passive tension, delayed onset muscle soreness (DOMS), decrements in strength and force production and increased efflux of intramuscular proteins into the blood (11, 26). The degree of damage and discomfort may be compounded over time and persist chronically, especially in individuals frequently engaging in vigorous exercise or those completing an overreaching phase (11, 26). As such, nutritional strategies have been proposed to mitigate the negative effects that may be experienced following strenuous resistance exercise. Amino acid supplementation, including branched-chain amino acids (BCAA), have been considered a potentially efficacious dietary intervention (34, 42, 45). Some evidence suggests BCAA supplementation may decrease skeletal muscle damage in response to intense resistance exercise (24, 46, 49) and promote subsequent recovery of muscle function (24, 28), however these findings remain inconclusive at present (2, 15, 28). Despite this lack of empirical consensus, BCAA supplementation is a popular practice among recreational exercisers and athletes (8, 33, 58). Branched-chain amino acids (leucine, isoleucine, and valine) are distinct among essential amino acids in that they are extrahepatically metabolized in skeletal muscle (22, 53). It has been suggested that intake of BCAA may reduce protein degradation and/or muscle enzyme release (8, 11, 34), though some of the underlying mechanisms remain unclear (11, 52). Moreover, if such benefits exist, it is unknown if a diet adequate in daily protein yields maximal benefits, making exogenous BCAA consumption superfluous (given sufficient intake of amino acids through the normal diet).

Recent investigations aimed at mitigating resistance exercise-induced damage and facilitation of recovery of muscle function using amino acid supplementation has produced consistent results (15, 28, 42, 45, 46, 57). It has been reported that 10 grams (g) of BCAAs twice per day for 12 days results in significantly attenuated plasma creatine kinase (CK) and enhanced recovery of maximal voluntary isometric contraction (MVIC) of knee extensors at 24 hours (hr) post-exercise. This dosage has also been associated with significantly lowered DOMS at 24 and 48 hr post-exercise when compared to a placebo group in 12 trained males following 100 drop-jumps (24). However, there were no significant differences between groups for vertical jump (24). And of great importance, daily protein intake was not controlled in this study, perhaps leading to discrepancies in overall amino acid intake. Similarly, three weeks of 6 g of BCAA daily yielded significantly lower serum CK levels 12 and 36 hr following 2 days of intense resistance exercise in 8 recreationally active males compared to placebo (49). It is worth noting, however, individuals consuming a daily protein intake above the recommended daily allowance (0.8 g/kg/d) were excluded from the study. Therefore, it is likely that BCAA supplementation aided in reaching a protein intake more appropriate for resistance exercisers, while the placebo group had insufficient amino acid intake. Jackman and colleagues (28) reported that compared to placebo treatment, 29.2 g of BCAA per day resulted in decreased DOMS at 48 and 72 hr in 24 non-resistance trained males after unilateral eccentric exercise. However, no differences in percent change for electrically stimulated maximal isometric force of the quadriceps, plasma CK, myoglobin, and interleukin-6 response between groups post-exercise were observed. Daily protein intake was controlled at 1.5 g/kg/d for subjects in both BCAA and placebo groups. Further,

Foure and coworkers (15) found that muscle soreness and MVC was not affected by 0.1 g/kg of BCAA ingested pre- and post-damaging neuromuscular electrostimulation exercise. Interestingly, the investigators observed that BCAA supplementation resulted in significantly greater plasma CK concentrations 3 and 4 days post-exercise compared to placebo in 26 recreationally active men. However, there were no differences immediately post exercise (IPE) and 1 and 2 days post-exercise. The BCAA group consumed a significantly greater quantity of daily protein during supplementation days, with the BCAA group ingesting an amount consistent with resistance training recommendations (1.5 g/kg/d), whereas the placebo groups' protein intake was below general recommendations (1.07 g/kg/d).

While some supportive data exist, the inconsistencies documented in numerous measures of muscular insult following damaging resistance exercise and subsequent recovery prevent any conclusive inferences regarding the efficacy of BCAA supplementation. Curiously, BCAA supplementation has been associated with reduced perceived soreness following intense resistance exercise (28, 42, 50), though a mechanism explaining the relationship between BCAA ingestion and perception of muscle soreness is not well established. Moreover, current evidence suggests the attenuation of DOMS (28, 42, 54), as well as efflux of biochemical markers of muscle damage in individuals supplementing with BCAA (24, 42, 49), does not necessarily occur with a concomitant enhancement of muscle function recovery (28, 42, 45). Adding to this complexity are the discrepancies in training state of study participants, damaging exercise protocols, and overall protein intake employed in the limited number of studies completed. The population most apt to supplement with BCAA to attenuate the negative

effects of intense resistance exercise are resistance trained individuals who are likely ingesting a moderate to high daily protein intake (1.2 – 2.4 g/kg/d) (39). The present study aimed to investigate the effects of BCAA supplementation on markers of muscle damage and recovery of muscle function in resistance trained males while adhering to a protein intake consistent with the recommended range for resistance training individuals.

METHODS

Participants:

Twenty young, resistance-trained (RT) males (age 22.3±1.5 yr, height 175.4±6.9 cm, and body mass 86.4±15.6 kg) were recruited for the study. The present study was approved by the institution's Human Research Review Committee. Participants were made aware of all procedures, including the risks and benefits, gave written consent and completed health history and physical activity questionnaires. All participants had several years (5.3±2.5 yr) of resistance exercise training, with an average self-reported training time of 7.3±2.1 hr per week. Participants were excluded if they were consuming creatine (within the past 6 months) and certain medications (non-steroidal anti-inflammatory or steroidal drugs). Individuals consuming protein supplements (e.g. whey, casein) were asked to refrain from taking these supplements one week prior to baseline assessment until completion of the study. Individuals utilizing treatments such cryotherapy or massage, past or current smokers, those without at least 1 yr of previous resistance training experience, and participants who had completed high-intensity eccentric squats in the last 4 months (repeated bout phenomenon) (9) were also excluded from the study. All participants refrained from unaccustomed or intense exercise 48 hr prior to testing, and were asked to refrain from all exercise and alcohol consumption 24 hr prior to testing

and throughout the entire testing period. Participant characteristics are shown in **Table 1**. Using methods previously described by Faul et al. (14) for estimating sample size for repeated measures designs, a minimum sample size of n = 9 was required for each group to reach a statistical power (1-b) of 0.80 based on the previous works of Jackman et al. (2010) and Howatson et al. (2012) (24, 28).

Table 1. Participant characteristics.

Characteristic	BCAA	PLCB
Subject #/group	10	10
Age (yr)	23.0 ± 1.2	21.5 ± 1.5
Height (cm)	177.6 ± 7.1	173.2 ± 6.2
Body Mass (kg)	86.6 ± 15.2	86.2 ± 16.8
Body Fat%	12.3 ± 3.8	11.7 ± 4.3
1RM Squat (kg)	154.8 ± 31.7	155.0 ± 32.0
RT Experience (yr)	5.6 ± 2.3	5.0 ± 1.9
Hours per week of RT	7.00 ± 2.30	7.65 ± 2.05

All values are mean ±SD. yr = years, cm = centimeters, kg = kilograms, 1RM = one repetition maximum, RT = resistance training, BCAA= branched-chain amino acid, PLCB= placebo

Experimental Design

Using a randomized, double-blind, placebo-controlled research design, participants were enrolled into either a branched-chain amino acid supplemented (BCAA) (MusclePharm BCAA 3:1:2 watermelon powder) or placebo (PLCB) group (maltodextrin) and performed one experimental muscle-damaging exercise trial **(Figure 1)**. Groups were matched for both body mass (+/- 5 kg; p=0.95) and one repetition maximum (1RM; +/- 5 kg; p=0.80). Following consent and initial screening (general health, supplementation history, and exercise history) participants were asked to complete preliminary testing consisting of a 1RM squat and familiarization of all performance measures (vertical jump, maximal voluntary isometric contraction, and 40% 1RM jump squat). Also on the day of initial screening, a registered dietician provided each participant with specific verbal and written directions and procedures for reporting a

detailed dietary intake, including information on how to record portions using household measures, preparation technique, and nutrient content descriptors (e.g. reduced-fat, light). During the two days prior to preliminary testing and throughout the entire experimental period, participants recorded their food intake and were instructed by a dietician how to follow a protein intake of 1.2 g/kg/d of body mass. Participants were asked to return in 48 hr for baseline testing of all performance measures and supplement distribution and counseling. All participants supplemented with either BCAA or PLCB (0.22 g/kg/d of body mass) for a total of 8 days, including a 5-day supplementation run-in phase prior to the muscle damaging exercise trial. An exercise protocol consisting of 4:1 second eccentric-concentric squats at each participant's 70% 1RM was completed on day five of supplementation in order to assess blood markers of muscle damage, recovery of exercise-performance variables, and perceived muscle soreness (DOMS) response immediately post-squat exercise (IPE), as well as at 1, 2, 4, 24, 48, and 72 hr post-exercise.

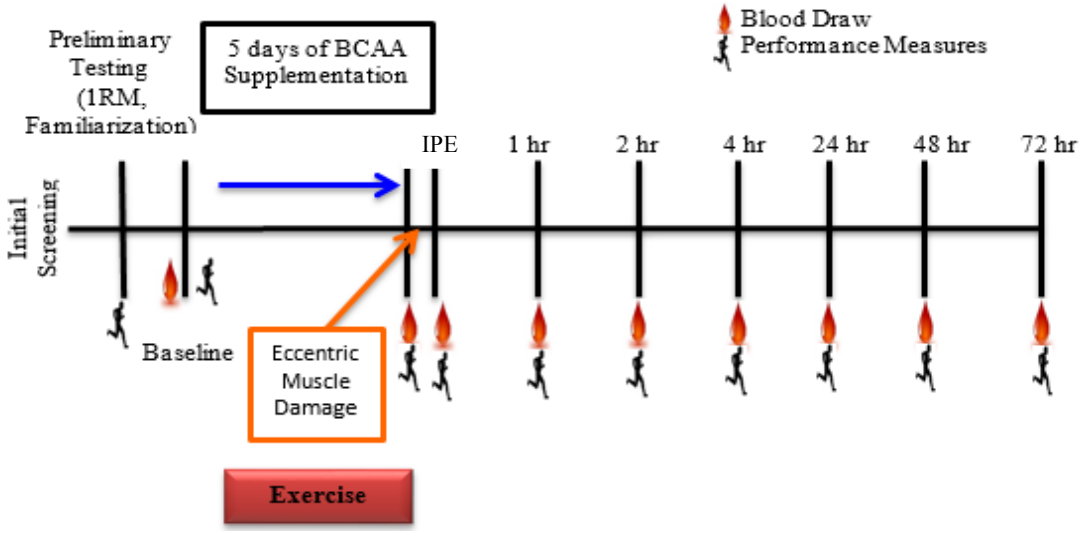

Figure 1. Study Overview. Performance measures consisted of vertical jump, maximal voluntary isometric contraction of the dominant leg quadriceps, and 40% 1RM jump squat. Blood was drawn for the collection of plasma (creatine kinase) and red blood cell lysate (reduced and oxidized glutathione). Supplements were consumed for a total of 8 days. The eccentric muscle damage protocol consisted of 10 sets of 8 repetition 4- second eccentric squat at each participant's 70% 1RM. *BCAA = branched-chain amino acids, 1RM = one repetition maximum, hr= hour, IPE= immediate post-exercise*

Preliminary Testing (1RM Testing) and Familiarization:

All participants were thoroughly familiarized with the study design; specifically, the diet and physical activity log requirements, blood draw timing and procedures, 1RM protocols, performance measures (vertical jump [VJ], maximal voluntary isometric contraction [MVIC] of the quadriceps, jump squat [JS]), and supplementation procedures. On the day of preliminary testing, participants first had their height and body mass measured, and body composition assessed using the skinfold technique. Participants were then asked to complete a self-selected 10 minute warm-up followed by 1RM assessment (procedure below). Each participant's 1RM was assessed in order to determine 40% and 70%1RM loads for the jump squat performance measure and squat-exercise load, respectively. Following 1RM assessment, participants were asked to rest for 10 minutes and then completed a thorough familiarization of the countermovement VJ, MVIC of the quadriceps muscle at 120 degrees of knee flexion, and 40%1RM jump squat performance measures in order to eliminate any learning effects on test performance during data collection. Test-retest reliability was determined for all performance variables during the familiarization sessions and was deemed acceptable if they were greater or equal to R=0.90. Such reliability has been shown to be essential for experimental sensitivity to nutritional interventions (18). Following the completion of preliminary testing and 1RM assessment, participants were asked to return 48 hr later for baseline assessment.

1 Repetition Maximum

The 1RM back squat testing was performed according to methods previously described (32). Following a 10 minute standardized, dynamic warm-up, each participant first performed a warm-up set of 8–10 repetitions at a light-weight (~50% of their

estimated (est) 1RM). A second warm-up consisted of a set of 3–5 repetitions with a moderate weight (~75% of $1RM_{est}$), and third warm-up included 1–3 repetitions with a heavy weight (~90% of $1RM_{est}$). After the warm-up, each participant's 1RM was tested by increasing the load during consecutive trials until the participants were unable to perform a proper lift using correct technique (90 degrees of knee flexion). The 1RM test was determined by 4-6 sets of one repetition, with 3- to 5 minutes of rest between attempts. Spotters were present to provide verbal encouragement and spotting to ensure safety of the subjects.

Baseline Assessment - Pre-Supplementation

Participants were asked to return to the laboratory 48 hr after initial screening, abstaining from alcohol and exercise, as well as caffeine (e.g. pre-workout supplements, coffee) 12 hr prior to baseline testing. On the day of baseline testing, a trained phlebotomist drew blood for the collection of plasma and red blood cell lysate for the baseline assessment of creatine kinase (CK) and glutathione (GSSG/tGSH), respectively. Participants then completed a 10 minute self-selected warm-up (same warm-up as initial testing), followed by baseline measurements of all performance variables (VJ, MVIC, 40% 1RM jump squat).

Anthropometric Measurements

Measurements of height, body mass, and percent body fat (BF%) by 3-site skinfolds (29, 55) were obtained to characterize the participants. Body mass was measured via a Tanita electronic scale (Model #3101, Arlington Heights, Illinois) to the nearest 0.1 kg. Two skinfold measurements on the right side of the body were obtained from three sites (chest, abdomen, and thigh) in serial fashion by the same investigator

utilizing Lange Calipers (Cambridge Scientific Industries, Cambridge, MD). Skinfold thickness was based on the average of the two trials. If the two skinfold measurements for a particular site varied by more than 0.5 millimeters (mm), the technician obtained a third measurement and the mean value of the two closest measurements was used. Body density was then calculated according to the Jackson and Pollock 3-site equation (29) and BF% was estimated according to Siri (55).

BCAA and Placebo Supplementation

Participants ingested 0.22 g/kg/d of BCAA (MusclePharm, Denver, CO; 7.16 g = 3 g leucine, 1 g isoleucine, 2 g valine) or maltodextrin (PLCB) in dry-powder form mixed with water (~175-350 ml) for a total of 8 days following baseline assessment. The supplements were separated into two doses per day, taken in the morning and evening. On the fifth day of supplementation, participants returned to the laboratory and completed the muscle-damaging, squat exercise protocol.

