Effects Of Marine Lipid Fraction PCSO-524™ Supplementation On Biochemical And Functional Measures Of Muscle Damage And Soreness In Untrained Men

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Abstract

Effects of marine lipid fraction PCSO-524™ supplementation on biochemical and functional measures of muscle damage and soreness in untrained men

INTRODUCTION: Intensive or unaccustomed eccentric exercise is known to cause exercise-induced muscle damage (EIMD) commonly resulting in delayed onset muscle soreness (DOMS). EIMD/DOMS can result in decrements in endurance and resistance exercise performance. Although many treatments have been investigated for the prevention and alleviation of EIMD/DOMS, success has been limited. PCSO-524™ has been found to attenuate symptoms of inflammatory diseases such as arthritis and asthma, and thus may be a viable treatment for EIMD/DOMS and may attenuate related exercise performance decrements.

PURPOSE: The purpose of this study was to examine whether or not PCSO-524™ supplementation could attenuate loss of range of motion, force loss, swelling, perceived pain through algometry and biochemical markers of inflammation, intracellular muscle proteins and oxidative stress after eccentric muscle damage.

METHODS: Subjects were thirty-two healthy, untrained males aged 18–26 who had not participated in a resistance training program in the past sixty days and did not exercise for more than three times per week for 30 minutes each session. Subjects supplemented for four weeks with either PCSO-524™ (400 mg/day) or placebo, after which time participated in a 20-minute downhill run (DHR) at -16% grade. Functional measures of DOMS were measured by algometry, midline thigh girth, and maximal voluntary contraction, following supplementation and 24, 48, 72 and 96 hours following eccentric exercise. Biochemical indicators of muscle damage such as Creatine Kinase-MM, Myoglobin, Slow skeletal troponin I, Fatty Acid Binding Protein 3, Interleukin-6, Tumor Necrosis Factor Alpha, 8-hydroxy-2' -deoxyguanosine (8-OHdG), and Cardiac Troponin I was measured pre-supplementation, post-supplementation and following DHR at 0, 2, 24, 48, 72 and 96 hours.

RESULTS: PCSO-524™ attenuated maximal voluntary contraction force loss significantly at 96 hours ($F = 8.140$ $p < .001$). PCSO-524™ attenuated loss of range of motion at 96 hours compared to baseline ($F = 8.236$ $p < .05$) for the PCSO-524™ group but did not change

for the placebo group. There was no significant change in muscle swelling from baseline as measured by thigh girth for either PCSO-524™ or placebo. There was no significant change in perceived muscle soreness as measured by algometry for either PCSO-524™ or placebo. There was significant attenuation of creatine kinase MM in the blood following EMD immediately ($t = 4.467$, $p < .001$; 95% CI = 63.0 – 169.2), 2 ($t = 5.076$, $p < .001$; 95% CI = 76.1 – 178.6), 24 ($t = 12.829$, $p < .001$; 95% CI = 562.5 – 775.6), 48 ($t = 11.477$, $p < .001$; 95% CI = 493.2 – 706.7), 72 ($t = 9.118$, $p < .001$; 95% CI = 359.8 – 567.5), and 96 hours ($t = 11.935$, $p < .001$; 95% CI = 574.4 – 811.6). Significant attenuation of myoglobin occurred 24 ($t = 4.334$, $p < .001$; 95% CI = 22.9 – 63.7), 48 ($t = 6.402$, $p < .001$; 95% CI = 67.7 – 131.1), 72 ($t = 5.340$, $p < .001$; 95% CI = 119.0 – 266.3), and 96 hours ($t = 3.272$, $p < .01$; 95% CI = 49.0 – 212.3) compared to placebo. Significant attenuation of skeletal muscle troponin I occurred in the PCSO-524™ group compared to placebo 2 ($t = 2.562$, $p < .05$; 95% CI = 0.9 – 8.0), 24 ($t = 3.561$, $p < .01$; 95% CI = 4.4 – 15.4), 48 ($t = 4.095$, $p < .001$; 95% CI = 4.7 – 14.0), 72 ($T = 3.222$, $p < .01$; 95% CI = 2.5 – 11.2), and 96 hours ($T = 2.177$, $p < .05$; 95% CI = 0.3 – 10.4) post EIMD. No significant effect of supplementation status on fatty acid binding protein appearance in the blood ($F = .418$ $p = .523$) post EMID. Significant attenuation of Interleukin-6 was observed 24 ($t = 3.619$, $p < .01$; 95% CI = 3.7 – 13.3), 48 ($t = 3.993$, $p < .001$; 95% CI = 5.8 – 17.8), 72 ($t = 3.621$, $p < .01$; 95% CI = 5.0 – 17.9), and 96 hours ($t = 3.076$, $p < .01$; 95% CI = 3.1 – 15.35) post EMID compared to placebo. A significant effect of time on TNF alpha concentration in the blood ($F = 132.012$ $p < .001$) was observed 24 ($t = 4.633$, $p < .001$; 95% CI = 11.1 – 28.5), 48 ($t = 4.942$, $p < .001$; 95% CI = 10.6 – 25.7), 72 ($t = 4.747$, $p < .001$; 95% CI = 11.3 – 28.5), and 96 hours ($t = 6.349$, $p < .001$; 95% CI = 16.8 – 32.8) post EIMD. No significant effect of time on 8-OHdG concentration in the blood ($F = 1.018$ p .419) was observed in either the PCSO-524™ group or placebo after EIMD. No significant effect of time was observed on cTn1 concentration in the blood ($F = .420$ $p = .889$) in either PCSO-524™ or placebo after EIMD.

CONCLUSIONS: Four weeks of PCSO-524™ supplementation does not improve swelling as determined by thigh girth; perceived pain by the use of algometry; appearance of Fatty Acid Binding Protein, 8-hydroxy-2' -deoxyguanosine (8-OHdG), and Cardiac Troponin I in the blood in

untrained males after downhill running compared to placebo. Four weeks of PCSO-524™ supplementation is effective at attenuating maximal voluntary contraction force loss; loss of range of motion; and reducing the appearance of creatine kinase-MM; skeletal muscle troponin I; interleukin-6; and Tumor Necrosis Factor- alpha in the blood in untrained males after downhill running.

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Chapter 1: Introduction

Exercise-induced muscle damage (EIMD) is an event that occurs after unaccustomed eccentric exercise and sustained metabolically demanding activities, resulting in a temporary rise in passive muscle tension, decrease in muscle force production (Ebbeling & Clarkson, 1989), increased perceptions of muscle soreness (Miles & Clarkson, 1994), localized swelling (Howell, Chleboun, & Conatser, 1993) (Howatson & Van Someren, 2008), decreased range of motion (Kazunori Nosaka & Clarkson, 1995) (McHugh et al., 1999) and greater concentrations of intramuscular proteins in blood (Howatson & Van Someren, 2008) (Stephan Sorichter et al., 1997). While the causes of muscle damage are unclear, literature points to mechanical damage, oxidative damage and a combination of both.

Mechanical damage to the muscle results from the disruption of contractile filaments of muscle tissue especially following eccentric exercise (D. J. Newham, D. A. Jones, & P. M. Clarkson, 1987). Myofibril structural changes post-eccentric exercise are evidenced by broadening or total disruption of z-lines (Cheung, Hume, & Maxwell, 2003) (Garrett & Kirkendall, 2000), myofilament loss, reduced numbers of mitochondria, disturbed A-band filaments (J. Friden, Sjostrom, & Ekblom, 1981) (J Friden, Kjörell, & Thornell, 1984), and extracellular matrix disruption due to inflammation (Stauber, Clarkson, Fritz, & Evans, 1990).

While the exact mechanism of loss of force is unknown, it has been hypothesized that the cause may lie in the tendon attachments, elastic muscle elements or loss of function of the excitation contraction coupling (Warren et al., 1993). Force loss is immediate, but temporary, with more intense bouts of exercise correlated to greater losses in magnitude requiring a longer duration until force production is restored to baseline levels (D. Newham, D. Jones, & P. Clarkson, 1987). Additionally, training status appears to play a role in the extent of muscle damage with untrained subjects experiencing a greater degree of EIMD/DOMS than trained individuals (Proske & Morgan, 2001).