Experimental Protocol (Muscle Damaging Exercise Visit)

Upon arrival to the laboratory, a trained phlebotomist collected the first (pre-exercise) of five blood samples, followed by pre-exercise assessment of VJ, MVIC, 40% 1RM jump squat, and perceived muscle soreness (DOMS). Participants then completed the muscle-damaging squat exercise protocol. Following completion of the exercise protocol, participants had their blood drawn, rated their current state of soreness, and completed all measures of exercise-performance IPE, 1, 2, 4, 24, 48, and 72 hr post-squat exercise. The same trained researcher was used for the collection of each participant's performance measures.

Muscle Damaging Protocol

A standardized bout of 4 second eccentric damaging resistance exercise involving 10 sets of 8 repetitions at 70% 1RM squats using a Smith machine was completed by all participants. The squat protocol consisted of a 4 to 1 eccentric to concentric rhythm. A stopwatch was started following the last repetition of each set and subjects were given 3 minutes of rest between all squat sets. Following completion of the squat protocol, participants then completed 5 sets of 20 consecutive (10 each leg) body-weight split jump repetitions with 2 minutes of rest between each set.

Markers of Exercise Performance/Muscle Function

Vertical Jump (VJ): Maximum countermovement VJ was assessed using a Vertec device (Perform Better, West Warwick, RI), an upright stand with horizontal plastic vanes. Participants were instructed to stand with their feet shoulder-width apart and flat on the ground directly beneath the Vertec. Participants were then instructed to reach up as high as possible with a single arm in order to measure standing reach height, defined as the height of the highest Vertec vane a participant is able to reach. Participants were then asked to complete a countermovement jump using both feet, while reaching up to touch the highest reachable vane. Each participant's standing height was then subtracted from their highest vane touched. Participants were allowed three jumps, with the highest jump recorded and used for statistical analysis.

Maximal Voluntary Isometric Contraction (MVIC): Maximum voluntary isometric strength of quadriceps of the dominant limb was measured by a dynamometer (Biodex Medical Systems, System 4, Shirley, NY). Participants sat upright with the chair's backrest inclined to 85°, with their knee placed in 120° of flexion. The axis (i.e.

lateral epicondyle of the femur) of the knee was aligned with the rotational axis of the dynamometer. For the test, participants were asked to perform a MVIC of the quadriceps muscles for 5 seconds and then rest for 1 minute. Participants completed this cycle three times, and the peak torque value (newton-meters) was recorded.

Jump Squat: Jump squat peak power (PP) was determined by taking the better of two maximal effort JS at 40% of each participant's 1RM with 1 minute of rest. All JS were performed using a Smith squat rack (Pro-Elite Strength Systems, Salt Lake City, UT). The depth of the countermovement during the JS was self-selected as previously described (13, 23). During the JS, subjects were asked to hold a bar across their shoulders and keep constant downward pressure on the bar so that it would not move independently of the body. Power production was determined via a Tendo Power Analyzer (TENDO PSA 310; Irmo, SC).

Perceived Soreness: Participants were asked to evaluate their perceived level of muscle soreness using the Visual Analog Scale (VAS). Soreness was assessed along a 10 cm scale (0 cm = no soreness, 10 cm = extreme soreness) for each time point (pre-exercise, IPE, 1, 2, 4, 24, 48, and 72 hr post-squat exercise) by drawing a line perpendicular to the continuum line extending from 0 to 10 cm. Soreness was evaluated by measuring the distance of each mark from 0 and rounded up to the nearest one-tenth of a centimeter (11).

Blood Markers

Blood Sampling and Analysis: Venous blood was collected at all time-points: pre-supplementation, pre-squat exercise, IPE squat exercise, and 1, 2, 4, 24, 48, and 72 hr post-exercise for the collection of plasma and red blood cell lysate.

Creatine Kinase: Plasma concentration of creatine kinase (CK) was measured as an indirect marker of muscle damage pre-exercise, as well as 4, 24, 48, and 72 hr post-exercise. Plasma CK concentrations were determined in duplicate using an enzymatic assay pre-supplementation, pre-exercise, as well as 4, 24, 48, and 72 hr post-exercise using commercially available reagents (Pointe Scientific, Canton, MI) and a spectrophotometer (Beckman Coulter, DU-520, Fullerton, CA) at a wavelength of 340 nanometers (nm).

Glutathione: Glutathione was measured using erythrocytes obtained from the blood samples taken pre-exercise, IPE, and 1, 2, 4, 24 hr post-exercise with a commercial kit (Kit #703002, Cayman Chemicals, Inc., Ann Arbor, MI). The red blood cells were lysed in four times the volume ice cold ultrapure water, centrifuged at 10,000 x g for 15 minutes at 4˚C, and then the supernatant was deproteinated in an equal volume of 0.1 g $^{mL-1}$ of metaphosphoric acid (MPA). The MPA and sample was vortexed and allowed to stand at room temperature for 5 minutes. The sample was then spun at 2,000 g for 2 minutes, the supernatant was removed and stored at -20 ˚C for the analysis of total glutathione (tGSH) and oxidized glutathione (GSSG) according to manufacturer guidelines. All samples were assayed in duplicate. The absorbance values were measured at 405 nm (iMark™ Microplate Absorbance Reader, Bio-Rad, Hercules, CA). Under the standardized conditions of the assay, the dynamic range is 0.5–16 µM tGSH and 0.25–8 µM GSSG. Ratio of GSSG/tGSH was calculated and reported as an indicator of oxidative stress as previously described by Goldfarb et al. (1, 16, 17).

Statistical Analysis

Statistical tests were conducted in R (version: 3.2.2; R Foundation for Statistical Computing; Vienna, Austria), using the 'afex' package (version 0.16-1). Separate mixed-effects (within-between) factorial ANOVAs (group x time) were used to assess the main and interaction effects for each reported dependent variable. Post-hoc pairwise comparisons were then used to investigate group differences across individual time-points, with the Bonferroni adjustment applied to correct for multiple comparisons. ANOVA models were evaluated for compliance with underlying model assumptions. Assumptions of sphericity were tested using Mauchly's test of sphericity and violations were corrected using the Greenhouse-Geisser correction factor. Unpaired t-tests were used to determine differences in years of previous resistance training experience, BM, and 1RM squat for BCAA and PLCB groups. The threshold for statistical significance was set *a priori* at $p \leq 0.05$ for all analyses.

Results

Participant characteristics are presented in **Table 1**. The groups were well matched, as there were no significant differences in previous resistance training experience (p=0.55), 1RM back squat (p=0.80), and body mass (p=0.95). All subjects successfully completed the 80 eccentric squats and 100 body-weight split jumps (50 each leg). Four individuals (two from each group) decreased the weight by 10 lbs in order to complete the eccentric squat exercise protocol (n=2: set 6; n=1 set 8; n=1 set 9). All performance measures (VJ, JS, and MVIC), perception of soreness, and blood measures showed time effects (p<0.05) for both PLCB and BCAA groups indicating that the eccentric exercise protocol effectively induced muscle damage.

Muscular Performance: Vertical Jump

Both groups demonstrated similar (p>0.05) VJ height prior to the eccentric exercise protocol (pre-exercise) (BCAA= 27.05±1.14 in; PLCB= 27.4±2.31 in). Vertical jump height was significantly lower for both BCAA and PLCB groups IPE, 1, 2, 4, 24, and 48 hr post-eccentric exercise (p<0.05); however, there were no group or interaction effects for vertical jump performance (Figure 2).

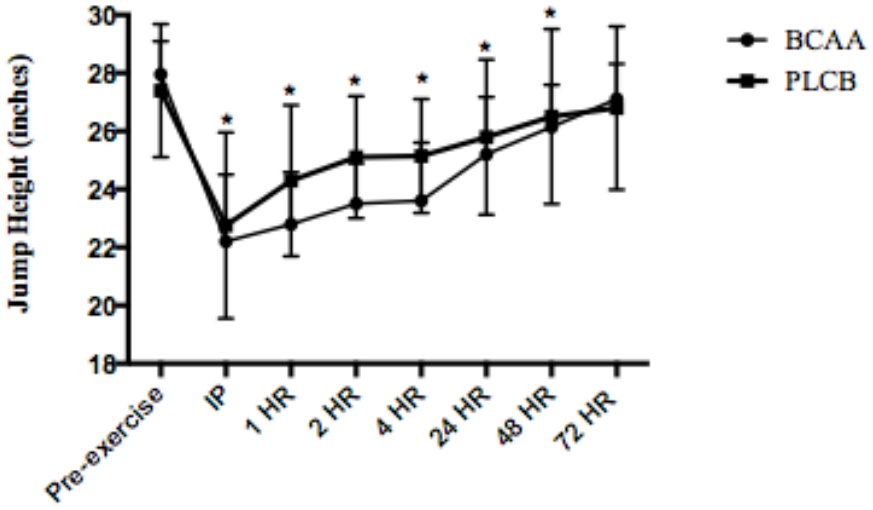

Figure 2. Mean (± standard deviation) jump height (inches) pre-eccentric exercise, immediate post (IP), 1, 2, 4, 24, 48, and 72 hours (hr) for resistance trained men supplementing with branched-chain amino acids or placebo (n = 20). * = significantly different from pre-exercise (p<0.05)

Muscular Performance: Maximal Voluntary Isometric Contraction

Both groups demonstrated similar (p>0.05) force output prior to the eccentric exercise protocol (pre-exercise) (BCAA= 305.30±89.70; PLCB= 279.70±59.90 Nm). Maximal voluntary isometric force output was significantly lower at all post-exercise time points for the PLCB group, while the BCAA group only displayed significantly

lower values IPE, 1, 2, and 4 hr post-eccentric exercise (p<0.05). Force output was not significantly different from baseline measures at 24 hr (p=0.18; BCAA: 270.8±68.8 Nm), 48 hr (p=0.11; BCAA: 271.4±45.8 Nm), or 72 hr (p=0.21; BCAA: 295.5±77.1 Nm), and no significant group-by-time effect was observed at any of the time points (p>0.05) (Figure 3).

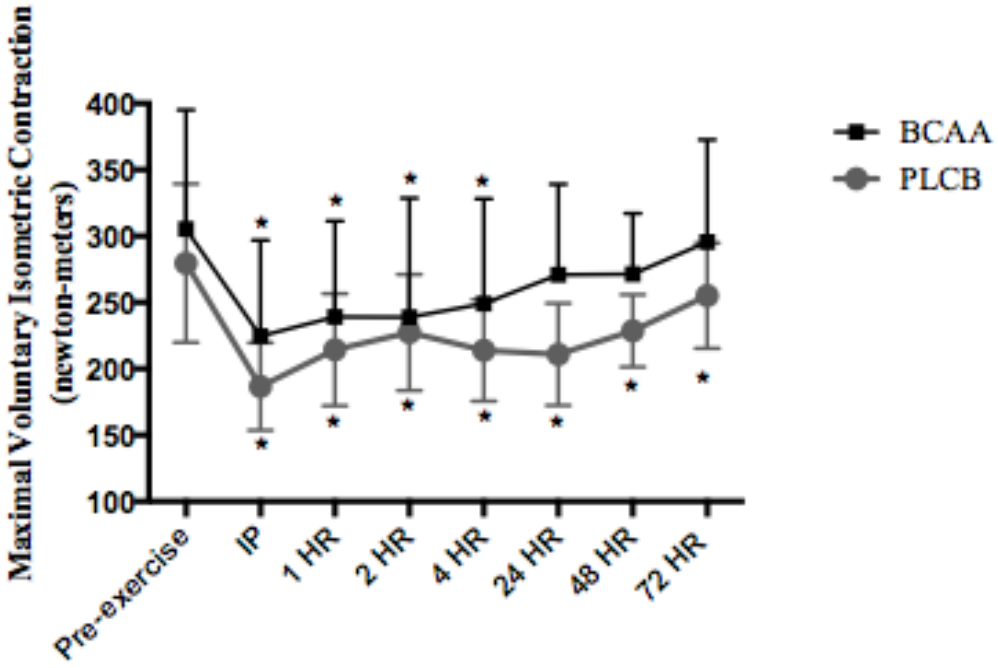

Figure 3. Mean (± standard deviation) force production (newton-meters) pre-eccentric exercise, immediate post (IP), 1, 2, 4, 24, 48, and 72 hours (hr) for resistance trained men supplementing with branched-chain amino acids or placebo (n = 20). * = significantly different from pre-exercise (p<0.05)

Muscular Performance: Jump Squat

Both groups demonstrated similar (p>0.05) peak power output as measured by the 40% 1RM JS prior to the eccentric exercise protocol (pre-exercise) (BCAA= 1392.90±344.10; PLCB= 1439.10±270.50 watts). Peak power output was significantly

lower for both BCAA and PLCB groups IPE, 1, 2, 4, 24, 48, and 72 hr following eccentric exercise (p<0.05); however, there were no group or interaction effects for JS performance (Figure 4).

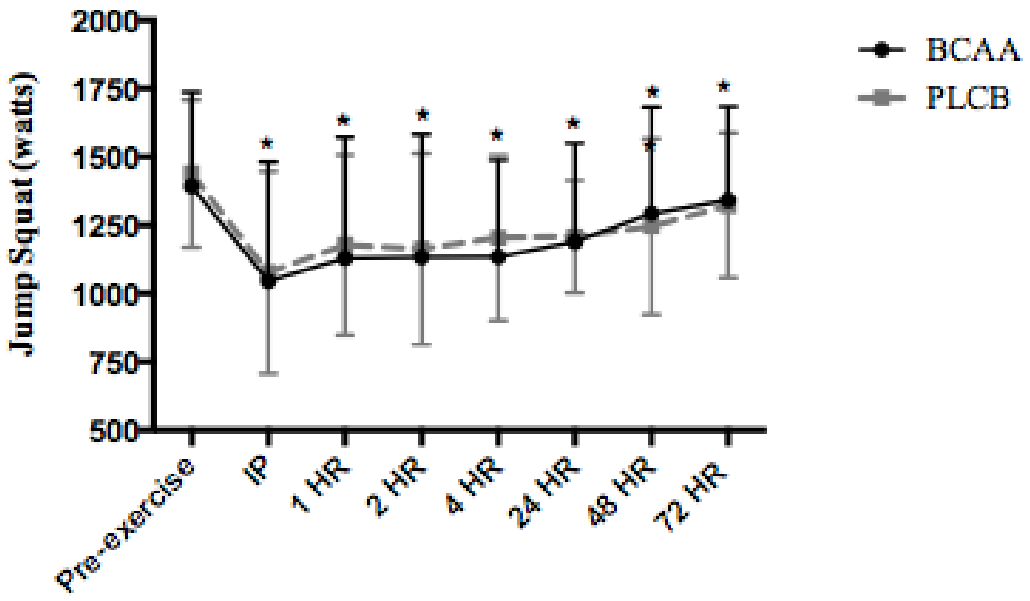

Figure 4. Mean (± standard deviation) peak power output (watts) pre-eccentric exercise, immediate post (IP), 1, 2, 4, 24, 48, and 72 hours (hr) for resistance trained men supplementing with branched-chain amino acids or placebo (n = 20). * = significantly different from pre-exercise (p<0.05)

Muscle Soreness

Both groups demonstrated similar pre-exercise perceived soreness ratings (BCAA= 0±0; PLCB= 0±0 cm). Perceived soreness was significantly elevated for both BCAA and PLCB groups IPE, 1, 2, 4, 24, 48, and 72 hr post-eccentric exercise (p<0.05), however the BCAA group reported significantly less soreness (p<0.01) at 48 hr (BCAA: 4.59±1.42; PLA: 7.14±1.65 cm) and 72 hr post-exercise (BCAA: 1.38±1.83 cm; PLA: 3.90±1.52 cm) (Figure 5).

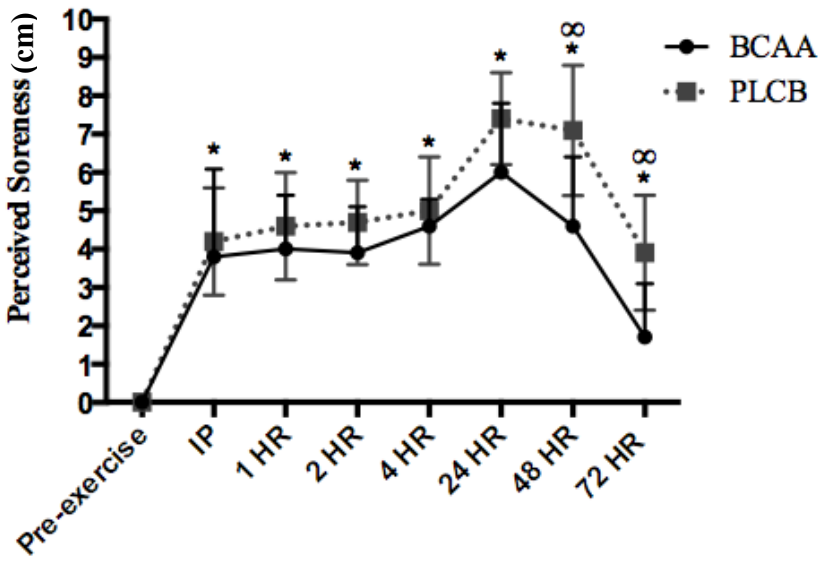

Figure 5. Mean (± standard deviation) perceived soreness rating pre-eccentric exercise, immediate post (IP), 1, 2, 4, 24, 48, and 72 hours (hr) for resistance trained men supplementing with branched-chain amino acids or placebo (n = 20). * = significantly different from pre-exercise (p<0.05); ∞ = significantly different from placebo group (p<0.05)

Blood Parameters: Creatine Kinase

Figure 6 displays the changes in plasma CK activity over the course of the experimental period. There were no significant differences between groups at pre-exercise (p>0.05; BCAA= 134.5±34.0; PLCB= 117.3±34.8 IU/L). Plasma CK concentrations were significantly elevated above baseline (p<0.001) in both BCAA and PLA groups at 4, 24, 48, and 72 hr post-exercise. While no significant group-by-time effect was detected for plasma CK (p=0.10), plasma CK levels were significantly lower for the BCAA group at 48 hr post-exercise (p=0.02; BCAA: 799.2±197.6; PLA: 1422.9±630.8 IU/L).

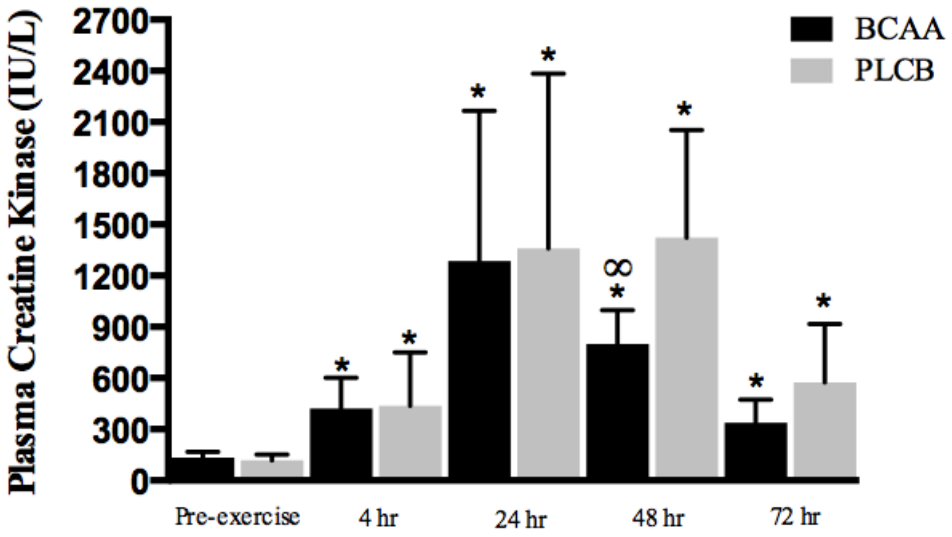

Figure 6. Mean (± standard deviation) plasma creatine kinase pre-eccentric exercise, 4, 24, 48, and 72 hours (hr) for resistance trained men supplementing with branched-chain amino acids (BCAA) or placebo (PLCB) (n = 20). * = significantly different from pre-exercise (p<0.001); ∞ = significantly different from placebo group (p=0.02)

Blood Parameters: Glutathione

Both groups demonstrated similar (p>0.05) glutathione redox ratio (GSSG/tGSH) prior to the eccentric exercise protocol (pre-exercise) (BCAA= 0.10±0.07; PLCB= 0.11±0.04). GSSG/tGSH was significantly higher for both BCAA and PLCB 1, 2, and 4 hr post-eccentric exercise (p<0.05); however, there were no group or interaction effects (Figure 7).

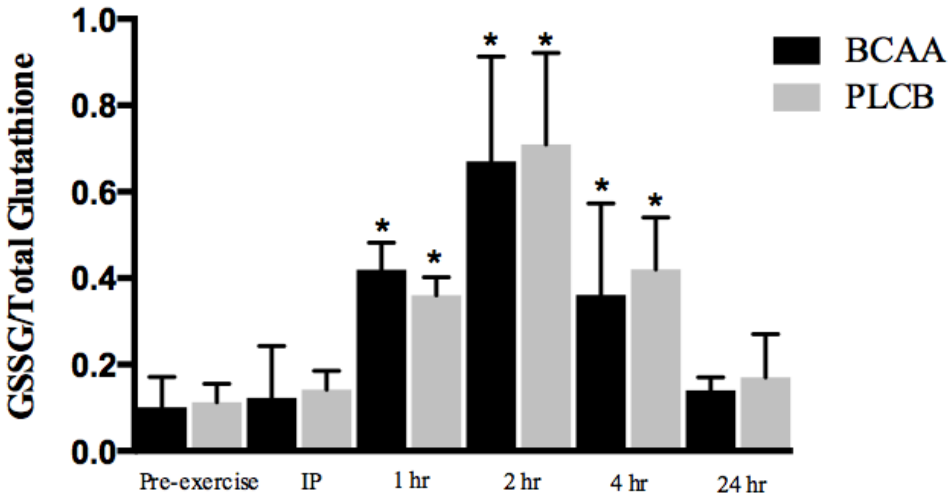

Figure 7. Mean (± standard deviation) glutathione redox ratio pre-eccentric exercise, immediately post (IP), 1, 2, 4, and 24 hours (hr) for resistance trained men supplementing with branched-chain amino acids (BCAA) or placebo (PLCB) (n = 20). * = significantly different from pre-exercise (p<0.05)

DISCUSSION

The aim of the present study was to examine the effect of BCAA supplementation on indices of muscle damage in resistance trained men consuming a strict protein diet plan. Our data demonstrate that BCAA supplementation (0.22 g/kg body weight) can reduce some of the negative effects of an acute bout of muscle-damaging eccentric resistance exercise by decreasing perceived muscle soreness, attenuating CK efflux, and enhancing recovery of muscle function. To date, this is the first study examining the potential of BCAA to mitigate damage and enhance recovery following acute squat eccentric exercise in resistance trained males undergoing strict dietary control. The ability of our eccentric squat exercise protocol to evoke skeletal muscle damage was indirectly evaluated by post-exercise changes in power production, isometric force loss, plasma CK concentrations, and participant's soreness ratings. It is evident from the significant time effects (4, 24, 41) and magnitude of response for each of these indices that muscle

damage was inflicted (27), allowing us to sufficiently study recovery up to 72 hours post-exercise. To our knowledge, this eccentric exercise protocol has never been used in previous research; however, our data support the efficacy of this protocol to induce muscle damage using a manner of training that may be used by resistance training individuals.

Proficient recovery procedures following strenuous, muscle-damaging exercise sessions are important for supporting training-induced adaptation and promoting quality subsequent exercise sessions. Amino acids have been shown to increase protein synthesis in the post-exercise period (3, 48) and their consumption by athletes and recreationally trained individuals is common practice as a means to enhance recovery. Research suggests greater protein synthetic rates and amino acid availability reduces damage to myofibrillar and cytoskeletal proteins, thereby helping to preserve force production abilities (39, 48).

The muscle force generating capabilities during the recovery period following eccentric exercise has been suggested to be one of the most reliable indices of muscle damage due to the relationship between muscle force and muscle function (27). Therefore, we chose to examine the effect of BCAA on recovery of muscle force production during an MVIC of the dominant leg quadriceps muscles, as well as to evaluate recovery of more ballistic movements. While we found no differences in vertical jump height or loaded jump squat peak power between groups, MVIC force recovery was significantly better at 24, 48, and 72 hours post for the BCAA group. These findings are similar to that of Howatson and colleagues (24) who reported a significantly lower decrement in MVIC force production, and recovery of force was greatest in trained males

supplementing with 20 g per day of BCAA; however, participants vertical jump height was unaffected. On the other hand, Foure et al. (15) report 7 g of BCAA per day had no effect on recovery of quadriceps MVIC in recreationally trained males following muscle damage. Likewise, in a study by Jackman et al. (28) no differences were detected in force producing capabilities in untrained males supplementing with 29.3 g of BCAA per day as measured by MVIC. Kirby and coworkers (31) examined the effect of 250 mg/kg of the BAA leucine on recovery of force production and vertical jump height. While no differences in jump height were detected, leucine attenuated mean peak force decrements across all post-exercise time points (up to 96 hours) in untrained males. Similar results from two cross-over investigations examining BCAA on recovery of muscle function in untrained individuals reported favorable outcomes of muscle function assessments (44, 51). However, these findings may be influenced by the repeated bout phenomena and should be considered a limitation (30, 37). While MVIC testing is a popular, valid and reliable measure of muscle function and recovery (38) it is important to note that the isometric movement associated with MVIC testing is distinctly different from several types of athletic performance movements. Though our data and others' work (20, 24) suggest enhanced recovery of force production in individuals consuming BCAA, no studies to date provide evidence that BCAA support recovery of more ballistic and functional movements (2, 24, 31).

While our data suggest a minor impact of BCAA on muscle function during recovery from muscle damaging exercise, the BCAA group reported significantly less soreness 48 and 72-hour post-exercise. This is in agreement with previous works (28, 56), suggesting the relationship between muscular function and soreness is not necessarily

inversely related. While no improvement in muscle function was detected, untrained men consuming a diet consisting of 1.5 g/kg/d of protein and 4 doses of 7.3 g BCAA per day reported significantly less soreness 72 hours post eccentric exercise (28). Similar findings by Howatson et al. (24) and Shimomura et al. (51) were reported 24 and 48-hours post resistance based muscle damaging exercise in individuals consuming 20 g per day BCAA and 100 mg/kg body mass, respectively. Further, there is evidence that mixed amino acid supplementation decreases perception of muscle soreness by 30% when ingested during recovery from muscle-damaging exercise (42). The mechanism(s) by which BCAA supplementation decreases muscle soreness cannot be deduced by our experimental study design, however, it has been suggested that enhanced glutamine production from BCAA degradation may be in-part responsible for these observations (7). Intense eccentric exercise results in significant increase in makers of inflammation (12). Previous research suggests the increases in inflammation heightens the sensitivity of muscle nociceptors (35), correlating with increased feelings of soreness (12, 35). Upon consumption, transamination of some BCAA to glutamate in order to synthesize glutamine may occur. Glutamine is consumed by inflammatory cells under inflammatory conditions (40). Nicastro and colleagues suggest that BCAA decrease the inflammatory status of damaged muscle by increased availability of amino acids as substrates for immune cells, glutamine in particular (40), however further research is needed to confirm this hypothesis.

We examined the effect of BCAA supplementation on one surrogate marker of muscle damage, CK, and one indirect index of oxidative stress, glutathione, during the recovery period. Efflux of CK into the blood is indicative of sarcolemma disruption (27).

Several studies suggest an effect of amino acid supplementation on CK efflux following muscle damaging exercise (8, 20, 24, 51). Plasma CK following our eccentric squat protocol was significantly elevated from pre-exercise levels in both groups; however, resistance trained men supplementing with BCAA demonstrated significantly lower values 48-hours post-exercise compared to the placebo group. Though non-significant, our data show the BCAA group's plasma CK levels were lower at all post-exercise time points when compared to those of the placebo. Numerous studies suggest amino acid supplements are effective at reducing CK efflux caused by damaging endurance or resistance exercise (8, 19, 24, 51). Following an acute bout of muscle damaging exercise, Howatson et al. (25) described significant reductions in plasma CK concentrations in well-trained, competitive rugby and national football players supplementing with 20 g of BCAA. Similarly, 0.4 g/kg of amino acids attenuated increase in plasma CK after 1 week of an overreaching program (47). A report from Shimomura investigating untrained women completing body weight squats and consuming 100 g BCAA/kg demonstrated lower CK values compared to that of a placebo group (51). Coombes and McNaughton (8) showed that increases in serum CK concentrations following 120 min cycling at 70% of each participant's maximal oxygen uptake were significantly lower for the males consuming 12 g per day of BCAA. Data from Ohtani and colleagues (43) demonstrated that 6.6 g/d of a mixed amino acid supplement attenuated increases in serum CK activity during recovery from strenuous long-distance running. However, following marathon performance, 5 g/d of BCAA did not attenuate changes in myoglobin, another indirect biochemical marker of muscle injury (e.g. sarcolemma disruption) (2). In the only study to date examining leucine supplementation alone on indirect measures of muscle damage,

250 mg/d was ineffective at attenuating increases in CK and myoglobin in untrained males who completed an acute bout of muscle damaging resistive exercise (31). While data exists to suggest BCAA supplements reduce the efflux of CK following a damaging or strenuous bout of exercise, the mechanism by which BCAA assist in repairing/preserving the muscle sarcolemma membrane has yet to be elucidated. Results from studies demonstrate that muscle cells have an efficient sarcolemma repair system to mediate response to local damage (27, 36). Small tears within the sarcolemma are typically sealed within seconds in healthy muscle. The protein dysferlin is a chief mediator of membrane resealing in muscle (36). Future research studies should be developed to examine the relationship between amino acid availability and sarcolemma remodeling.

Glutathione is a sensitive marker of oxidative stress (16). Reduced glutathione (GSH) is considered to be one of the most important scavengers of reactive oxygen species (ROS), and its ratio with oxidized glutathione (GSSG) is a commonly used marker of oxidative stress (5, 16). Glutathione plays a pivotal role in antioxidant defense, protecting the body from oxidative damage that may occur following strenuous exercise (21). Glutathione is known for proficiently scavenging reactive oxygen species in order to prevent increases in oxidative stress and damage (6). When oxidative stress levels are high, the reduced GSH is oxidized to form glutathione disulfide (GSSG) and accumulation of GSSG occurs (6). Following eccentric exercise, participants from both the experimental and placebo groups demonstrated significant increases in erythrocyte GSSG:GSH ratio 1, 2, and 4 -hours post-exercise, indicating significant oxidative stress in both groups and no effect of BCAA. This may be a result of the selected participants

training history, as chronic resistance training has been shown to increase basal concentrations of GSH, allowing trained individuals to more effectively defend against oxidative damage. Previous work by D'Antona et al. demonstrated a relationship between BCAA supplementation and reduced reactive oxygen species in cardiac and skeletal muscle of mice, though the exact mechanism by which this occurs is unknown (10). Future research should examine both markers of oxidative stress and antioxidants post-exercise in individuals supplementing with BCAA in order to determine this relationship.