Muscle damage results in increased swelling due to an influx of fluid into the muscle cell as evidenced by MRI (Shellock, Fukunaga, Mink, & Edgerton, 1991). Swelling is most pronounced 6 days post-muscle damaging exercise, and is thought to occur either due to connective tissue injury, increased capillary permeability, muscle cell protein degradation, or a combination of factors. (Mair et al., 1992) (Takahashi et al., 1994). Swelling is thought to be responsible for the increase in the sensation of pain due to fluid influx into the damaged muscle and subsequent increase in pressure on nociceptors (Crenshaw, Thornell, & Friden, 1994).

Muscle damage results in increased levels of intracellular proteins in the blood such as skeletal muscle troponin 1, fatty acid binding protein, myoglobin (Mb) and inflammatory mediators such as interleukin-6 (IL-6) and tumor necrosis factor (TNF-α). It is thought that intracellular proteins diffuse out of the cell into the surrounding tissue and capillaries due to either sheer stress or free radical damage to the membrane following eccentric exercise (W. Evans & Cannon, 1991). These proteins attract macrophages and T-cells, which release inflammatory mediators that amplify the initial muscle injury through increased release of activation of phospholipases, proteases, and reactive oxygen species causing inflammation (MacIntyre, Reid, & McKenzie, 1995) (P. Calder & Grimble, 2002). These mediators, in addition to cytokines, such as IL-6, coordinate the immune response at the site of injury. IL-6 is a pro-inflammatory cytokine involved in lipid peroxidation which degrades the cell membrane (Hinson, Williams, & Shacter, 1996). TNF-α is another important mediator of the inflammation process with elevated expression in injured muscle fibers (R. A. Collins & Grounds, 2001) and necessary for muscle repair (Y.-P. Li, 2003). Treatment strategies have sought to minimize inflammation inhibiting the inflammatory pathways and immune cell activation by the use of NSAIDs and antioxidants such as fish oil (P. Calder & Grimble, 2002) . NSAIDs inhibit inflammatory pathways, such as COX-2, while fish oil, which contains n-3 PUFAs such as eicosapentaeoic (EPA) and docosahexaenoic acid (DHA) inhibit lymphocyte proliferation and the inflammatory cascade (Dubois et al., 1998; Trebble et al., 2003). However inflammation may provide a vital role in muscle repair as performance is inhibited when the inflammatory process is impaired (Almekinders, 1999). Free radicals can also

be generated from inflammatory cells responding to increases in markers of cellular damage such as TNF-α, Myoglobin (Mb) and creatine kinase (CK) released into the blood from ruptured cells. Inflammatory cells increase rates of tissue destruction and remodeling through the recruitment of macrophages through increased concentrations of cytokines and interleukins (Tiidus, 2008).

Therapies seeking to minimize EIMD/DOMS have typically inhibited COX2 enzymes with the use of over the counter non-steroidal anti-inflammatory drugs (NSAIDs) such as Tylenol or Aleve. NSAIDs inhibit prostaglandin formation which are important mediators for the inflammatory response (Burian & Geisslinger, 2005). Studies have found that NSAID supplementation post-eccentric muscle exercise can improve contractile function and reduce circulating levels of inflammatory cells and intercellular muscle enzymes (Howatson & Van Someren, 2008; Mishra, Friden, Schmitz, & Lieber, 1995). However, chronic NSAID use has been linked to gastric bleeding, fluid retention, hypertension and acute renal failure if taken too close to exercise (Boulter, Noakes, & Hew-Butler, 2011; Derry & Loke, 2000).

While metabolism of the components in fish oil such as EPA and DHA are less inflammatory and used in the same metabolic pathway as NSAIDs, studies are inconclusive on whether fish oil supplementation on EIMD/DOMS treatment is effective (Macaluso et al., 2013). For example, 1.8g/day omega 3 supplementation proved effective in ameliorating delayed onset muscle soreness in untrained males after eccentric knee exercise (Bakhtiar Tartibian, Behzad Hajizadeh Maleki, & Asghar Abbasi, 2009). However, it has been shown that n-3 PUFA supplementation at 1.8g/day for 30 days was not effective in attenuating the acute phase inflammatory response or functional measures of muscle damage after 50 repetitions of elbow flexion exercise in 22 healthy male subjects (Lenn et al., 2002).

In addition to the immune system, metabolic processes such as the electron transport chain within the mitochondria generate free radicals which damage the muscle sarcolemma phospholipids through lipid peroxidation, causing a loss of membrane integrity ultimately leading

to cell death (Davies, Quintanilha, Brooks, & Packer, 1982). Lipid peroxidation can be quantified by using fatty acid binding protein which selectively binds to the products of lipid peroxidation and is involved in intracellular membrane transfer (B. A. McMahon & Murray, 2010) (Weisiger, 2002). It has been found that preventing free radical formation resulted in decreased sensations of muscle damage with the extent of cellular damage quantified using 8-Oxo-2'-deoxyguanosine (8-OHdG) as a marker of DNA oxidative damage (A. R. Collins, Dusinská, Gedik, & Sětina, 1996) (Mastaloudis et al., 2004). 8-OHdG production results from the interaction between free radicals and DNA which may result in purine-pyrimidine base substitution mutations linked to inflammation (de Souza-Pinto et al., 2001).

Treatment of oxidative damage can be minimized through consumption of food and supplements rich in antioxidants such as vitamin E, C and beta carotene (Powers, Ji, & Leeuwenburgh, 1999). These compounds react with the free radicals and neutralize them, thus preventing damage to the cell membrane (Machlin & Bendich, 1987). Additionally, damaging reactions can be attenuated with consumption of foods rich in n-3 PUFAs such as eicosapentaenoic acid (EHA) and docosahexaenoic acid (DHA) due to their ability to be preferentially oxidized instead of the phospholipids that comprise the membrane (Thompson et al., 2001). DHA is an n-3 PUFA that is a structural component of the phospholipid bilayer that can be synthesized from alpha-linolenic acid or obtained directly from fish oil. DHA is a carboxylic acid with a 22-carbon chain and six cis double bonds with the first double bond located at the third carbon from the omega end (Guesnet & Alessandri, 2011). Eicosapentaenoic acid (EPA) is an n-3 PUFA which acts as a precursor for prostaglandin-3, thromboxane-3, and leukotriene-5 groups (Schmitz & Ecker, 2008). The number and placement of the double bonds in the EPA and DHA contribute to it's anti-inflammatory properties compared to n-6 PUFAs (arachidonic acid) when oxidized by free radicals during lipid peroxidation (Mori et al., 2000).

Supplementing with n-3 PUFAs, such as EPA and DHA, have been used to treat inflammation in patients with certain chronic inflammatory diseases such as arthritis and asthma (James &

Cleland, 1997; Mickleborough, Lindley, Ionescu, & Fly, 2006). Cellular membrane incorporation of polyunsaturated fatty acids, particularly EPA and DHA, decrease release of pro-inflammatory mediators such as 2-series prostaglandins and 4–series leukotrienes (Eriksen, Sandvik, & Bruusgaard, 1996).

Prostaglandins and leukotrienes are a subfamily of inflammatory mediators known as eicosanoids that are signaling molecules that mediate inflammation, fever and pain and derived from either n-3 or n-6 PUFAs from the phospholipids of the cell membrane. While products of both n-3 or n-6 PUFAs are generally considered inflammatory, n-3 PUFAs are less inflammatory than n-6 PUFAs (De Meester, Watson, & Zibadi, 2013). Prostaglandin synthesis proceeds along the cyclooxygenase-1, and cyclooxygenase-2 pathways while leukotriene biosynthesis proceeds along the 5, 12, and 15-lipoxygenase pathways in response to cytokines, growth factors and other inflammatory stimuli (Ballou, Botting, Goorha, Zhang, & Vane, 2000). Prostaglandins contribute to pain by directly activating pain receptors by increasing levels of cyclic AMP and enhancing nociceptor sensitization (Rueff & Dray, 1993). It has been shown that diets high in n-3 PUFAs reduce the effect of inflammatory diseases such as asthma and arthritis by affecting eicosanoid production (Harizi, Corcuff, & Gualde, 2008) (Kremer et al., 1985) (P. Calder & Grimble, 2002).