The results of this investigation demonstrate that supplementing a controlled diet of 1.2 g/kg of protein with 0.22 g/kg body mass of BCAA results in decreased perceptions of soreness in recreationally trained individuals. However, BCAA supplementation in this fashion provides a minimal protective effect on attenuating other indirect makers of muscle damage following eccentric-based resistance exercise. While BCAA may aid in the maintenance of isometric muscle function following muscle damage, this ergogenic effect may be trivial as there was no effect on dynamic measures of muscle function. Since the majority of recreational individuals and athletes will most likely engage in subsequent exercise sessions that consist of dynamic movements rather than isometric contractions, the ability of BCAA to maintain force output during isometric contractions lacks applicability. Therefore, when consumed with a diet adequate in daily protein (1.2 g/kg/d) it appears BCAA effects on indirect measures of muscle recovery are reduced.

Although we feel this study has substantial external validity, results should be interpreted with caution as we were unable to use a cross-over study design due to the repeated bout effect associated with eccentric exercise. Also, while we included strict

dietary instructions, we were unable to prepare and administer meals to participants. Future research studies should be done to examine the effect of BCAA supplements on recovery from exercise in individuals consuming a daily protein intake lower than the recommended range for resistance training individuals.

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CHAPTER 4

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

This research team was the first to examine the impact of branched-chain amino acid (BCAA) supplements on recovery from an acute bout of eccentric resistance exercise in recreationally trained males undergoing strict dietary guidelines. This study team is also the first to investigate eccentric exercise and BCAA supplementation on the autophagic and heat shock response. Specifically we investigated: 1) if branched-chain amino acid supplementation (BCAA) enhances recovery from acute, eccentric resistance exercise as measured by indirect markers of muscle damage, including muscular performance measures, blood-borne makers of skeletal muscle injury, and delayed onset muscle soreness, and 2) the impact of acute, eccentric resistance exercise on markers of autophagy (microtubule-associated protein light chain 3 [LC3] and Sequestosome 1 [p62]) and heat shock protein 70 (HSP70) during recovery, and whether this response is different in resistance trained males supplementing with BCAA versus placebo. The review manuscript found in Chapter 2 entitled "Branched-Chain Amino Acid Supplementation and Recovery: An Evidence-Based Review of Both Endurance and Resistance-Based Research" encompasses all of the peer-reviewed literature to date (9 studies) examining BCAA and recovery from strenuous exercise. This paper demonstrates that, though BCAA play a major role in protein anabolism, their effectiveness on recovery from strenuous activity remains unconfirmed.

The research manuscript entitled, "Effect of Branched-Chain Amino Acid Supplementation on Markers of Muscle Damage and Recovery Following Acute Eccentric Resistance Exercise" found in Chapter 3 provides evidence that BCAA supplementation may mitigate soreness and efflux of creatine kinase (CK), though their ability to attenuate decrements in muscular performance are modest.

The data found in Chapter 4 is a part of an ongoing study in our laboratory. Thus far, it appears muscle-damaging resistance exercise significantly upregulates autophagy in PBMCs. Specifically, we observed decreases in p62/β-actin and LC3II/β-actin up to 4 hours (hr) post-exercise suggesting autophagosome degradation. This may be attributed to increased oxidative stress activity, as measured by the increased glutathione concentrations in the same timeframe. We also demonstrate that HSP70 is significantly increased above baseline 48-72 hr post-muscle damaging exercise. These time-course observations support the previous work of Dokladny et al., suggesting HSP70 expression significantly inhibits autophagy activity. Though we can only speculate, this may the consequence of increased mTOR activation by HSP. We found no effect of BCAA supplementation on either of these systems.

Conclusions

Branched-chain amino acid supplementation may play a role in recovery of indirect markers of muscle damage following strenuous, eccentric exercise. However, the impact on muscular performance during the recovery period is negligible. We conclude the following regarding our study of BCAA supplementation versus placebo consumed by resistance-trained males adhering to a strict diet consisting of 1.2 grams per kilogram of protein a day:

- Branched-chain amino acids resulted in decreased perceptions of soreness following muscle damaging resistance exercise.
- The impact of BCAA on indirect biochemical markers of muscle damage is modest.
- Isometric force production recovered faster in BCAA supplemented males; however, this ergogenic effect may be trivial as there was no effect on dynamic measures of muscle function.

From our secondary study of autophagy and heat shock protein response in the same population, we have identified that strenuous, eccentric exercise increases autophagy activation, as demonstrated by a significant decrease in p62/β-actin and LC3/β-actin protein expression in PBMCs up to 4 hr post-exercise. Exercise-induced autophagy has been demonstrated in multiple tissues, including skeletal, adipose, and brain tissue , but this is the first study to demonstrate that autophagy is upregulated in PBMCs after an acute bout of intense resistance exercise (He, Sumpter, & Levine, 2012). In addition, we also found a significant upregulation in HSP70 at 48 and 72 hr post-exercise. Though we can only speculate, these data support the hypothesis by Dokladny et al. that heat shock proteins alter autophagic activity, where autophagy is activated in the early hours after an acute stressor, followed several days later by the increase in HSP70 control. It has been suggested that HSP70 acts as a cellular switch transitioning from an initial degradation phase to a building and protein synthesis phase (Dokladny, Myers, & Moseley, 2015).

Recommendations

Several questions remain regarding the value of BCAA supplementation. We did not find any benefit of BCAA on dynamic muscular function during recovery; however, we suggest future research examine sport-specific dependent variables (e.g. sprinting) or ask subjects to complete a follow up bout of exercise (e.g. 1 mile run, squat repetitions to failure) in the recovery period following strenuous exercise. This will provide more insight as to the effects of BCAA on muscular function during recovery using activities that are more practical for recreationally training individuals or athletes.

Further examination of systemic markers in the proximal hr post-exercise of oxidative stress, cytokines, and catabolic hormones (i.e. cortisol) may provide more understanding into the impact of muscle damaging resistance exercise on the increased activity of autophagy and HSP70 in PBMCs, as these factors have been previously reported to impact both autophagy and HSP (Vainshtein & Hood, 2016). We also recommend completing a similar study in skeletal muscle, as this tissue undergoes significant protein breakdown during eccentric exercise and may provide additional understanding of autophagy and HSP responses (Ebbeling & Clarkson, 1989, 1990; Hyldahl et al., 2015). To further elucidate the relationship between HSP70 and autophagy, we suggest the study of the protein mTOR (mammalian target of rapamycin), as HSP70 has be purported to regulate autophagy via mTOR activation (Dokladny et al., 2013). Further, it would be interesting to compare the autophagy and HSP response following resistance exercise in both skeletal muscle (i.e. local tissue) and PBMCs (i.e. systemic tissue). In addition, increased gene expression of HSP70 has previously been reported in trained individuals. It would be interesting to study the HSP70 and autophagy

response in both trained and untrained individuals at multiple time-points during recovery.

APPENDICES

A. Synopsis of Secondary Study: *Data from an ongoing study examining autophagy and heat shock protein response.*

B. Informed Consent

C. Flyer

D. Health History Questionnaire

E. Email Script

F. Data Collection Sheets

G. Dietary Food Log

H. Visual Analog Scale (10 cm) (DOMS)

Appendix A

Secondary Study

BACKGROUND

The molecular mechanisms underlying initiation of autophagy have been extensively studied; however, the relationship between exercise and autophagy regulation is largely unexplored (Cuervo, 2004; Dokladny et al., 2015; Fan et al., 2016; Grumati et al., 2011; Klionsky et al., 2016; Moller et al., 2015; Schwalm et al., 2015; Smuder, Kavazis, Min, & Powers, 2011; Wohlgemuth, Seo, Marzetti, Lees, & Leeuwenburgh, 2010). While it is well known that exercise is associated with numerous health benefits, including protection against metabolic disorders, sarcopenia, cardiovascular disease, cancer, and neurodegenerative disease (ACSM, 2014; Garza, Wason, & Zhang, 2015), the evidence for autophagy as a key mechanism of this phenomenon is beginning to surface (McCormick, McLain, Ulrich, Dokladny, & Mermier, 2016; Mooren & Kruger, 2015; Vainshtein & Hood, 2016). Autophagy, translated as "self-eating", is an evolutionary-conserved cellular housekeeping pathway responsible for degradation of intracellular constituents including aged and dysfunctional organelles, and damaged and misfolded proteins (Levine & Klionsky, 2004; Levine & Kroemer, 2008; Mooren & Kruger, 2015). Macroautophagy (herein referred to as autophagy) is a form of autophagy that regulates the bulk degradation of cytosolic components by sequestering undesirable proteins and organelles to the lysosome through an intermediate double membrane bound vesicle, known as the autophagosome, which fuses with the lysosome to form an autophagolysosome (Todde, Veenhuis, & van der Klei, 2009).

Autophagy activation is initiated in response to a multitude of factors including energetic insufficiency and metabolic disturbances (Cui, Yu, Wang, Gao, & Li, 2013;

Rubinsztein, Marino, & Kroemer, 2011; Wohlgemuth et al., 2010), oxidative stress and inflammation (Vainshtein & Hood, 2016), and exercise, which elicits a number of positive autophagic signals at the cellular level, including those mentioned above. (Nair & Klionsky, 2011). Data supporting the importance of autophagy for maintaining cellular homeostasis in response to exercise have been growing in both human and animal models (Dokladny et al., 2013; He et al., 2012; Nair & Klionsky, 2011; Smuder et al., 2011); however, to date, human studies are scarce. Jamart and colleagues reported an increase in gene expression of autophagy related markers (e.g. autophagy related proteins- ATG4B and ATG12, and microtubule light chain-3 [LC3-II]) following ultra-endurance running performance in humans (Jamart et al., 2012). Additional research in mice has demonstrated that strenuous endurance exercise (10 $m \cdot min^{-1}$; running speed increased by 0.5 $m \cdot min^{-1}$ every minute for 40 min and then by 1 $m \cdot min^{-1}$ until exhaustion) increased autophagic markers LC3-II and p62, especially near exhaustion (Pagano, Py, Bernardi, Candau, & Sanchez, 2014). Grumati and associates reported that exercise to fatigue in rodents upregulated autophagy, as illustrated by the changes in LC3-II/LC3-I ratio and LC3-II expression (Grumati et al., 2011). While research on exercise and autophagy is still in its infancy, investigations of autophagy and resistance type exercise are scant. Luo and colleagues demonstrated that rats who chronically resistance trained for nine weeks demonstrated reduced protein expression of LC3-II/LC3-I ratio, reduced p62 protein levels, and increased levels of autophagy regulatory proteins, including Beclin 1, Atg5/12, Atg7, and the lysosomal enzyme cathepsin (Luo et al., 2013). These data support the work of Fry and colleagues who also demonstrate that levels of autophagy are not significantly different in younger and older individuals following acute resistance

exercise consisting of 8 sets of 10 repetitions at 70% one repetition maximum (1RM) (Fry et al., 2013).

Heat shock proteins (HSP) are involved in the restoration of damaged cellular proteins and have been shown to possess an essential role in cellular response to exercise (Dokladny et al., 2015). Exercise elicits several stressors that activate the HSP response, including oxidative stress and inflammation (de Moura, Lollo, Morato, Carneiro, & Amaya-Farfan, 2013; Fittipaldi, Dimauro, Mercatelli, & Caporossi, 2014; Lollo, Moura, Morato, & Amaya-Farfan, 2013; Mikkelsen et al., 2013; Peart, Kirk, Madden, Siegler, & Vince, 2013). The effect of exercise on HSP regulation has been documented in both animals and humans (de Moura et al., 2013; Fehrenbach & Niess, 1999; Fittipaldi et al., 2014); however, less is known as to the relationship between HSP and autophagy.
It has been suggested that autophagy and HSP (i.e. HSP70) respond to exercise in a biphasic manner, insofar as autophagy increases during the initial degradation phase of exercise (i.e. during or immediately post), followed by an increase in HSP during the latter stages of recovery signaling a transition to a building and protein synthesis phase (Dokladny et al., 2015). Increased expression of HSP70 is thought to inhibit autophagy through the mammalian target of rapamycin complex 1 (mTORC1)/protein kinase B (Akt) pathway (Dokladny et al., 2013), however more work is needed to confirm this hypothesis.

Moreover, amino acids, including branched-chain amino acids (BCAA), serve as potent inhibitors of autophagy, acting through the mTORC1 pathway. mTORC1 inhibits autophagy by directly interacting with autophagy-related proteins (Atgs), ultimately preventing autophagosome formation (Meijer, Lorin, Blommaart, & Codogno, 2015;

Todde et al., 2009). Specifically, hyperphosphorylation of Atg13 and Atg1 (known as ULK1 in mammals) by mTORC1 prevents the association of these proteins, which is required to initiate autophagosome formation (Meijer et al., 2015). This interaction is of particular interest in that mTORC1 is also upregulated by mechanical stress, such as that elicited during resistance exercise in skeletal muscle (Marcotte, West, & Baar, 2015) and likely plays a key role in the autophagic response to resistance-based exercise. Though literature regarding the activity of autophagy in response to resistance training is scarce, Glynn et al. showed that 10 sets of 10 repetitions at 70% 1RM using leg extension did not result in any change in LC3bII protein content, or the LC3bII/LC3bI ratio at 1 hr post-exercise (Glynn et al., 2010). This lack of response has been evidenced in others studies (Fry et al., 2013; Smiles et al., 2015). These studies highlight both the novel exercise- and tissue-specific autophagic response to an acute bout of exercise.

The anabolic effect of BCAA, leucine in particular, is multidimensional. In addition to decreasing autophagic activity (Deldicque, Theisen, & Francaux, 2005; Kanazawa et al., 2004; Sugawara, Ito, Nishizawa, & Nagasawa, 2009), these amino acids are also capable of increasing protein synthesis through the upregulation of mTORC1 activity (Marcotte et al., 2015; Meijer et al., 2015). Further, BCAAs must be present for other mTORC1 signaling sources (i.e. mechanical load, growth factors) to effectively result in increased protein synthesis, as they are required for the translation of proteins from mRNA (Blommaart, Luiken, Blommaart, van Woerkom, & Meijer, 1995; Crozier, Kimball, Emmert, Anthony, & Jefferson, 2005; Stipanuk, 2007; Tipton & Wolfe, 1998).

PURPOSE

The purpose of this secondary study is two-fold: 1) To determine if acute muscle-damaging resistance exercise affects autophagy and heat shock protein responses acutely and at multiple time points over 72 hr post-exercise; 2) To determine if there is an impact of BCAA supplementation on markers of autophagy and heat shock protein in individuals who perform a muscle-damaging bout of resistance exercise.

METHODS

Participants:

Twenty young, resistance-trained (RT) males (age 22.3±1.5 yr, height 175.4±6.9 cm, and body mass 86.4±15.6 kg) were recruited for the study. The present study was approved by the institution's Human Research Review Committee. Participants were made aware of all procedures, including the risks and benefits, gave written consent and completed health history and physical activity questionnaires. All participants had several years (5.3±2.5 yr) of resistance exercise training, with an average, self-reported training time of 7.3±2.1 hr per week. Participants were excluded if they were consuming creatine (within the past 6 months) and certain medications (non-steroidal anti-inflammatory or steroidal drugs). Individuals consuming protein supplements (e.g. whey, casein) were asked to refrain from taking these supplements one week prior to baseline assessment until completion of the study. Individuals utilizing treatments such cryotherapy or massage, past or current smokers, those without at least 1 year of previous resistance training experience, and participants who had completed high-intensity eccentric squats in the last 4 months were also excluded from the study due to the repeated bout phenomenon (Coratella, Chemello, & Schena, 2015). All participants refrained from

unaccustomed or intense exercise 48 hr prior to testing, and were asked to refrain from all exercise and alcohol consumption 24 hr prior to testing and throughout the entire testing period. Participant characteristics are shown in **Table 1**.

Table 1. Participant characteristics.

Characteristic	BCAA	PLCB
# of Subjects	10	10
Age (yr)	23.0 ± 1.2	21.5 ± 1.5
Height (cm)	177.6 ± 7.1	173.2 ± 6.2
Body Mass (kg)	86.6 ± 15.2	86.2 ± 16.8
Body Fat%	12.3 ± 3.8	11.7 ± 4.3
1RM Squat (kg)	154.8 ± 31.7	155.0 ± 32.0
RT Experience (yr)	5.6 ± 2.3	5.0 ± 1.9
Hours per week of RT	7.00 ± 2.30	7.65 ± 2.05

All values are mean±SD. yr = years, cm = centimeters, kg = kilograms, 1RM = one repetition maximum, RT = resistance training, BCAA= branched-chain amino acid, PLCB= placebo

Experimental Design:

Using a randomized, double-blind, placebo-controlled research design, participants were enrolled into either a branched-chain amino acid supplemented (BCAA) (MusclePharm BCAA 3:1:2 watermelon powder) or placebo (PLCB) group (maltodextrin) and performed one experimental muscle-damaging exercise trial**.** Groups were matched for both body mass (+/- 5 kg; p=0.95) and one repetition maximum (1RM; +/- 5 kg; p=0.80). Following consent and initial screening (general health, supplementation history, and exercise history) participants were asked to complete preliminary testing consisting of a 1RM squat. Also on the day of initial screening, a registered dietician provided each participant with specific verbal and written directions and procedures for reporting a detailed dietary intake, including information on how to record portions using household measures, preparation technique, and nutrient content

descriptors (e.g. reduced-fat, light). During the two days prior to preliminary testing and throughout the entire experimental period, participants recorded their food intake and were instructed by a dietician how to follow a protein intake of 1.2 g/kg of body mass. Participants were asked to return in 48 hr for a baseline blood draw and supplement distribution and dietary counseling. All participants supplemented with either BCAA or PLCB (0.22 gram per kilogram of bodyweight) for a total of 8 days, including a 5-day supplementation run-in phase prior to the muscle damaging exercise trial. An exercise protocol consisting of 4:1 second eccentric-concentric squats at each participant's 70% 1RM was completed on day five of supplementation. Autophagy and heat shock protein responses were assessed via peripheral blood mononuclear cells (PBMCs) collection immediately post-squat exercise (IPE), as well as at 1, 2, 4, 24, 48, and 72 hr post-exercise.

Preliminary Testing (1RM Testing) and Familiarization:

All participants were thoroughly familiarized with the study design; specifically, the diet and physical activity log requirements, blood draw timing and procedure, and 1RM protocol. On the day of preliminary testing, participants first had their height and body mass measured, and body composition assessed using the skinfold technique. Participants were then asked to complete a self-selected 10-minute warm-up followed by 1RM assessment (procedure below). Each participant's 1RM was assessed in order to determine 70%1RM load for the squat-exercise protocol. Following the completion of preliminary testing and 1RM assessment, participants were asked to return 48 hr later for baseline assessment.

One Repetition Maximum

The 1RM back squat testing was performed according to methods previously described (Kraemer, 1995). Following a 10-minute standardized, dynamic warm-up, each participant first performed a warm-up set of 8–10 repetitions at a light-weight (~50% of their estimated (est) 1RM). A second warm-up consisted of a set of 3–5 repetitions with a moderate weight (~75% of $1RM_{est}$), and third warm-up included 1–3 repetitions with a heavy weight (~90% of $1RM_{est}$). After the warm-up, each participant's 1RM was tested by increasing the load during consecutive trials until the participants were unable to perform a proper lift using correct technique (90 degrees of knee flexion). The 1RM test was determined by 4-6 sets of one repetition, with 3- to 5-min of rest between attempts. Spotters were present to provide verbal encouragement and spotting to ensure safety of the subjects.

Baseline Assessment - Pre-Supplementation

Participants were asked to return to the laboratory 48 hr after initial screening, abstaining from alcohol and exercise, as well as caffeine (e.g. pre-workout supplements, coffee) 12 hr prior to baseline testing. On the day of baseline testing a trained phlebotomist drew blood for the collection of peripheral blood mononuclear cells and red blood cell lysate.

Anthropometric Measurements

Measurements of height, body mass, and percent body fat (BF%) by 3-site skinfolds (Jackson & Pollock, 1978; Siri, 1993) were obtained to characterize the participants. Body mass was measured via a Tanita electronic scale (Model #3101, Arlington Heights, Illinois) to the nearest 0.1 kg. Two skinfold measurements on the right

side of the body were obtained from three sites (chest, abdomen, and thigh) in serial fashion by the same investigator utilizing Lange Calipers (Cambridge Scientific Industries, Cambridge, MD). Skinfold thickness was based on the average of the two trials. If the two skinfold measurements for a particular site varied by more than 0.5 millimeters (mm), the technician obtained a third measurement and the mean value of the two closest measurements was used. Body density was then calculated according to the Jackson and Pollock 3-site equation (Jackson & Pollock, 1978) and BF% was estimated according to Siri (Siri, 1993).

BCAA and Placebo Supplementation

Participants ingested 0.22 g/kg of body weight of BCAA (MusclePharm, Denver, CO; 7.16 g = 3 g leucine, 1 g isoleucine, 2 g valine) or maltodextrin (PLCB) in dry-powder form mixed with water (~175-350 ml) for a total of 8 days following baseline assessment. The supplements were separated into two doses per day, taken in the morning and evening. On the fifth day of supplementation, participants returned to the laboratory and completed the muscle-damaging, squat exercise protocol.

Experimental Protocol (Muscle Damaging Exercise Visit)

Upon arrival to the laboratory, a trained phlebotomist collected the first (pre-exercise) of five blood samples. Participants then completed the muscle-damaging squat exercise protocol. Following completion of the exercise protocol, participants had their blood drawn, IPE, 1, 2, 4, 24, 48, and 72 hr post-squat exercise.

Muscle Damaging Protocol

A standardized bout of 4-second eccentric damaging resistance exercise involving 10 sets of 8 repetitions at 70% 1RM squats using a Smith machine was completed by all

participants. The squat protocol consisted of a 4 to 1 eccentric to concentric rhythm. A stopwatch was started following the last repetition of each set and subjects were given 3 minutes of rest between all squat sets. Following completion of the squat protocol, participants then completed 5 sets of 10 consecutive (each leg) body-weight split jump repetitions with 2 minutes of rest between each set.

Blood Sampling and Analysis

Venous blood (~10 ml) was collected at all time-points: pre-supplementation, pre-squat exercise, IPE, 2, 4, 24, 48, and 72 hr post-exercise for the collection of peripheral blood mononuclear cells and red blood cell lysate.

Markers of Autophagy and Heat Shock Proteins

Blood samples (~10 mL) were collected into EDTA treated tubes to isolate PBMCs before supplementation, pre-exercise, IPE, 2, 4, 24, 48, and 72 hr after the completion of the squat exercise. Following PBMC isolation, proteins were analyzed via Western Blot analysis. Markers of autophagy included LC3-II, LC3-I and p62 protein. Heat shock protein response was analyzed via changes in HSP70 protein expression. All proteins were standardized to β-actin.

Autophagy Measurements

LC3 and p62 are commonly used markers of autophagic activation and were measured in this study pre-supplementation, pre-exercise, IPE, 2, 4, 24, 48, and 72 hr post-exercise. When autophagy is activated, the conversion of LC3-I, the cytosolic form of LC3, to LC3-II, the autophagosome membrane-bound form of LC3, is increased. LC3-II is associated with the completed autophagosome; its disappearance may be reflective of autophagosome degradation. The ratio of LC3-II to LC3-I may be used to further infer

autophagic flux. Additionally, measurement of the rate of disappearance of p62, an adapter protein that serves to carry protein cargo to be degraded to the phagophore (Moller et al., 2015) is indicative of autophagosome degradation (Klionsky et al., 2016). Degradation of LC3-II and p62, as well as the ratio of LC3-II to LC3 I are indicative of increased autophagic flux under normal (non-inhibitory) conditions (Klionsky et al., 2016).

Immunoblot analysis

Cells were lysed in a modified RIPA buffer (Tris-HCl 8.0 pH; Invitrogen, 15568-025); 0.5M EDTA (Invitrogen, 15568-020); 1.5M NaCl (Sigma-Aldrich, S9888) 1% Triton X 100 (Sigma-Aldrich, X100); and freshly added protease (ThermoScientific, 78430) and phosphatase (ThermoScientific, 7842) inhibitors. HSP70, LC3, SQSTM1/p62, and β-actin were resolved by electrophoresis in a 12% polyacrylamide gel (Bio-Rad, 456-144). Proteins were transferred to cellulose membranes (Bio-Rad, 162-0094) then blocked in tris buffered saline (150 mM NaCl, pH 8.0) containing 0.2% polysorbate (Tween 20; Bio-Rad, 170-6531) detergent and 5% powdered milk (Bio-Rad, 170-6404). Membranes were then incubated in tris-buffered saline containing 0.2% polysorbate detergent and 5% bovine serum albumin (Sigma-Aldrich, A9418) with primary antibodies including: LC3 (Sigma-Aldrich, L7543), SQSTM1/p62 (Abcam, ab56416), HSP70 (Enzo, P08107) and β-actin (Sigma-Aldrich, A5441). Primary antibodies were detected by horseradish peroxidase-labeled secondary antibody (Goat anti-rabbit; Cell Signaling, 7074s; Goat anti-mouse; Cell Signaling, 70076s) binding, which was detected using Santa Cruz Western blotting luminol reagents (Santa Cruz Biotechnology, Santa Cruz, CA) using the ChemiDoc XRS+ (Bio-Rad, Hercules, CA).

Image analysis and statistical methods: Adobe Photoshop (version 6, Adobe Systems Incorporated) was used to quantify immunoblot band intensity.

Glutathione

Glutathione was measured using erythrocytes obtained from the blood samples taken pre-exercise, IPE, and 1, 2, 4, 24 hr post-exercise with a commercial kit (Kit #703002, Cayman Chemicals, Inc., Ann Arbor, MI). The red blood cells were lysed in four times the volume ice-cold ultrapure water, centrifuged at 10,000 x g for 15 minutes at 4˚C, and then the supernatant was deproteinated in an equal volume of 0.1 g $^{mL-1}$ of metaphosphoric acid (MPA). The MPA and sample was vortexed and allowed to stand at room temperature for 5 minutes. The sample was then spun at 2,000 g for 2 minutes; the supernatant was removed and stored at -20 ˚C for the analysis of total glutathione (tGSH) and oxidized glutathione (GSSG) according to manufacturer guidelines. All samples were measured in duplicate. The absorbance values were measured at 405 nm (iMark™ Microplate Absorbance Reader, Bio-Rad, Hercules, CA). Under the standardized conditions of the assay, the dynamic range is 0.5–16 µM tGSH and 0.25–8 µM GSSG. Ratio of GSSG/tGSH was calculated and reported as an indicator of oxidative stress as previously described by Goldfarb et al. (Aad et al., 2015; Goldfarb, Bloomer, & McKenzie, 2005; Goldfarb, Patrick, Bryer, & You, 2005).

Statistical Analysis

Statistical tests were conducted in R (version: 3.2.2; R Foundation for Statistical Computing; Vienna, Austria), using the 'afex' package (version 0.16-1). Separate mixed-effects (within-between) factorial ANOVAs (group x time) were used to assess the main

and interaction effects for each reported dependent variable. Post-hoc pairwise comparisons were then used to investigate group differences across individual time-points, with the Bonferroni adjustment applied to correct for multiple comparisons. ANOVA models were evaluated for compliance with underlying model assumptions. Assumptions of sphericity were tested using Mauchly's test of sphericity and violations were corrected using the Greenhouse-Geisser correction factor. The threshold for statistical significance was set *a priori* at $p \leq 0.05$ for all analyses.

RESULTS

LC3 PROTEIN EXPRESSION

Between Groups

There was no significant difference for LC3-II/β-actin protein expression between PLCB and BCAA groups' pre-supplementation and pre-exercise *(p*>0.05). No statistical differences were observed when examining LC3-II/β-actin protein expression at any measured time point between groups (*p*>0.05; Figure 1A and Figure 1B).

Within Groups

Expression of LC3-II/β-actin protein was significantly lower relative to pre-exercise for the PLCB (Figure 1A) group 2 hr (*p*<0.01) and 4 hr (*p*<0.01) post-exercise. No within group differences were detected for the BCAA group (Figure 1B).

Combined BCAA and Placebo

When subjects are combined, expression of LC3-II/β-actin protein was significantly lower relative to pre-exercise 2 hr (*p*<0.001) and 4 hr (*p*<0.001) post-exercise (Figure 1C).

LC3 II/I RATIO PROTEIN EXPRESSION

Between Groups and Within Groups

There was no significant within or between differences for LC3II/I protein expression (*p*>0.05) (Figure 2A and 2B).

Combined BCAA and Placebo

No differences were detected when treatment conditions were combined for expression of LC3 II/I protein (*p*>0.05) (Figure 2C).

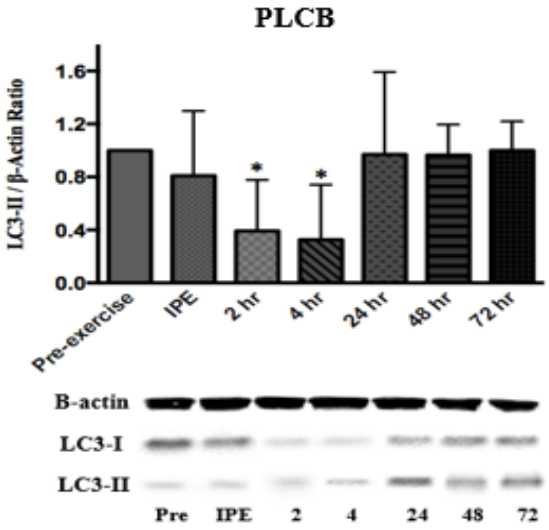

Figure 1A.

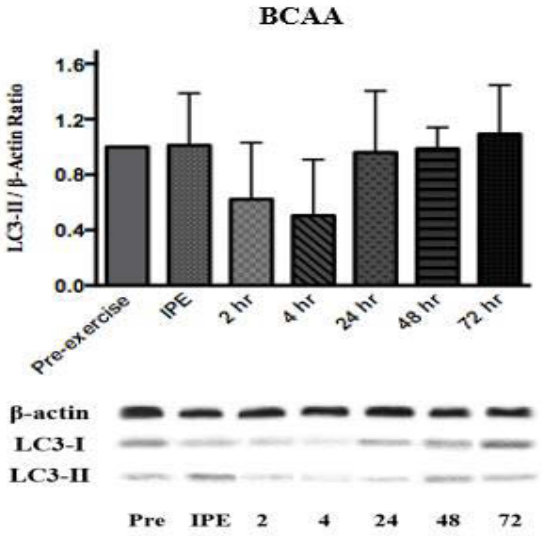

Figure 1B.