Chemical analysis has elucidated that the New Zealand green-lipped mussel (NZGLM) known as *Perna canaliculus*, contains EPA and DHA, 91 different polyunsaturated and furan fatty acids (Wolyniak, Brenna, Murphy, & Sinclair, 2005). The EPA and DHA composition is approximately 13% EPA, 21% DHA, 30% cholesterol and contains up to 91 polyunsaturated fatty acids including 5,9,12,15-octodecatretraenoic acid, 5,9,12,16-nondecatertraenoic acid, 7,11,14,17-eicosatetraenoic acid, 5,9,12,15,18-heneicsapententaenoic acid, oleic acid, stearic acid, palmitoleic acid, palmitic acid and myristic acid (Wolyniak, et al., 2005). PCSO-524™ (Lyprinol/Omega XL) is a supplement manufactured from the New Zealand Green Lipped Mussel that has been shown to reduce airway inflammation and bronchoconstriction in asthmatics,

attenuate colon inflammation in irritable bowel syndrome, decrease joint inflammation in rheumatoid arthritis patients, and reduce ADHD symptoms in children (Kean et al., 2013; Mickleborough, Vaughn, Shei, Davis, & Wilhite, 2013; Tenikoff, Murphy, Le, Howe, & Howarth, 2005; Zawadzki, Janosch, & Szechinski, 2013a).

Additionally, PCSO-524™ contains furan fatty acids which have been shown to possess greater anti-inflammatory properties than EPA and DHA alone by quenching the lipid peroxidation processes in the cell by the free radical scavenging properties of the furan ring in *Dehalococcoides* bacterial strains (Teixeira, Cox, & Egmond, 2013; White et al., 2005). Free radical scavengers reduce pro-inflammatory lipid peroxidation products such as leukotriene B4 (LTB4) in human monocytes and LTB4 and eicosanoids such as 5-Hydroxyeicosatetraenoic acid (5(S)-HETE) in human neutrophils, inhibit the COX-1 and COX-2 enzymes, prevent interleukin tumor necrosis factor and interferon synthesis. (Wakimoto et al., 2011). Ingestion of PCSO-524™ in osteoarthritic patients reduced perceptions of pain compared to fish oil (Zawadzki, Janosch, & Szechinski, 2013b).

Additionally, PCSO-524™ has shown potent anti-inflammatory activity in rat paw edema assays at a concentration lower than fish oil, which contained abundant EPA (M. Whitehouse, 2001). PCSO-524™ has been shown to be more effective than fish oil in reducing symptoms of experimentally induced inflammatory bowel disease (Tenikoff, et al., 2005). This suggests that the anti-inflammatory effects of PCSO-524™ may not be solely due to the EPA and DHA content, since the n-3 PUFA content of PCSO-524™ compared to fish oil is significantly lower (Tenikoff, Murphy, Le, Howe, & Howarth, 2005). Since none of the fatty acids are unique to PCSO-524™, it is possible that that additional ingredients such as the 91 polyunsaturated fatty acids and furan components act together with n-3 PUFAs to attenuate the inflammatory response (Wakimoto, et al., 2011).

Important to this study, runners supplemented with PCSO-524™, experienced less DOMS following a 30-kilometer run than placebo (Baum, Telford, & Cunningham, 2013). However, Pumpa et al. (2011) demonstrated that 8 weeks of supplementation with 200 mg/day of PCSO-524™ did not affect performance measures (Visual analogue scale, algometry, and force production) and biochemical markers (IL-6 IL-10, TNF-alpha, C reactive protein, myoglobin, and creatine kinase) compared to placebo (Pumpa, Fallon, Bensoussan, & Papalia, 2011). Therefore, the data are equivocal as to whether PCSO-524™ can attenuate the functional and biochemical markers of muscle damage.

Statement of the Problem

It has been shown that EPA and DHA supplementation attenuate acute tissue inflammation and minimize DOMS. DOMS and inflammation are two of the symptoms of EIMD which when exacerbated through chronic training may result in greater tissue damage and decreased strength gains. PCSO-524™ is a marine extract from the New Zealand Green Lipped Muscle, and has been shown effective in attenuating inflammation in chronic inflammatory diseases such as asthma, rheumatoid arthritis, and irritable bowel syndrome. PCSO 524's™ effectiveness in reducing inflammation may be due not only to its EPA and DHA content, but also its furan fatty acids and up to 91 different lipids which are thought to integrate into the membrane and provide a less inflammatory substrate and absorb reactive oxygen species thereby minimizing tissue damage. To date, there has been no research investigating the ability of PCSO-524™ (Lyprinol®/Omega XL®) to attenuate the functional and biochemical measures of EIMD/DOMS in untrained males after a continuous bout of eccentric aerobic exercise.

Hypotheses

Hypothesis 1: The percent change in force production from baseline for subjects that consumed PCSO-524™ will be higher compared to those that consumed placebo.

Hypothesis 2: The percent change in quadriceps range of motion from baseline for subjects that consumed PCSO-524™ will be higher, compared to placebo.

Hypothesis 3: The percent change in size of the midline of the quadriceps will be less in subjects that supplemented with PCSO-524™ than placebo.

Hypothesis 4: The percent change in the perception of pain at the quadriceps from baseline measured by algometry in subjects that supplemented with PCSO-524™ will be less than compared to placebo.

Hypothesis 5: Supplementing untrained men with PCSO-524™ for 4 weeks will lessen the appearance of creatine kinase in the blood post eccentric muscle damage compared to placebo.

Hypothesis 6: Supplementing untrained men with PCSO-524™ for 4 weeks will lessen the appearance of myoglobin in the blood post eccentric muscle damage compared to placebo.

Hypothesis 7: Supplementing untrained men with PCSO-524™ for 4 weeks will lessen the appearance of skeletal muscle troponin I in the blood post- eccentric muscle damage compared to placebo.

Hypothesis 8: Supplementing untrained men with PCSO-524™ for 4 weeks will lessen the appearance of fatty acid binding protein in the blood post- eccentric muscle damage compared to placebo.

Hypothesis 9: Supplementing untrained men with PCSO-524™ for 4 weeks will lessen the appearance of interleukin-6 in the blood post eccentric muscle damage compared to placebo.

Hypothesis 10: Supplementing untrained men with PCSO-524™ for 4 weeks will lessen the appearance of TNF alpha in the blood post eccentric muscle damage compared to placebo.

Hypothesis 11: Supplementing untrained men with PCSO-524™ for 4 weeks will lessen the appearance of cardiac muscle troponin I in the blood post- eccentric muscle damage compared to placebo.

Purpose of the Study

The purpose of this study was to evaluate whether chronic PCSO-524™ supplementation in untrained males attenuates exercise induced muscle damage following eccentric muscle exercise; specifically reduction in range of motion, perceived muscle soreness, cytokines, muscle proteins, and inflammatory mediators.

Delimitations

Untrained males were recruited from the Indiana University classifieds and tested in a laboratory setting with environmental conditions carefully controlled. Muscle damaging exercise was performed in a laboratory setting on a treadmill at a predetermined speed. Subjects were asked to refrain from consuming fish oil, NSAIDS, or marine related products during the course of the study. Additionally, subjects were requested not to consume alcohol or caffeine for at least 24 hours prior to lab testing. Additionally subjects maintained their normal diet and were asked not to exercise more than 30 minutes a day, three days a week while refraining from performing any eccentric exercise. Blood draws were performed at the same time during subsequent days.

Limitations

Since personal histories were self-reported, subjects could have misrepresented their background or exercise habits in order to gain access to the study. Additionally, since supplementation was performed away from the lab and self reported, subjects might not have consumed the required number of capsules during the supplementation period. Subject pain perception might have varied between visits and while spots were marked to ensure consistent measurement, some subjects repeatedly washed markers off which may have resulted in marks not being reapplied in exactly the same location on subsequent days. For range of motion the goniometer relied on a correct point of view and alignment, even slight variations of the instrument may result in large deviations.