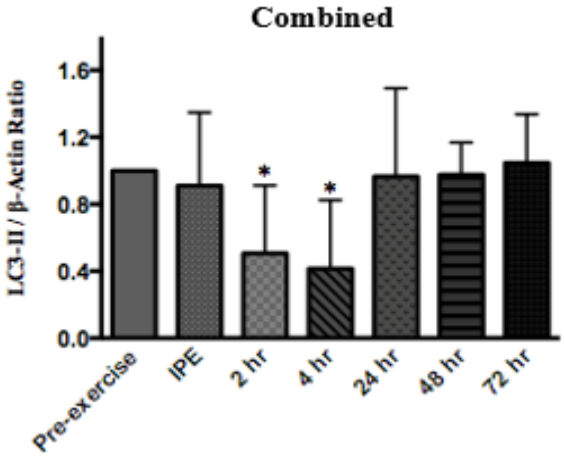

Figure 1C.

Figure 1. PBMCs were collected from resistance trained males consuming a placebo supplement (A; n=10) and branched-chain amino acid supplement (B; n =10). Figure C displays all twenty participants. Cells were harvested and prepared for Western blot analysis to measure the relative expression of LC3and β-actin (an internal loading control). Quantification of relative protein content was completed using densitometric values obtained using Photoshop software and normalized to β-actin and set to 1 for control condition. Data represent means ± standard deviation (A-C). *, indicates statistical significance ($p \leq 0.01$) within the respective group compared to pre-exercise.

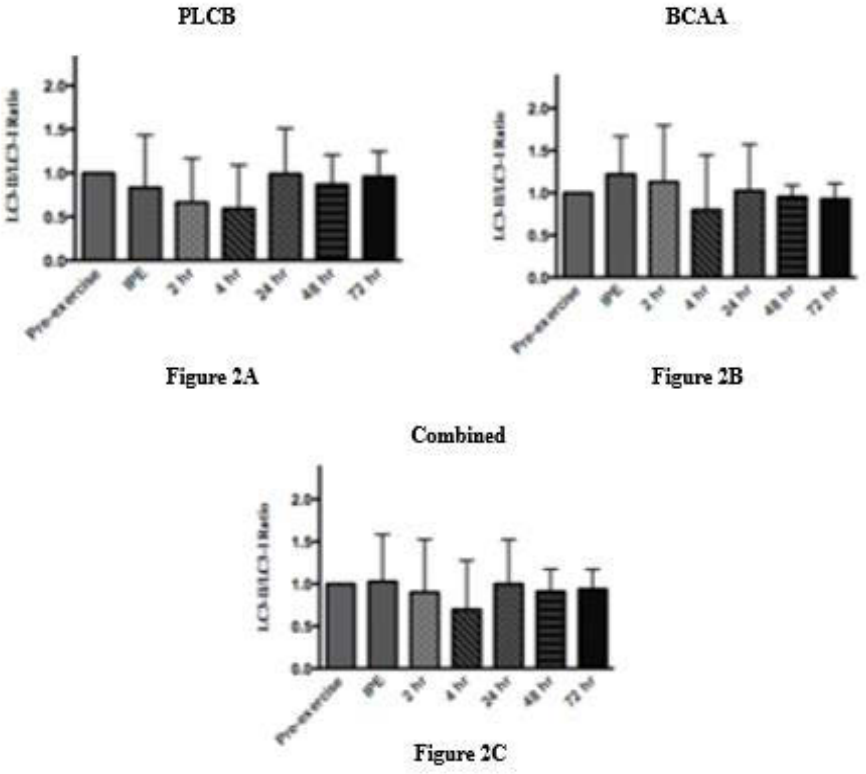

Figure 2. PBMCs were collected from resistance trained males consuming a placebo supplement (A, n=10) and branched-chain amino acid supplement (B; n =10). Figure C displays all twenty participants. Cells were harvested and prepared for Western blot analysis to measure the relative expression of both LC3-II and LC3-I. β-actin was used as the internal loading control. Quantification of relative protein content was completed using densitometric values obtained using Photoshop software and normalized to β-actin and set to 1 for control condition. Data represent means ± standard deviation (A-C). No differences were found within or between groups for LC3II/I ratio within or between groups, as well as when groups were combined (p>0.05).

p62 PROTEIN EXPRESSION

Between Groups

There was no significant difference for p62/β-actin protein expression between PLCB and BCAA groups' pre-supplementation and pre-exercise ($p>0.05$). No statistical differences were observed when examining p62/β-actin protein expression at any measured time point between groups ($p>0.05$; Figure 3A and Figure 3B).

Within Groups

Expression of p62/β-actin protein was significantly lower relative to baseline for the PLCB group IPE ($p<0.01$), 2 hr ($p<0.001$), and 4 hr ($p<0.001$) post-exercise and rose significantly above pre-exercise levels 24 hr post-exercise ($p=0.03$) (Figure 3A). Branched-chain amino acid p62/β-actin protein expression was significantly lower 2 hr ($p<0.001$) and 4 hr ($p<0.001$) post-exercise and increased significantly above pre-exercise levels 24 hr post-exercise ($p=0.03$) (Figure 3B).

Combined BCAA and Placebo

When subjects are combined, expression of p62/β-actin protein was significantly lower relative to pre-exercise IPE ($p<0.001$), 2 hr ($p<0.001$), and 4 hr ($p<0.001$) post-exercise and increased significantly compared to pre-exercise levels 24 hr post-exercise ($p<0.001$) (Figure 3C).

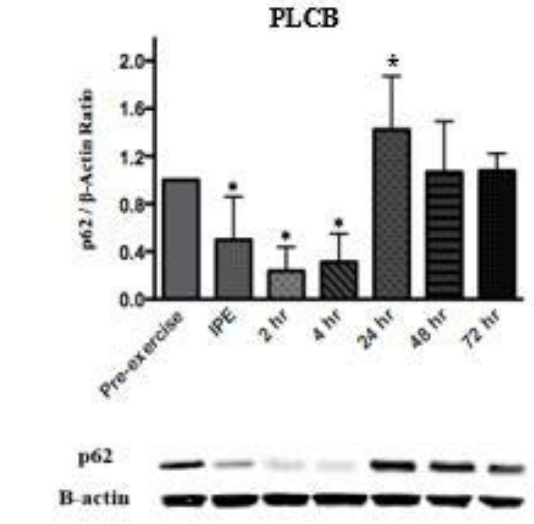

Figure 3A.

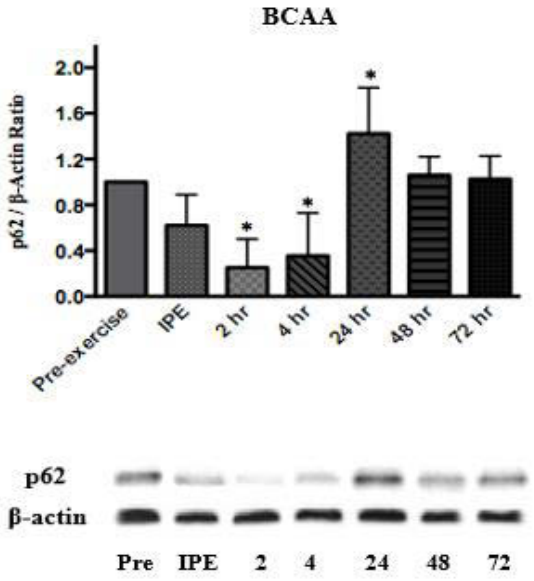

Figure 3B.

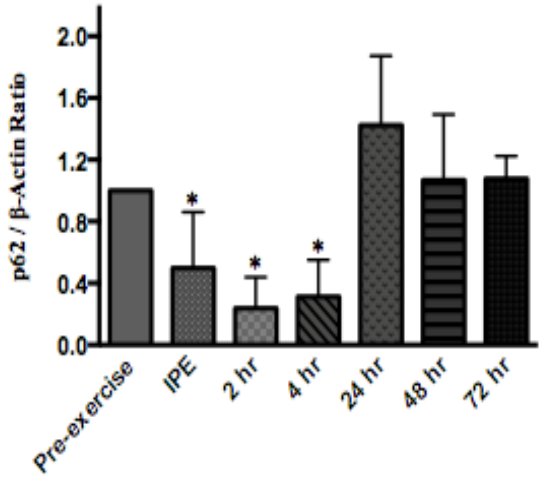

Figure 3C.

Figure 3. PBMCs were collected from resistance trained males consuming a placebo supplement (A; n=10) and branched-chain amino acid supplement (B; n =10). Figure C displays all twenty participants. Cells were harvested and prepared for Western blot analysis to measure the relative expression of p62 to β-actin (an internal loading control). Quantification of relative protein content was completed using densitometric values obtained using Photoshop software and normalized to β-actin and set to 1 for control condition. Data represent means ± standard deviation (A-C). *, indicates statistical significance ($p \leq 0.05$) within the respective group compared to pre-exercise

HSP70 PROTEIN EXPRESSION

Between Groups

There was no significant difference for HSP70 protein expression between PLCB and BCAA groups pre-supplementation and pre-exercise ($p>0.05$). No statistical differences were observed when examining HSP70 protein expression at any measured time point between groups ($p>0.05$; Figure 4A and Figure 4B).

Within Groups

HSP70 protein expression was significantly elevated relative to baseline for both PLCB (Figure 4A) and BCAA (Figure 4B) groups 48 hr post-exercise (PLCB: $p<0.01$; BCAA: $p<0.01$) and 72 hr post-exercise (PLCB: $p<0.01$, BCAA: $p<0.01$).

Combined BCAA and Placebo

When subjects are combined, expression of HSP70 protein was significantly higher relative to pre-exercise 48 hr ($p<0.001$) and 72 hr ($p<0.001$) (Figure 4C).

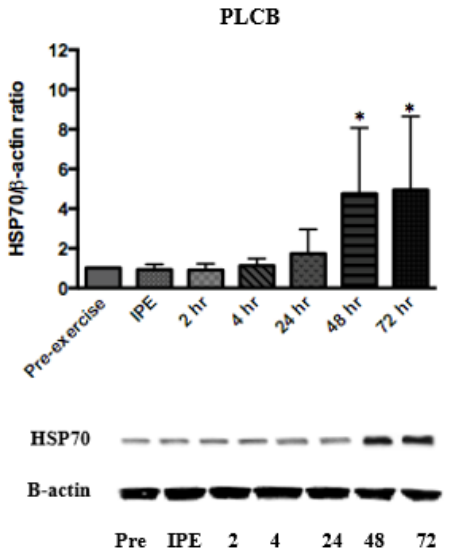

Figure 4A

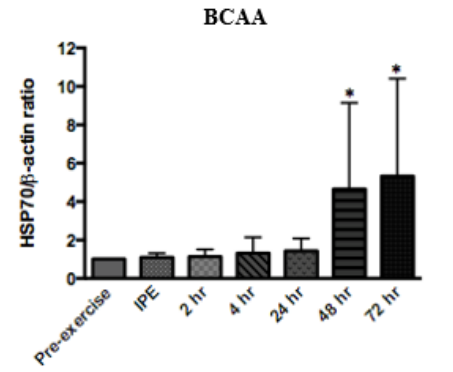

Figure 4B

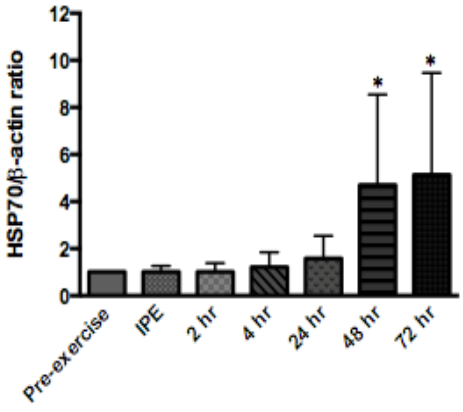

Figure 4C

Figure 4. PBMCs were collected from resistance trained males consuming a placebo supplement (A; n=10) and branched-chain amino acid supplement (B; n =10). Figure C displays all twenty participants. Cells were harvested and prepared for Western blot analysis to measure the relative expression of heat shock protein 70 (HSP70) to β-actin (an internal loading control). Quantification of relative protein content was completed using densitometric values obtained using Photoshop software and normalized to β-actin and set to 1 for control condition. Data represent means ± standard deviation (A-C). *, indicates statistical significance (p<0.01) within the respective group compared to pre-exercise.

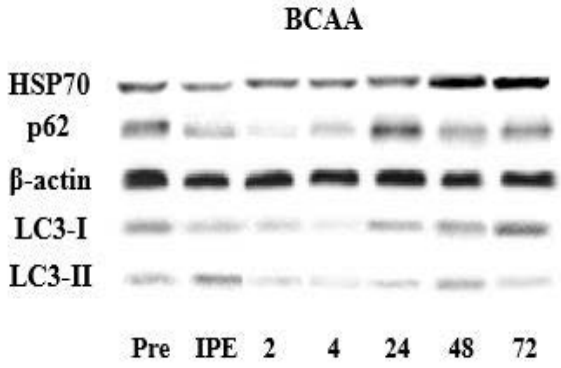

Figure 5. Representative Western blot of a branched-chain amino acid supplemented individual who completed an acute bout of eccentric resistance exercise. *HSP70= heat shock protein 70, p62 = sequestosome 1, β-actin = beta-actin, LC3 = microtubule light chain 3*

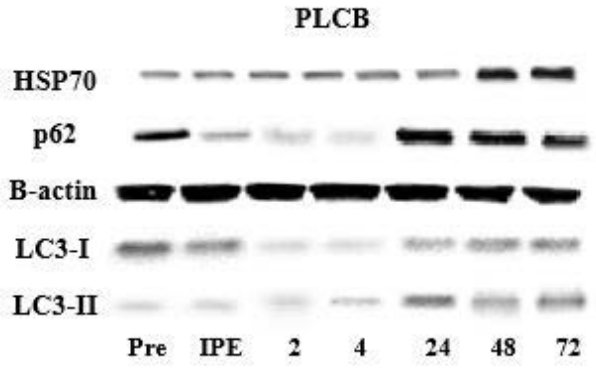

Figure 6. Representative Western blot of a placebo supplemented individual who completed an acute bout of eccentric resistance exercise. *HSP70= heat shock protein 70, p62 = sequestosome 1, β-actin = beta-actin, LC3 = microtubule light chain 3*

Glutathione

Both groups demonstrated similar (p>0.05) glutathione redox ratio (GSSG/tGSH) prior to the eccentric exercise protocol (pre-exercise) (BCAA= 0.10±0.07; PLCB= 0.11±0.04). GSSG/tGSH was significantly higher for both BCAA and PLCB 1, 2, and 4 hr post-eccentric exercise (p<0.05); however, there were no group or interaction effects (Figure 7).