Assumptions

Subjects did not change their diet or exercise habits throughout the study. Subjects did not change their perception of pain throughout the study. The treadmill protocol was a valid and sufficient method in inducing exercise induced muscle damage. Subjects were motivated to complete the study and were rested and hydrated prior to their visit. Algometry tests were subjective and the amount of pain perception by each subject did not vary between lab visits. Blood draws were performed in such a way that no significant hemolysis occurred.

Definition of terms

8-OHdG: 8-Hydroxyguanosine is a nucleoside marker of oxidative stress. Its appearance is thought to be the result of DNA damage as a result of free radical exposure (Wu, Chiou, Chang, & Wu, 2004).

Akt/mTOR/S6K1: A signaling cascade which plays an important role both in cell survival (Gingras et al., 2007b).

COX: Cyclooxygenase is an enzyme that creates prostanoids, including prostaglandins, prostacyclin and thromboxane. These products contribute to pain and inflammation (Seibert et al., 1995).

DAG: Diglycerol A Glyceride consisting of two fatty acid chains covalently bonded to a glycerol molecule through ester linkages. DAG is a second messenger signaling lipid, that is produced by the hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) by the enzyme phospholipase C which results in inositol trisphosphate (IP3) (Hardie, 2003).

NF-κB: A protein complex that controls the transcription of DNA. NF-κB is thought to be involved in cellular responses to stimuli such as stress, cytokines, and free radicals. NF-κB regulates the immune response (Shih, Tsui, Caldwell, & Hoffmann, 2010).

p70s6k: a serine/threonine kinase that acts downstream of PIP3 and phosphoinositide-dependent kinase-1 in the PI3 kinase pathway and is activated by mTOR inducing protein synthesis at the ribosome. Physical exercise has been shown to phosphorylate p70s6k which results in protein synthesis (Pearson et al., 1995).

PCSO-524™: A marine lipid extract from the New Zealand green-lipped mussel (Perna canaliculus). The extract contains 30 polyunsaturated fatty acids including the essential n-3 PUFAs such as DHA and EPA, furan fatty acids and up to 91 polar lipids (Wakimoto, et al., 2011).

PIP2: Phosphatidylinositol 4,5-bisphosphate is a minor phospholipid component of cell membranes. PIP2 is enriched at the plasma membrane where it is a substrate for a number of important signaling proteins in the IP3/DAG pathway (Czech, 2000).

PIP3: Is a phospholipid that resides on the plasma membrane that activates downstream signaling components, such as protein kinase AKT, which activates downstream anabolic signaling pathways required for cell growth and survival (Czech, 2000).

PLA2: A class of enzymes that recognize and catalytically hydrolyze the sn-2 acyl bond of phospholipids releasing arachidonic acid that undergoes modification into active compounds by cyclooxygenase enzymes resulting in formation of eicosanoids that include prostaglandins and leukotrienes that can act as pro and anti-inflammatory mediators (Balsinde, Balboa, Insel, & Dennis, 1999).

PUFA: Fatty acids have 2 or more cis double bonds that are separated from each other by a single methylene bridge. Fatty acid notation can be is X:Y where X is the length of the carbon chain and Y is the number of methylene bridges in the compound (C. M. Williams & Burdge, 2006).

ROM: Range of Motion is the angular distance that a limb may normally travel while attached to another (H. M. Clarkson, 2000).

ROS: Chemically reactive molecules containing oxygen which are formed as the byproduct of metabolism and play important roles in cell signaling and homeostasis and increase dramatically during times of cellular stress which can cause damage to cellular structures (Kane et al., 1993).

Chapter 2: Literature review

PCSO-524™

The green-lipped mussel is native to New Zealand and an integral part of the diets of coastal indigenous tribes and is thought to contribute to a lower incidence of arthritis compared to inland tribes due to its anti-inflammatory properties. Recent attempts to purify the compound to create a supplement for arthritis sufferers proved difficult due to the instability of the active components resulting in products with no anti-inflammatory capacity (Galliard, 1970). Purification was finally achieved through the use of supercritical fluid extraction and the resulting compound is known as PCSO-524™. PCSO-524™ is consumed in capsule form under the tradename Lyprinol® and Omega XL® with each capsule containing 50 mg PSCO-524™ lipid extract, 100 mg olive oil and 0.225 mg vitamin E (d- α -tocopherol). PCSO-524™ contains a multitude of lipid classes including triglycerides, sterol esters, polar lipids, free fatty acids, and sterols. Ninety-one fatty acid components have been identified in PSCO-524™ including, palmitic acid, 5,9,12,16-nondecatertraenoic acid, 5,9,12,15,18-heneicsapententaenoic acid, myristic acid, 5,9,12,15-octodecatretraenoic acid, palmitoleic acid, stearic acid, oleic acid and 7,11,14,17-eicosatetraenoic acid. Omega fatty acids were the most abundant unsaturated fatty acids in PSCO-524™ with 140 mg/mL eicosapentaenoic acid (EPA, 20:5) and 87 mg/mL docosahexaenoic acid (DHA, 22:6) present in the purified extract (Murphy, Mooney, Mann, Nichols, & Sinclair, 2002; Wolyniak, et al., 2005).

PCSO-524™ has been investigated with positive effects in both acute and chronic inflammatory diseases, such as asthma, osteoarthritis in rats, inflammatory bowel disease models in mice and acute inflammation such as muscle soreness after a 30 kilometer run in humans (Baum, et al., 2013; Lello, Liang, Robinson, Leutenegger, & Wheat, 2012; Tenikoff, et al., 2005; Zawadzki, Janosch, & Szechinski, 2013c).

Lello at al (2012) investigated PCSO-524™ supplementation in asthmatic children aged 6 to 13 years old. 71 children participated in the 16-week, double blind placebo-controlled study and received either 2 capsules daily Lyprinol® or placebo and were maintained on an as-needed inhaled corticosteroid. The study found that PCSO-524™ supplementation decreased the percentage of children reporting trouble with their asthma symptoms at three months of treatment (97% vs. 76% p = 0.057). PCSO-524™ attenuated mild and moderate asthma exacerbations. The study found that PCSO-524™ has a positive effect for asthma sufferers and should be considered a nutritional supplement for children with moderate asthma and possibly a supplement to conventional asthma therapy.

Zawadzki at al. (2013) demonstrated that PCSO-524™ supplementation attenuates pain to a greater extent compared to fish oil supplementation alone. 47 patients completed the randomized blind study with 22 patients supplementing with 1200 mg/day (4 capsules) PCSO-524™ and 25 subjects supplementing with fish oil (4 capsules; 150 mg/capsule; total n-3 PUFA 18% EPA and 12% DHA). Patients supplemented with PCSO-524™ showed a statistically significant improvement compared with patients who took fish oil. PCSO-524™ supplementation resulted in lower pain scores within four weeks, improvement in health and disease, and increased blood cell count. Patients that supplemented with fish oil showed significantly less improvement and a greater level of physical discomfort during the study.

Investigating the potential of PCSO-524™ supplementation on DOMS symptoms, Baum et al. (2013) found after a 30 kilometer run by 32 nonprofessional distance runners, compared to no

changes in the control group while runners supplemented with PCSO-524™ had significant attenuation of muscle soreness following their post-treatment run compared to the pre-treatment run. Median baseline pain scores for the control group did not differ from post-treatment pain scores in the PCSO-524™ group. Runners that trained under 3 times a week experienced the greatest benefit in attenuated DOMS perception suggesting that PCSO-524™ supplementation may be effected by training status and suited for untrained rather than trained athletes due to lack of conditioning.

Additionally, other studies found that subject-training status was important for the study outcome as evidenced by Pumpa et al. (2013) who showed PCSO-524™ was ineffective at attenuating the symptoms of DOMS effective in 20 well-trained male subjects. Each group of 10 subjects consumed an olive oil capsule (placebo) or 200 mg of PCSO-524™ daily for eight weeks. After the supplementation period, muscle damage was induced with each subject performing 5 bouts of 8 minutes running downhill at a −10% gradient with 2 minutes level walking between bouts at 80% maximal heart rate. The results suggest that PCSO-524™ supplementation at 200 mg/day was ineffective at attenuating the symptoms of DOMS with no significant differences between groups on functional measures such as perception of pain, algometry and biochemical markers such as Interleukin-1, Interleukin-6, Interleukin-10, C-reactive protein, creatine kinase, tumor necrosis factor-α, and myoglobin spaced 4, 24, 48, 72 and 96 hours after muscle damage (Pumpa, et al., 2011).