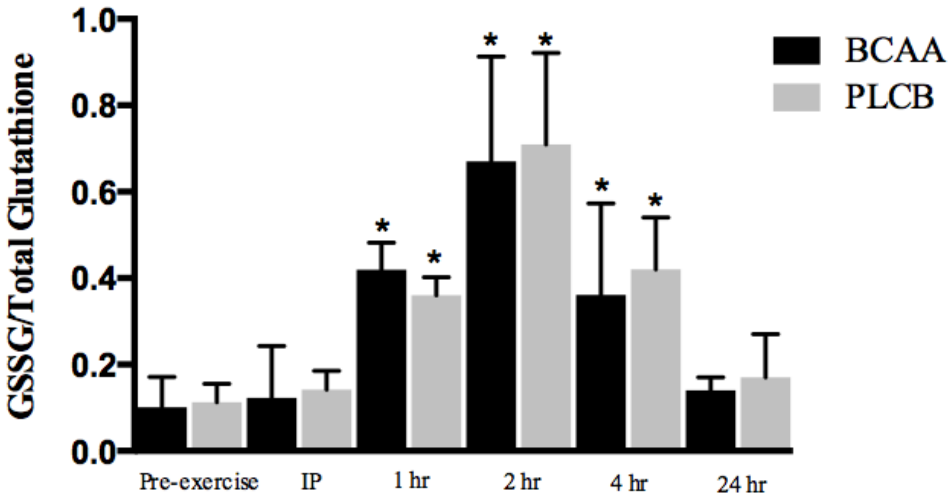

Figure 7. Mean (± standard deviation) glutathione redox ratio pre-eccentric exercise, immediately post (IP), 1, 2, 4, and 24 hours (hr) for resistance trained men supplementing with branched-chain amino acids (BCAA) or placebo (PLCB) (n = 20). * = significantly different from pre-exercise (p<0.05)

We conclude this eccentric exercise protocol induced muscle damage by the significant increase in plasma creatine kinase and perceived soreness ratings demonstrated by the participants of this study (Chapter 3).

CONCLUSIONS

- Supplementation with BCAA has no effect on autophagic flux and heat shock protein response following an acute bout of muscle damaging exercise in PBMCs.
    - It is well know that BCAA, leucine in particular, stimulate mTOR and protein synthesis (Moberg et al., 2016). mTOR is a negative upstream regulator of autophagy as it senses glucose and amino acid starvation and is intrinsically linked to metabolism and protein turnover. Supplementing individuals with BCAA undergoing strict dietary control was hypothesized to increase amino acid availability; however, there was no impact of BCAA on autophagy or HSP response at the tested dose of BCAA supplementation. This may be attributed to participants consuming a diet that is adequate in protein (1.2 g/kg/d); therefore, amino acid availability is adequate.
- Damaging acute eccentric exercise resulted in significant increases in autophagy activity in the early hours post-exercise up to 4 hr for both groups.
- Damaging acute eccentric exercise resulted in a significant increase in heat shock protein response 48 and 72 hr post-exercise for both groups.
- Glutathione (GSSG/tGSH) was significantly increased in both BCAA and PLCB groups 1, 2, and 4 hr post-exercise. Though we can only speculate, the elevated glutathione response up to 4 hr is indicative of high oxidative stress. This response coincides with the increased activation of autophagy, speculating oxidative stress may play a role in the post-exercise autophagic activity.

- Antioxidants play important roles in preventing endogenous oxidative damage. Glutathione, the endogenously produced, thiol containing tri-peptide, plays an important role in antioxidant defense and redox homeostasis.
- The degradation and recycling process by autophagy serves the purpose of decreasing the cellular energy cost for the maintenance of cellular structure and function, and attenuating further propagation of cellular damage. Autophagy not only incudes the clearance of damaged proteins, but can also contributes to the antioxidant defense response (Dodson, Darley-Usmar, & Zhang, 2013; Giordano et al., 2014; Levonen, Hill, Kansanen, Zhang, & Darley-Usmar, 2014; Yang & Klionsky, 2010).
- Inhibition of mTOR has also been found to play a role in autophagy initiation by reactive oxygen species (ROS) (Alexander et al., 2010; Zhang et al., 2013). Previous reports suggest TSC1/2 (tuberous sclerosis complex ½) localizes to peroxisomes through binding to peroxisomal biogenesis factor (PEX) 19 and 5, and can be activated by ROS to inhibit mTOR activities and induce autophagy.

- These data also support the previous work of Dokladny et al. (2015) suggesting HSP70 plays a role in the transition from an initial degradation phase to a building and protein synthesis phase following exercise; however, this is purely speculation as we did not measure any direct measures of protein synthesis.
  - In a review by Dokladny et al., acute exercise raises HSP70 expression but the magnitude is dependent on intensity and type (Dokladny et al., 2015).

- Examination of HSP70 response to acute, moderate intensity, endurance exercise demonstrates no change in PBMC HSP70 expression (A. J. Ryan, Gisolfi, & Moseley, 1991; K. K. Ryan et al., 2011). However, exhaustive endurance exercise (i.e. half marathon) demonstrates significant increases in HSP expression in leukocytes (Fehrenbach et al., 2000). Similarly, exhaustive treadmill exercise and vigorous cycling augments HSP70 protein expression in leukocytes that lasts up to 48 hr post exercise (Fehrenbach, Niess, Veith, Dickhuth, & Northoff, 2001; Niess et al., 2002; Taylor et al., 2012) .
- Dokladny et al. suggest that, "recovery from and adaptation to exercise represents a single, unified physiological response resulting from cooperation between autophagy and the heat shock response, two protein management systems." This model is characterized by an increase in autophagy immediately post-exercise in order to sequester and repair damaged proteins, followed by increased HSP activity that initiate the refolding and building phase. This may be attributed to HSP's association with mTOR activity.
- Previous work demonstrates overexpression of HSP70 in cell culture models significantly inhibits starvation-induced autophagy and that this inhibition correlates with an increase in mTOR and Akt activity (Dokladny et al., 2013).

- As a continuation of this study, mRNA will be assessed for LC3, p62, and HSP70 to assess transcriptional activity.

APPENDIX B

The University of New Mexico
Consent to Participate in Research

14-280

Acute Resistance Exercise and Dose-Response Impact of Branched-Chain Amino Acid Supplementation on Muscle Fiber Breakdown, Oxidative Stress and Autophagy

10/23/2014

Purpose and General Information

You are being asked to participate in a research study that is being done by Christine Mermier, Ph.D, who is the Principal Investigator and her associates, from the Department of Health, Exercise and Sport Sciences. This research is studying the effect of branched-chain amino acid (BCAA) supplements on muscle strength and performance and on markers of stress (creatine kinase, oxidative stress, autophagy) and muscle fiber breakdown. Branched-chain amino acids are important nutrients that must be consumed in our diets and are the building blocks of protein and have various functions related to energy production during and after exercise.

You are being asked to participate in this study because you are a healthy (no cardiac, pulmonary or metabolic disease, and no current orthopedic injury) man or woman between the ages of 18-45 years who currently includes weight-lifting as a part of their exercise program. Up to sixty people will be recruited to take part in this study at the University of New Mexico.

This form will explain the research study, and will also explain the possible risks as well as the possible benefits to you. We encourage you to talk with your family and friends before you decide to take part in this research study. If you have any questions, please ask one of the study investigators.

What will happen if I decide to participate?

If you agree to participate, the following things will happen:

You will visit the Exercise Physiology Lab on six occasions.

Pre-Visit 1: If you agree to be in the study, you will first sign this combined consent/HIPAA form and then complete medical history and physical activity questionnaires. Your answers to these questionnaires will be kept in a locked room and only members of the research team will see them. You will also be asked to complete a liver function test (LFT). This test is done in the blood and is used to monitor or diagnose liver damage or disease. You will be asked to donate a small blood sample (1 ml less than one-half teaspoon). This sample will be sent to, a local professional laboratory with scientists who will analyze your blood. This blood sample will only be labeled with your birthdate and not your name; therefore, it will be completely confidential and only you and the research team will know your results. If our physician determines that your

liver function test is abnormal, you will not be able to participate in the study, but he will advise you of the test results.

If the screening questionnaires and LFT qualify you for the study, you will be scheduled for your first exercise session. You will arrive at the lab fasted (no food or drink other than water) for 8-10 hours and having refrained from heavy exercise for 48 hours prior to performing the first exercise session.

Visit 1 (Exercise Session 1)

You will be tested to find your one repetition max (1RM) for the squat exercise, which is the most weight you can lift no more than one time. The squat exercise will require you to have a bar loaded with weight resting on your shoulders while you lower your body by bending your hips, knees and ankles similar to sitting on a chair. You will first warm up with light weights, and rest will be given between lifts. After the warm-up, you will complete one-repetition sets in progressively increasing loads to determine the highest amount of weight that can be successfully performed using standard technique. Two minutes rest will be given between each set of exercise. A repetition will be deemed successful if you are able to squat down to a depth where your knee forms a 80-90° knee angle as determined visually by the investigators.

After determination of your 1RM (on the same day), you will be randomized (meaning you have a one in five chance to be assigned to any of the groups) into one of the five study groups below:

a) No-exercise, placebo supplemented group (the placebo will consist primarily of carbohydrate that will have the same color, texture and flavor as other groups)

b) High intensity eccentric exercise, placebo supplemented group

c) High intensity eccentric exercise, 10 grams/day BCAA supplemented group

d) High intensity eccentric exercise, 20 grams/day BCAA supplemented group

e) High intensity eccentric exercise, 30 grams/day BCAA supplemented group

You will take your assigned supplement according to the prescribed schedule and amounts for five complete days prior to being scheduled for study visit 2. You will be given instructions on how to properly consume the supplement or placebo, how to complete a four-day dietary record, and instructions to fast overnight and refrain from heavy exercise for 48 hours before your next visit. We will then schedule you for your next visit. Visit 1 will take approximately 1-2 hours.

Branched-Chain Amino Acid Supplement and Placebo Details

The BCAA product consists of a 3:1:2 ratio of leucine (1,791.50 mg), valine (1,194.33 mg), and isoleucine (597.17 mg). Leucine, valine, and isoleucine are branched-chain amino acids.

The placebo for this study is a mixture of maltodextrin (2,750.00 mg), silicon dioxide (272.50 mg), malic acid (318.00 mg), sucralose (77.50 mg), and watermelon flavor with color (164 mg).

Both products are identically flavored and will have similar color, texture, foaming, and consistency. Thus, you should not be able to tell them apart. The flavor of both products is watermelon.

Visit 2:

Visit 2 will be scheduled five full days after Visit 1 to allow time for you to complete the food record and supplementation protocol. You will arrive at the lab fasted and having refrained from heavy exercise.

--We will first measure your height and weight without shoes, and body fat will be estimated with a bioelectrical impedance analyzer (a small device held with both hands for 30 seconds).

--The first of six blood samples (30 ml or 2 tablespoons each time) will then be collected by a trained phlebotomist from a vein in your arm.

--If you are in one of the groups requiring exercise, you will complete 10 sets of 8 repetitions at a challenging amount of resistance using the squat exercise. For each repetition you will be required to slowly lower your body to a full squat position. You will rest for three minutes rest between each set. Upon completing the squats, you will complete five sets of 10 consecutive split jump repetitions and rest for 1 minute between each set of jumps. The entire exercise session will take approximately 45 minutes.

--A total of six blood samples will be collected on visit 2 (pre, 0, 1, 2, 4 and 8 hours post-exercise).

--At the same time point when visit 2 blood samples are collected (with the exception of immediately post-back squat exercise), you will be asked to rate your perceived level of soreness, complete a vertical jump test, complete a squat jump test, and complete a leg extension exercise that measures your ability to produce maximal force with your quadriceps (muscles above your knee on the front of your leg).

Each of these exercise sessions will take approximately 45 minutes. You will continue to fast until four hours after the squat exercise, and then you will be given two food bars and a bottle of sports drink. You can have as much water as you want throughout the visit. Participants randomized to the no exercise group will do everything described above (fasting, 1RM, blood draws, jump testing etc.), except for the back squat exercise protocol.

You will be asked to remain in the lab through the four hour time point and encouraged (but not required) to remain in the lab until the 8 hour time point. You will be asked to come back to the lab 20 minutes before the last (8 hr) blood collection that is scheduled to occur 8 hours after completing the exercise bout.

This entire study visit is anticipated to take 11 hours, including the time between the last two blood draws.

You will continue to take your assigned supplement as instructed by the research team after completion of visit 2.

Visits 3 - 5: Like visit 2, you will observe a 10-hour fast. You will be asked to return to the lab 24, 48 and 72 hours after completing the visit 2 exercise bout or 24, 48 and 72 hours after the initial blood draw in visit 2 for the no-exercise group. You will continue to take your assigned supplement as instructed by the researchers each day upon completion of your study visit until visit 5 is completed or for a total of 8 days.

Again, you will be asked to provide a fasting blood sample (30 ml or 2 tablespoons) and complete a perceived soreness assessment, vertical jump, squat jump, and a leg extension exercise that measures your ability to produce maximal force with your quadriceps muscles. Completion of visit 5 will complete your study participation. Study visits 3-5 are anticipated to take 60 minutes each.

Over the course of the study, you will be asked to provide eleven blood samples of 30 ml (2 tablespoons) over a period of four days. This equals a total of 300 ml (20 tablespoons), which is less than a pint (30 tablespoons) or, the amount of blood you give when you donate blood.

How long will I be in this study?

Participation in this study will take a total of 16 hours over a period of two weeks.

What happens to my blood after it is drawn?

Once the blood has been drawn into the tubes, your blood will be tested for mRNA and protein content. Your blood levels of creatine kinase and oxidative stress will also be examined. Only researchers who have been properly trained will have access to your blood.

mRNA is messenger RNA (mRNA). mRNA is a molecule that carries copies of instructions for the assembly of amino acids into proteins from DNA (deoxyribonucleic acid) to the cells of your body. **This is considered genetic testing when we analyze your blood's mRNA content, however we will not be looking for specific risks of diseases. The purpose of this testing is to find the amount of mRNA and whether it relates to the supplement you are taking.** Creatine kinase and oxidative stress are markers of stress and muscle fiber breakdown.

Studying your mRNA and protein content, as well as creatine kinase and oxidative stress blood levels is important in our study of branched chain amino acids and autophagy.

What are the risks or side effects of being in this study?

There are risks of stress, emotional distress, inconvenience and possible loss of privacy and confidentiality associated with participating in a research study.

There are risks associated with maximal strength testing including the following: brief feelings of nausea, lightheadedness, muscle cramps, muscle soreness or dizziness. There is also the risk of musculoskeletal injury (muscle or bone injury), such as a herniated disc, when performing a 1 repetition maximum squat. The risk of a cardiac event occurring during maximal exercise is 6 in 10,000.

There are minor risks associated with blood draws including temporary pain and discomfort from the needle stick, a risk of bruising, and feeling faint or light-headed. This risk will be minimized by using trained and experienced phlebotomists. We will be asking you to donate 300 ml of blood over a two week time period. Potential side effects of donating this amount of blood include not being able to donate blood (outside of blood drawn for the study) over the time span of your participation in the study and not being able to schedule a surgery during your participation in the study. We recommend that you do not schedule a surgery during your enrollment in the research study and do not donate blood during this time period.

Branched-chain amino acids appear to be safe for most people. Though unlikely, side effects such as fatigue and loss of coordination have been reported. The use of BCAAs has been linked with lung failure and higher death rates when used in patients with amyotrophic lateral sclerosis (ALS).

Possible drug interactions when taking BCAAs may include those taking medications for diabetes including glimepride (Amaryl), glyburide (DiaBeta, Glynase PresTab, Micronase), insulin, pioglitazone (Actos), rosiglitazone (Avandia), chlorpropamide (Diabinese), glipizide (Glucotrol), and tolbutamide (Orinase). Other potential interactions with BCAAs include Levodopa (for Parkinson's disease), Diazoxide (for low blood sugar), and medications for inflammation (Corticosteroids).

There are risks of hunger and discomfort from having to fast overnight and until four hours after the exercise session for Visit 2. You may also feel bored or inconvenienced waiting between blood draws during Visit 2.