PCSO-524™'s effectiveness may have beneficial effects on muscle damage and recovery processes by minimizing the inflammatory response and DOMS that occurs following EIMD in untrained individuals. There are numerous histological changes that occur inside the muscle cell after it has been damaged, including toxic intracellular proteins which leak out of the cell into the microenvironment which attract macrophages which release reactive oxygen species causing further damage to both injured and healthy muscle cells. Additionally there is an increase in cytosolic calcium due to increased sarcoplasmic reticulum permeability resulting in

Phospholipase A2 activation which cleaves unsaturated membrane phospholipids to initiate the arachidonic acid cascade which is an eicosanoid precursor including four-series leukotrienes (LT4) and two-series prostaglandins (PGE2) catalyzed by cyclooxygenase and 5-lipoxygenase. The arachidonic acid cascade culminates in membrane damage if lipid peroxidation is excessive. Additionally elevated levels of calcium cause rapid damage of force generating myofilaments (Duncan, 1988).

PCSO-524™'s anti-inflammatory ability for inhibition of both cyclooxygenase-2 and 5-lipoxygenase inhibiting leukotriene B4 (LTB4) and prostaglandin production has been demonstrated in both human polymorphonucleocytes and neutrophils (M. W. Whitehouse et al., 1997). Additionally PCSO-524™ contains furan fatty acids that scavenge reactive oxygen species produced from activated neutrophil and cellular metabolic processes and have been shown to have antioxidant effects (Ishii, Okajima, Koyamatsu, Okada, & Watanabe, 1988). It is hypothesized that furan fatty acids have a tetraalkyl-substituted furan ring, which is susceptible to oxidation, and the subsequent ring opening generates dioxoene, which is highly reactive and further converted to other compounds. Dioxenes are extremely reactive, causing total inhibition of products of the arachidonic acid pathway at concentrations as low as 20 μ M (Graff, Gellerman, Sand, & Schlenk, 1984). These naturally but rarely detected fatty acids show a potent radical-scavenging ability and are essential constituents of plants and algae and have recently started to become appreciated as a key link between the cardio protective effects of a diet high in EPA and DHA (Spiteller, 2005). For example, PCSO-524™ had greater anti-inflammatory activity in a rat model of adjuvant-induced arthritis than the same dose of EPA and DHA alone. (Wakimoto, et al., 2011).

Process of Muscle Damage

Muscle damage, which can occur as a result of eccentric muscle action, is postulated to follow five phases: the initiating event, early structural changes, necrosis and degeneration, regeneration, and protection from further insults. Muscle damage has been shown to be localized and only affect a small percentage of muscle fibers in skeletal muscle (H Kuipers, Drukker,

Frederik, Geurten, & Kranenburg, 1983). Initial events in muscle damage can be observed in the first hours following eccentric exercise with the affected site experiencing muscle swelling, contractual protein loss such as desmin and troponin, followed by phagocytic cell invasion (Armstrong, 1990; Stephan Sorichter, et al., 1997).

The first phase of exercise induced skeletal muscle injury is the initiating event. While this phase is poorly understood, hypothetical postulations have centered on either physical or metabolic events. The role that physical damage plays into the initiating event has been postulated to be caused by three factors. First, force production due to eccentric exercise generally exceeds maximal isometric force by 100% (Woledge, Curtin, & Homsher, 1985). Secondly, the area of force to active fiber ratio during eccentric contractions compared to isometric contractions is greater (T. A. McMahon, 1984). Finally, it has been shown that the number of cross bridges decreases as lengthening velocity of muscle increases (1984). The metabolic initiating mechanism follows that the muscle is not damaged by mechanical but by disruption of metabolic processes such as lack of ATP availability that induces intracellular calcium overload. Support for this theory has been offered by studies that have induced structural changes similar to exercise induced muscle damage in resting muscle in rats after ischemia (Mäkitie & Teräväinen, 1977). However, studies have shown that greater energy expenditure in rats does not correlate with increasing muscle damage suggesting that muscle damage does not correlate with metabolic demands (J Komulainen, Takala, & Vihko, 1995).

The initial event also produces temporary force loss, which occurs immediately after the initial event and lasts several days following muscle-damaging exercise (Clarkson, Nosaka, & Braun, 1992). The cause of force loss has been attributed to damage to the sarcoplasmic reticulum delaying the restoration of normal calcium levels and as a consequence impaired force generation (Clarkson, et al., 1992). The second hypothesis to explain force loss is the loss of overlap between the actin and myosin filaments due to the longer distance that sarcomeres must travel during downhill running (Eston, et al., 1995). Force loss during eccentric exercise is

correlated to the relative muscle length with greater force losses occurring at longer muscle lengths (Newham, et al., 1988).

Regardless of whether the initiating event is physical or metabolic, the next step in the initiating event is disturbance in intracellular calcium homeostasis. For example, downhill walking was shown to concentrate calcium in damaged muscles of rats (Duan, Delp, Hayes, Delp, & Armstrong, 1990). Cytosolic calcium accumulation has been shown to be the result of either membrane damage through micropuncture or passive muscle stretch in rats (Armstrong et al., 1993; Carpenter & Karpati, 1989). Increased free cytosolic calcium accumulation has been implicated in disruption of mitochondrial ATP production and sarcoplasmic reticulum, increased protease and phospholipase activity (Armstrong, Warren, & Warren, 1991; Reddy, Etlinger, Rabinowitz, Fischman, & Zak, 1975).

After accumulation of intracellular calcium, muscle swelling is one of the most visible responses to muscle damage after the initial event. Swelling is the result of an influx of water into the muscle cell and exacerbated after eccentric muscle damage (Jan Friden, Sfakianos, Hargens, & Akeson, 1988). Regulation of water volume is complex and depends on increased capillary permeability, which allows serum proteins to leak into the cell. The resulting water influx and resulting muscle swelling is a prerequisite for fiber necrosis and is proportional to the magnitude of muscle damage (J. Komulainen & Vihko, 1995). Swelling has been implicated in the perception of delayed onset muscle soreness (DOMS) after exercise. It has been suggested that swelling creates an increase in local tissue pressure in tight compartments (Bobbert, Hollander, & Huijing, 1986). However, it has been shown that DOMS preceded cellular water influx in mice after eccentric exercise suggesting that swelling is not the cause of DOMS (Bar PR, 1994).

Early structural changes are observable almost immediately after exercise cessation. Changes to the muscle include myofilament disruption through Z line streaming which has been hypothesized to result from fracturing of much thinner actin myofilaments (J Friden, Sjjöström, & Ekblom, 1981)

and cell cytoskeleton disruption through fracturing of desmin linker proteins which occurs within 15 minutes of eccentric exercise in rabbits (Lieber, Thornell, & Friden, 1996). For example, desmin loss after eccentric rabbit dorsiflexion exercise was observed in a significant portion of muscle fibers across the muscle that is rapidly repaired up to 72 hours following eccentric exercise (J Friden & Lieber, 2001). Additional changes in histological manifestations of exercise induced muscle damage include enlarged mitochondria (Gollnick & King, 1969), myofibril rupture (W. T. Stauber, Fritz, & Dahlmann, 1990), and subsarcolemmal vacuolization (Kuipers, et al., 1983) with short periods of eccentric exercise producing no changes to the ultrastructure or the metabolic processes of the mitochondria. During this period, the mitochondrial energy production decreases as evidenced by an increase in hydrogen ion concentration between the inner and outer membrane and a mitochondrial temperature decrease (Jennings & Ganote, 1976; Kloner, Ganote, Reimer, & Jennings, 1975).