For more information about risks and side effects, ask the investigator.

How will my information be kept confidential?

We will take measures to protect the security of all your personal information, but we cannot guarantee confidentiality of all study data.

Information contained in your study records is used by study staff. The University of New Mexico Human Research Review Committee (HRRC) that oversees human subject research and/or other entities may be permitted to access your records. There may be times when we are required by law to share your information. However, your name will not be used in any published reports about this study.

What are the benefits to being in this study?

There will be no direct benefits to you. However, it is hoped that information gained from this study will help us understand the impact of branched-chain amino acid supplementation on outcomes related to high-intensity eccentric exercise in humans.

What other choices do I have if I do not want to be in this study?

Participation in this study is voluntary, so the only option is not to enroll in the study.

What are the costs of taking part in this study?

There are no direct costs other than your time and a possibility of parking fees if you choose to park on or near the UNM campus.

Will I be paid for taking part in this study?

Yes. You will be paid $10 for completion of each of the five study visits. You will be paid a total of $50 if you complete the study. Payment will occur in the form of gift cards.

What will happen if I am injured or become sick because I took part in this study?

If you are injured or become sick as a result of this study, UNMHSC will provide you with emergency treatment, at your cost.

No commitment is made by the University of New Mexico Health Sciences Center (UNMHSC) to provide free medical care or money for injuries to participants in this study.

In the event that you have an injury or illness that is caused by your participation in this study, reimbursement for all related costs of care will be sought from your insurer, managed care plan, or other benefits program. If you do not have insurance, you may be responsible for these costs. You will also be responsible for any associated co-payments or deductibles required by your insurance. It is important for you to tell the investigator immediately if you have been injured or become sick due to taking part in this study. If you have any questions about these issues, or believe that you have been treated carelessly in the study, please contact the Human Research Review Committee (HRRC) at the (505) 272-1129 for more information.

How will I know if you learn something new that may change my mind about participating?

You will be informed of any significant new findings that become available during the course of the study, such as changes in the risks or benefits resulting from participating in the research or new alternatives to participation that might change your mind about participating.

Can I stop being in the study once I begin?

Your participation in this study is completely voluntary. You have the right to choose not to participate or to withdraw your participation at any point in this study without affecting your future health care or other services to which you are entitled.

HIPAA Authorization for Use of Your Protected Health Information (HIPAA)

As part of this study, we will be collecting health information about you. This information is "protected" because it is identifiable or "linked" to you.

Protected Health Information (PHI)

By signing this Consent Document, you are allowing the investigators and other authorized personnel to use your protected health information for the purposes of this study. This information may include: height, weight, age, %body fat, results of 1RM testing, blood analysis results, and medical and exercise history. You will be asked to let us know if you are taking certain dietary supplements (i.e., caffeine and creatine) or anabolic steroids which may make you ineligible to participate.

In addition to researchers and staff at UNMHSC and other groups listed in this form, there is a chance that your health information may be shared (re-disclosed) outside of the research study and no longer be protected by federal privacy laws. Examples of this include disclosures for law enforcement, judicial proceeding, health oversight activities and public health measures.

Right to Withdraw Your Authorization

Your authorization for the use of your health information for this study shall not expire unless you cancel this authorization. Your health information will be used as long as it is needed for this study. However you may withdraw your authorization at any time provided you notify the UNM investigators in writing.

To do this, please send letter notifying them of your withdrawal to:

Christine Mermier

MSC 04 2610

1 University of New Mexico

Albuquerque New Mexico 87131

Please be aware that the research team will not be required to destroy or retrieve any of your health information that has already been used or shared before your withdrawal is received.

Refusal to Sign

If you choose not to sign this consent form and authorization for the use of your PHI, you will not be allowed to take part in the research study.

Whom can I call with questions or complaints about this study?

If you have any questions, concerns or complaints at any time about the research study, Christine Mermier, or her associates will be glad to answer them at 505-277-2658, Monday through Friday 8:00 a.m. to 5:00 p.m.

If you need to contact someone after business hours or on weekends, please call and ask for Trisha VanDusseldorp at 641-295-2799.

If you would like to speak with someone other than the research team, you may call the UNMHSC HRPO at (505) 272-1129.

Whom can I call with questions about my rights as a research participant?

If you have questions regarding your rights as a research participant, you may call the UNMHSC HRPO at (505) 272-1129. The HRPO is a group of people from UNM and the community who provide independent oversight of safety and ethical issues related to research involving human participants. For more information, you may also access the IRB website at http://hsc.unm.edu/som/research/hrrc/irbhome.shtml.

CONSENT AND AUTHORIZATION

You are making a decision whether to participate in this study. Your signature below indicates that you read the information provided (or the information was read to you). By signing this consent form, you are not waiving any of your legal rights as a research participant.

I have had an opportunity to ask questions and all questions have been answered to my satisfaction. By signing this consent form, I agree to participate in this study. A copy of this consent form will be provided to you.

_____	_____	_____
Name of Adult Subject (print)	Signature of Adult Subject	Date

INVESTIGATOR SIGNATURE

I have explained the research to the participant and answered all of his/her questions. I believe that he/she understands the information described in this consent form and freely consents to participate.

_____
Name of Investigator/ Research Team Member (type or print)

_____	_____
(Signature of Investigator/ Research Team Member)	Date

APPENDIX C.

Male and Female Subjects Needed for *Branched Chain Amino Acid* Supplementation research project at the UNM Exercise Physiology Lab!
HRPO# 14-280

What is the study about? This research is studying the effect of branched-chain amino acids on muscular strength and performance, as well as markers of stress and muscle fiber breakdown.

Who can volunteer? Males and females between the ages of 18-45 yrs who have previous weight-training experience.

What will I be asked to do if I participate? We will measure your body fat %, 1RM, vertical jump, jump squat, and other performance variables. Multiple blood draws required. Total time required: 16 hrs. over a period of two weeks.

Is there any compensation for completing this study?
Yes, all subjects who complete the study will receive $10.00 per exercise session in the form of a gift card (**Total: $50**).

Who can I contact for more information?

Trisha VanDusseldorp, 641-295-2799, tvandusseldorp@unm.edu

Jeremy McCormick, 505-350-8370, aeneid@unm.edu

version 2 10-23-14

APPENDIX D.

HEALTH HISTORY QUESTIONNAIRE **(RESEARCH ONLY 5/20/02)**

Subject #_____ Date___/___/___

*Phone #: home*_____ *cell*_____

Date of Birth ___/___/___Age____ Gender____ Ethnicity_____ Phone _____

Address
(home)_____zip_____

email_____

Primary health care provider and health insurance_____
(Only for information/emergency contact)
Person to contact in case of emergency:
name_____**phone #**_____

♦♦

MEDICAL HISTORY

Self-reported: Height_____ Weight_____
Physical injuries:_____

Limitations_____

Have you ever had any of the following cardiovascular problems? Please check all that apply.

Heart attack/Myocardial Infarction	____	Heart surgery	____	Valve problems	_____
Chest pain or pressure	____	Swollen ankles	____	Dizziness	_____
Arrhythmias/Palpitations	____	Heart murmur	____	Shortness of breath	_____
Congestive heart failure	____				

Have you ever had any of the following? Please check all that apply.

Hepatitis/HIV	_____	Depression	_____	Cancer (specify type)	_____
Rheumatic fever	_____	High blood pressure	_____	Thyroid problems	_____
Kidney/liver disease	_____	Obesity	_____	Total cholesterol >200 mg/dl	_____
Diabetes (specify type)	_____	Asthma	_____	HDL cholesterol <35 mg/dl	_____
Emphysema	_____	Stroke	_____	LDL cholesterol >135 mg/dl	_____
				Trygylcerides>150 mg/dl	_____

Do immediate blood relatives (biological parents & siblings **only**) have any of the conditions listed above? If yes, list the problem, and family member age at diagnosis.

____

Is your mother living? Y N Age at death______ Cause___________________
Is your father living? Y N Age at death______ Cause___________________

Do you currently have any condition not listed that may influence test results? Y N

Details____

____

Indicate level of your overall health. Excellent ____ Good ____ Fair ____ Poor_____

Are you taking any medications, vitamins or dietary supplements now? Y N

If yes, what are they?____

Do you have allergies to any medications? If yes, what are they?

____

Are you allergic to latex? Y N

Have you been seen by a health care provider in the past year? Y N

If yes, elaborate

____

Have you had a prior treadmill test? Y N. If yes, when?______________ What were the results?

____

____

Have you ever experienced any adverse effects during or after exercise (fainting, vomiting, shock, palpitations, hyperventilation)? Y N If yes, elaborate.____

♦♦

LIFESTYLE FACTORS

Do you now or have you ever used tobacco? Y N If yes: type ________________

How long?______ Quantity____/day Years since quitting_______________

How often do you drink the following?

Caffeinated coffee, tea, or soda _______oz/day Hard liquor _______oz/wk Wine _______oz/week

Beer _______oz/wk

Indicate your current level of emotional stress. High____ Moderate ____ Low____

♦♦

PHYSICAL ACTIVITY/EXERCISE

Physical Activity

Minutes/Day (*Weekdays*) Minutes/Day (*Weekends*)

_____/_____ average _____/_____ average

Do you train in any activity (eg. jogging, cycling, swimming, weight-lifting)?

Y N

How well trained are you? _____

Vigorous Exercise (>30 Minute sessions)

_____Minutes/hours a week

♦♦

WOMEN ONLY

Please check the response that most closely describes your menstrual status:

_____ Post-menopausal (surgical or absence of normal menstrual periods for 12 months)

_____ Eumenorrheic – Normal menstrual periods (~every 28 days)

_____ Amenorrheic – Absence of normal menstrual periods for at least 3 months

_____ Oligomenorrheic – Irregular menstrual periods with occasional missed cycles.

This subject meets the medical screening criteria to participate in this study? Y

_____

Name of Investigator/ Research Team Member (print)

_____ _____

(Signature of Investigator/ Research Team Member) Date

_____

Name of Principal Investigator (print)

_____ _____

(Signature of Principal Investigator) Date

APPENDIX E.

My name is Trisha VanDusseldorp / Jeremy McCormick and I am a researcher in the Health, Exercise and Sports Sciences department at the University of New Mexico. I am emailing you to notify you of a research study we are conducting in which you may be an eligible participant. The research study is examining the effect of the branched-chain amino acids on changes in muscular strength and performance as well as markers of stress. If you are a healthy male or female between the ages of 18 – 45 who has previous weight training experience you may be eligible to participate. Throughout the study you will have your body fat %, maximal strength, vertical jump and other performance measures determined. It is estimated this study will require 5 visits to the research laboratory and require you to be at the laboratory a total of approximately 16 hours. You will be eligible to receive compensation for your participation. Please contact Trisha VanDusseldorp at 641-295-2799 (tvandusseldorp@unm.edu), Jeremy McCormick at 505-350-8370 (aeneid@unm.edu) or Christine Mermier, PhD at cmermier@unm.edu if you are interested in participating and would like to learn more about the study.

APPENDIX F

Acute Resistance Exercise and Dose-Response Impact of Branched Chain Amino Acid Supplementation on Muscle Damage, Oxidative Stress and Autophagy

Subject Number: Study Number:_____

Visit 1 Date:_____ Fasted (y/n): _____ Blood Draw (check): ☐

Ht (cm):_____ Standing Reach (in):_____

Wt (kg): _____ Vertical Jump (in): Trial 1:_____ Trial 2:_____ Trial 3: _____

BF%: _____ Squat Jump: Trial 1:_____ Trial 2:_____

1RM (y/n):_____ MVC: _____ ENTER AND SAVE ANTHROPOMETRIC

1 RM (if applicable):_____ SETTINGS IN BIODEX (check): ☐

SEE SEPARATE DATA SHEET FOR VISIT 2!!!

Visit 3 Date:_____ Fasted (y/n): _____ Blood Draw (check): ☐

Standing Reach (in):_____

Vertical Jump (in): Trial 1:_____ Trial 2:_____ Trial 3: _____

Squat Jump: Trial 1:_____ Trial 2:_____

MVC: _____

Visit 4 Date:_____ Fasted (y/n): _____ Blood Draw (check): ☐

Standing Reach (in):_____

Vertical Jump (in): Trial 1:_____ Trial 2:_____ Trial 3: _____

Squat Jump: Trial 1:_____ Trial 2:_____

MVC: _____

Visit 5 Date:_____ Fasted (y/n): _____ Blood Draw (check): ☐

Standing Reach (in):_____

Vertical Jump (in): Trial 1:_____ Trial 2:_____ Trial 3: _____

Squat Jump: Trial 1:_____ Trial 2:_____

MVC: _____

Acute Resistance Exercise and Dose-Response Impact of Branched Chain Amino Acid Supplementation on Muscle Damage, Oxidative Stress and Autophagy

Subject Number: Study Number:_____

Visit 2 Date:_____	Fasted (y/n): _____ Standing Reach (in):_____
70% of 1RM: _____	
Pre-blood draw: ◻	Vertical Jump (in): Trial 1:_____ Trial 2:_____ Trial 3: _____
	Squat Jump: Trial 1:_____ Trial 2:_____
Tubes:	MVC: _____

Squat Exercise (check): Set 1◻ Set 2 ◻ Set 3 ◻ Set 4◻ Set 5 ◻ Set 6 ◻ Set 7 ◻ Set 8◻ Set 9◻ Set 10◻
Split Squat Jumps (check): : Set 1◻ Set 2 ◻ Set 3 ◻ Set 4◻ Set 5 ◻

Post-Exercise	Vertical Jump (in): Trial 1:_____ Trial 2:_____ Trial 3: _____
Post-exercise blood draw: ◻	Squat Jump: Trial 1:_____ Trial 2:_____
Tubes:	MVC: _____

1 hr Post Exercise	Vertical Jump (in): Trial 1:_____ Trial 2:_____ Trial 3: _____
1 hr blood draw: ◻	Squat Jump: Trial 1:_____ Trial 2:_____
Tubes:	MVC: _____

2 hr Post Exercise	Vertical Jump (in): Trial 1:_____ Trial 2:_____ Trial 3: _____
2 hr blood draw: ◻	Squat Jump: Trial 1:_____ Trial 2:_____
Tubes:	MVC: _____

4 hr Post Exercise	Vertical Jump (in): Trial 1:_____ Trial 2:_____ Trial 3: _____
4 hr blood draw: ◻	Squat Jump: Trial 1:_____ Trial 2:_____
Tubes:	MVC: _____

8 hr Post Exercise	Vertical Jump (in): Trial 1:_____ Trial 2:_____ Trial 3: _____
8 hr blood draw: ◻	Squat Jump: Trial 1:_____ Trial 2:_____
Tubes:	MVC: _____

Technitian Initials:_____

APPENDIX G

University of New Mexico

Subject ID _____

INSTRUCTIONS

1. Record everything you eat for 5 days (including one weekend day). If you eat pretzels, record how many. If you eat a bag of chips, record the number of ounces. For drinks, record the number of cups or ounces. Record everything you drink except water.
2. Record the Food, Amount, Brand Name, and Preparation Methods. For example: baked vs. fried chicken; 1 cup of rice; 2 teaspoons of margarine; 1 cup of 2% milk; McDonald's, Healthy Choice, or Frosted Flakes.
3. Record immediately after eating. Waiting until that night may make it difficult to remember all foods and quantities.

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

APPENDIX H

University of New Mexico

Exercise Physiology Laboratory

Perceived Muscle Soreness Rating

Directions:

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

Time point / Testing Session: _____ Date: _____

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