Secondary changes to the muscle ultrastructure occur as soon as two hours post eccentric muscle damage and include proteolytic and lipolytic breakdown of muscle proteins and membrane lipids which are mediated by the damaged muscle cell (Armstrong, 1990). Following autolytic destruction, neutrophils invade the area and phagocytize dead tissue and release cytotoxic factors such as reactive oxygen species which accelerate tissue destruction and alter the basement membrane permeability promoting further invasion of inflammatory cells to the damaged area (Movat, Cybulsky, Colditz, Chan, & Dinarello, 1987; Vihko, Rantamäki, & Salminen, 1978). In addition to neutrophils, monocytes differentiate, invade and phagocytize dead tissue after 24 hours of strenuous exercise in humans (Hikida, Staron, Hagerman, Sherman, & Costill, 1983). These changes cause an efflux of proteins into the extracellular space such as lactate dehydrogenase and creatine kinase following exercise (Armstrong, et al., 1983). These cytoplasmic enzymes are large and are normally unable to cross the cell membrane (Maughan et al., 1989). The increase in intracellular protein concentration in the blood may result from the disruption of the sarcolemma, allowing the intercellular proteins to diffuse down their concentration gradients and out of the cell (Maughan, et al., 1989). These events, while

traumatic for the cell, may benefit tissue regeneration by producing regeneration components that help tissue reconstruction through necrotic fiber phagocytosis and autophagocytosis of surviving fibers (Salminen, 1985).

Degeneration necrosis of the muscle tissue is followed by the regeneration phase that occurs four to six days after the initial damage. Muscle cell repair begins with satellite cell activation in exercised muscle with growth factors controlling myoblast-myotube fusion that results in myofiber maturation and proliferation of mitochondria (Florini, Ewton, & Magri, 1991; Myllyla, Salminen, Peltonen, Takala, & Vihko, 1986). In the soleus muscle in rats, maximal satellite cell proliferation occurs 24 hours after an acute bout of eccentric exercise (Darr & Schultz, 1987). Additionally, the muscle adapts as evidenced by an increase in sarcomere number in muscle fibers leading to a shift in the optimum length of the muscle for active tension protecting the muscle against future injuries (Proske & Morgan, 2001).

The damage repair process appears to provide resistance to future insults in both animals and humans (P. M. Clarkson, Byrnes, Gillisson, & Harper, 1987; Schwane & Armstrong, 1983). It is believed that training creates a minimization of morphological changes, reduced inflammation, preservation of maximal force as evidenced by decreases in plasma levels of intracellular muscle proteins, and DOMS attenuation compared to the untrained state (J Friden, Sjöström, & Ekblom, 1983; Sacco & Jones, 1992). These adaptations appear to be the result of improved coordination and reorganization of the muscle fiber contractile apparatus due to increases in sarcomere length, synthesis of Z band proteins and reinforcement of intermediate filaments such as desmin (1983).

Quantifying muscle damage has proceeded through a number of indirect methods such as estimation of pain, decrease in maximal muscle force, and impaired range of motion (P. M. Clarkson, Nosaka, & Braun, 1992; Stauber, et al., 1990; Stauber WT & B., 1988). It has been

shown that muscle damage correlates well with some intracellular proteins such as troponin, fatty acid binding protein, myoglobin, and creatine kinase.

Troponin is a protein found only in cardiac and skeletal muscle that is integral to muscle contraction and is an often-used diagnostic marker of myocardial infarction and heart muscle cell death. The binding of calcium to troponin causes a conformational change exposing binding sites allowing myosin-actin cross bridge formation, allowing contraction of the muscle. Troponin isoforms differ between muscle tissues with cTN1 the predominate form in cardiac muscle and sTN1 found in skeletal muscle. Skeletal muscle troponin I has been used very rarely in exercise studies due to the difficulty of ELISA preparation. However the advent of commercially available kits has made performing these assays much easier and much less time consuming. The first study to use skeletal troponin I as an indicator of muscle damage was Storichter et. al. (1997) who found it to be a highly reliable indicator of muscle damage (Stephan Sorichter, et al., 1997).

Fatty Acid Binding Protein 3 (FABP3) is a 15 kilo Dalton cytoplasmic protein with highest concentrations found in cardiac and type I skeletal muscle fibers. FABP3 plays a role in fatty acid metabolism by transporting fatty acids from the cell membrane to mitochondria for oxidation and plays a role in regulating cell growth and proliferation (Kleine, Glatz, Van Nieuwenhoven, & Van der Vusse, 1992). FABP3 is released from cardiac myofibrils following cardiac muscle damage by ischemic episodes and is used with troponin to diagnose myocardial infarction (Azzazy, Pelsers, & Christenson, 2006). Recently Pritt et. al. (2008) found that FABP3 was the most useful individual biomarker for skeletal muscle damage based on concordance, sensitivity, and predictive value and had greater diagnostic value than creatine kinase and aspartate transaminase (Pritt et al., 2008).

Myoglobin is an iron and oxygen-binding protein found in cardiac, type I and type II skeletal muscle tissue and any appearance in the blood is attributed to muscle cell membrane rupture.

Myoglobin is a diagnostic marker for muscle injury, making it a potential marker for myocardial infarction. However since myoglobin is cleared from the kidney this assumes the rates for production and clearance are equal. Unequal rates affect the myoglobin plasma concentration resulting in an over or underestimation of the extent of EMID/DOMS (Tiidus, 2008).

Creatine Kinase has been used as a marker of muscle damage and is an enzyme that catalyses the conversion of creatine and consumes adenosine triphosphate to create phosphocreatine and adenosine diphosphate and has many different isoforms found in a variety of tissues (Goldblatt, 1969). Typical measured values of creatine kinase in the blood are between 10 - 120 micrograms per liter (mcg/L) which peaks 12-24 hours post eccentric muscle damage (Byrnes et al., 1985). A specific isoform of creatine kinase, CK-MM is found in skeletal muscle and has been shown to be an indicator of muscle damage in males after eccentric biceps contractions despite no change in total creatine kinase levels (Apple, Hellsten, & Clarkson, 1988). Creatine kinase's ability to accurately estimate the extent of EIMD/DOMS has been called into question by recent studies. For example, there are large differences between older and younger men in the extent of muscle damage determined by muscle biopsy following eccentric exercise despite no significant difference in creatine kinase activity (Manfredi et al., 1991). Further, Van der Meulen et. al. (1991) found that creatine kinase levels overestimate tissue damage as determined by muscle biopsy in rats after a downhill run (Van der Meulen, Kuipers, & Drukker, 1991).

Strategies to minimize EMID

Since muscles are subjected to extreme stress during eccentric exercises, minimization of damage has centered on increasing membrane flexibility to maintain a balance between a rigid, ordered inflexible structure and completely fluid nonviscous liquid. Membrane flexibility enhances and increases membrane integrity retaining toxic cellular contents inside the cell and thereby attenuating the inflammation response. Unsaturated fatty acids increase membrane flexibility while saturated fatty acids increase rigidity. It has been shown that incorporation of n-3 PUFAs into the ER of yeast cells increased resistance to cellular stress (Deguil et al., 2011). Forces

found in exercising muscle such as shear stress exert various metabolic effects on endothelial cells by up regulating genes controlling membrane fluidity through fatty acid catabolism and membrane incorporation (Qin et al., 2007). Cell membranes have optimal fluidity for function and act as shock absorbers thereby distributing force evenly minimizing fractured cells and cytoplasmic leakage (Chien, 2008). Supplementation of n-3 PUFAs has resulted in a decrease in intracellular biomarkers and inflammatory biomarkers in resting men suggesting increased membrane integrity (Richard J Bloomer, Douglas E Larson, Kelsey H Fisher-Wellman, Andrew J Galpin, & Brian K Schilling, 2009).

Exercise related muscle damage and free radical mechanisms

There are many potential sites for free radical generation during exercise as well as many enzymes that assist in free radical neutralization in skeletal muscle. Since the mitochondria is a large producer of reactive oxygen species, oxidative skeletal muscle fibers are subjected to large changes in oxygen flux during aerobic exercise which has been thought to increase skeletal muscle tissue damage (Sen, 1995). Minimization of reactive oxygen species is enhanced in skeletal muscles through incorporation of antioxidants and enzymes in skeletal muscle tissue. Current literature suggests free radical generation is increased during exhaustive aerobic exercise but does not significantly cause damage to the surrounding muscle tissue. However skeletal muscle is very susceptible to secondary damage caused by the immune system during eccentric exercise and secondary tissue damage caused by the resulting immune response (Reznick, Packer, Sen, Holloszy, & Jackson, 1998).

DNA in muscle cells is under the continuous assault of reactive oxygen species and lipid peroxide radicals, which can interact with DNA causing damage which leads to mutations and if severe enough, cell death. DNA's vulnerability to reactive oxygen species results from prevalence of double bonds in DNA bases that radicals can easily add to and labile hydrogen atoms that can be abstracted through the deoxyribose sugar backbone, leaving a radical formation at the reacted carbon on the DNA strand which reacts with molecular oxygen leading to a DNA strand break (Povirk, 1996). In order to maintain proper function, DNA is repaired by various protein kinases resulting in 8-Oxo-2'-deoxyguanosine (8-OHdG) which is most often used to assess the extent of

DNA damage in skeletal muscle (Park & Ames, 1988). For example Hartman el. (1994) found that DNA damage in rats by running on a treadmill for 45 minutes peaked at 24 hours after running and returned to normal levels 72 hours postexercise (Hartmann, Plappert, Raddata, Grünert-Fuchs, & Speit, 1994). Regular exercise such as swimming and distance running has been shown to decrease levels of nuclear 8-OHdG along with increases of plasma antioxidant concentrations (Inoue, Mu, Sumikawa, Adachi, & Okochi, 1993; Pilger et al., 1997). It seems that DNA damage from radicals can only be detected when exercise is exhaustive such as marathons and ultramarathons (Gomez-Cabrera et al., 2006; Keizo Umegaki, Daohua, Sugisawa, Kimura, & Higuchi, 2000). Sacheck al el (2003) studied 12 weeks of vitamin E (1000 IU/day) supplementation on biomarkers of oxidative stress in healthy men. Muscle damage was induced at -15% downhill running for 45 min at 75% VO2max and blood samples were obtained at baseline, 0, 6, 24, and 72 hours postexercise to determine the extent of DNA damage. The study found that leukocyte 8-OHdG concentrations were unaffected by exercise in both control and experimental groups.

Prostaglandin metabolism generates reactive oxygen species through intermediates activated during excessive contractile activity (A McArdle, Edwards, & Jackson, 1991). A precursor to prostaglandins, arachidonic acid, can be activated through lipoxygenase enzymes and generates reactive oxygen species which have been implicated in skeletal muscle damage in female Wistar rats (Jackson, Wagenmakers, & Edwards, 1987).

However the role of prostiglandins in exercise muscle damage is not clear. Kuipers et. al. (1985) used Flurbiprofen (90 mg/day), a cyclooxygenase-inhibiting drug, to assess the role of prostaglandin metabolism on subjective symptoms of soreness and eventual structural changes in muscle during a 30 minute cycle ergometry session at 80% VO2max. Muscle biopsies taken at baseline, immediately after, and 24 hours after exercise were used to examine muscle structural changes in conjunction with plasma levels of muscle enzymes. No significant enzyme release was noticed in any of the subjects and structural changes were restricted to the mitochondria. No

statistical difference in subjective soreness was observed between groups suggesting that at this workload and mode of exercise prostaglandin metabolism does not play a major role in exercise-induced muscle soreness and structural damage.

It has been suggested that failure of muscle calcium homeostasis following exercise results in the free radical formation through activation of calcium dependent proteases such as Calpain and phospholipase A2 which cleave intracellular muscle and membrane phospholipids respectively. Additionally the mitochondria eventually fail to produce ATP resulting in increased superoxide production from metabolic free radical generation due to calcium overload (Anne McArdle & Jackson, 1997). However these mechanisms are not believed to be the primary cause of muscle damage as failure of calcium homeostasis occurs secondary to an increase in free radical activity in biological tissue (Anne McArdle & Jackson, 1997).

The most likely cause of muscle tissue damage after endurance exercise by reactive oxygen species occurs by invading macrophages and other phagocytic white cells from the blood and interstitial space (Armstrong, 1986). The phagocytic process results in substantial amounts of oxygen radical release and has been shown to be essential for efficient preparation of muscle tissue regeneration and destruction of necrotic tissue (Fantone & Ward, 1985). Since this response is non specific and occurs in biological tissue, an increase in reactive oxygen species does not necessarily correlate with an increase in tissue damage and must be used in consort with other markers of muscle damage such as inflammatory markers and intracellular proteins to assess the extent of post exercise damage (Cannon et al., 1991).

Inflammatory process

Inflammation is a general response to tissue injury such as muscle damage and designed to minimize further damage and promote healing. Symptoms of inflammation include pain, swelling, redness, and reduced function. The process of inflammation (figure 1) following muscle damage is characterized by an influx of fluid and neutrophils into the tissues within the first few hours following injury.

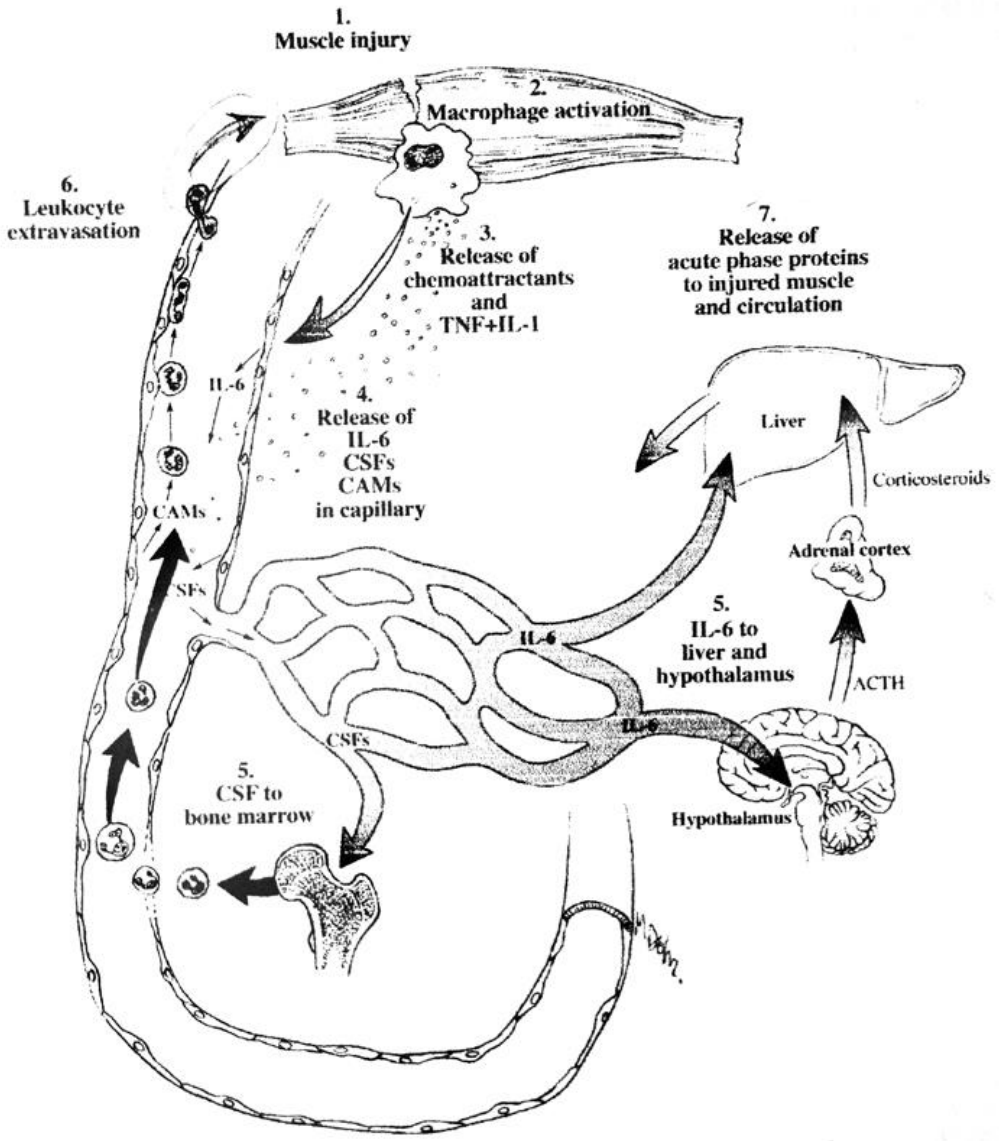


Figure 1: Proposed inflammatory sequence: taken from Garrett et al. (2000)

Next macrophages are recruited to the injured site, remove cellular debris, and secrete chemokines and cell adhesion molecules that aide in the amplification and ultimately termination of the inflammatory response. Cytokines serve as emergency signalling molecules that coordinate signaling among many different types of cells including leukocytes, stem cells, progenitor cells, endothelial cells, and hepatocytes. TNF-α, IL-1, and IL-6 are three important cytokines in muscle damage. TNF-α is a 212-amino acid-long transmembrane protein that regulates immune cells and has been shown to induce cell death, fever, and inflammation with

disregulation resulting in various inflammatory diseases such as cancer and inflammatory bowel disease (Brynskov et al., 2002; Locksley, Killeen, & Lenardo, 2001). TNF α and IL-1 are produced first by macrophages resident in the injured tissue and are strong inducers of IL-6 production (Mastorakos, Chrousos, & Weber, 1993). IL-6 is a signaling protein when produced by monocytes or macrophages and elicits a pro-inflammatory response while muscle production elicits an anti-inflammatory response and is independent of a preceding TNF-alpha response or NF-kB activation (Brandt & Pedersen, 2010). Plasma concentrations of IL-6 increase up to 100-fold from baseline post eccentric exercise resulting in a delayed peak and a much slower decrease of plasma IL-6 during recovery (Bente K Pedersen, 2013).

Muscle biopsies showed IL-6 production increased within the muscle immediately after strenuous exercise such as high intensity interval or marathon running (Nehlsen-Cannarella et al., 1997; Papanicolaou et al., 1996). Neutrophils are the first leukocyte population to respond to the cytokine “alarm call” and are responsible for debris phagocytosis by binding to compliment immunoglobulins; release lysosomal proteases which degrade intramuscular proteins; and damage lipid membranes with reactive oxygen species produced by NADPH oxidase (Epstein & Weiss, 1989). These actions, if left uncontrolled can cause disease since they can target both diseased and healthy tissue. These destructive processes are regulated by acute phase proteins such as C-reactive protein which are produced in the liver in response to eccentric exercise (Colley, Fleck, Goode, Muller, & Myers, 1983). The magnitude of tissue damage is correlated with C-reactive protein release with 88 kilometer ultra marathon runners experiencing levels equivalent to a small cardiac infarction (Strachan, Noakes, Kotzenberg, Nel, & De Beer, 1984). Accumulations of neutrophils in muscle fibers have been reported after high force eccentric contractions within minutes after injury with resolution occurring within five to seven days (Fielding et al., 1993; Round, Jones, & Cambridge, 1987). Neutrophil activity is modified by exercise with oxidative capacity decreased and phagocytic capacity increased in response to strenuous exercise (Pyne, 1994; Rincon, 1994). Leukocyte recruitment into injured tissue from the vasculature is accomplished through cell adhesion molecules, which arrest leukocyte

movement in the vasculature and facilitate cell invasion. During strenuous exercise it has been found that cells leave circulation more readily due to the up regulation of certain cell adhesion molecules such as VCAM-1 and ICAM-1 (L. Smith et al., 2000).

After macrophage infiltration the final phase in the inflammatory process is necrosis, which releases intracellular contents into the microenvironment resulting in an exacerbated inflammatory response (Cohen, 1993). Necrosis is circumvented by apoptosis which is a sequential destruction of the cell where membrane integrity is never lost and the microenvironment is protected from harmful intracellular components such as hydrogen peroxide, enzymes, and neutrophil granules with cytokines such as IL-6 promoting cells apoptosis in neutrophils resulting in eicosanoid and cytokine production cessation (Afford, Pongracz, Stockley, Crocker, & Burnett, 1992; Epstein & Weiss, 1989).

Strategies to minimize Inflammation

As far back as the Grecian times, myrtle leaves and willow bark were used to minimize the undesirable effects of inflammation (McMurry, 2004). In modern times, athletes have sought to minimize inflammation and maximize training by using NSAIDs. NSAIDs minimize inflammation by acting as non-selective inhibitors of the enzyme cyclooxygenase (COX). COX catalyzes prostaglandin and thromboxane formation from arachidonic acid which is an unsaturated fatty acid precursor cleaved from the cell membrane by phospholipase A2. Arachidonic acid is non polar and as a consequence readily diffuses in and out of cells and serves as both an intra and intercellular messenger (J. Williams, Errington, Lynch, & Bliss, 1989). Prostaglandins also serve as signaling molecules that mediate the inflammatory process and are released from skeletal muscle that has been subjected to excessive contractile activity (A McArdle, et al., 1991). Prostaglandin metabolism generates reactive oxygen species (ROS) which can lead to membrane destruction (Halliwell & Gutteridge, 1985). Additionally, extracellular arachidonic acid leaking from damaged muscle cells can serve to activate neutrophils resulting in tissue destruction by lipid peroxidation through ROS formation. Lipid peroxides form due to the generation of ROS by the mitochondria and pro-oxidant enzymes on the neutrophils such as

NADPH oxidase that degrade the lipids of the cell membrane as evidenced by increased concentrations of intracellular lipid peroxides, which are products normally formed from polyunsaturated fatty acids reacting with free radicals (Jenkins, Martin, & Goldberg, 1983). It is believed that ROS activates the NF-kB pathway which initiates the third phase of phagocytic destruction by the immune system (Whitehead, Yeung, & Allen, 2006). NF-kB is a transcription factor that regulates pro inflammatory cytokine expression such as TNF-alpha, IL-1, and IL-β (Kumar & Boriek, 2003) (Whitehead, et al., 2006). TNF-alpha can cause a positive feedback loop in the mitochondria leading to a chain reaction of ROS production causing increased lipid peroxidation and inflammation (Reid & Li, 2001). ROS can be produced by inflammatory cells and can be decreased by the release of nitric oxide by vascular endothelial cells (Privat et al., 1997). While multiple versions of the COX enzyme exist, only COX2 is believed to play a role in inflammation. NSAID treatment has been successfully used to minimize inflammation and DOMS in athletes following eccentric muscle damage (J. M. Peterson et al., 2003). However, inhibiting the inflammatory process with NSAIDs may not be beneficial to long term training performance and can lead to satellite cell quiescence and attenuated performance gains following training (Mikkelsen et al., 2009).

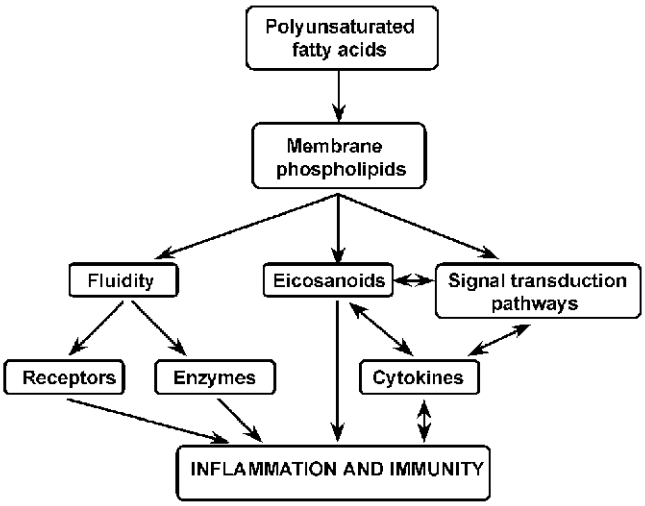


Figure 2 Mechanisms whereby polyunsaturated fatty acids might exert effects on inflammation and immunity.

Figure 2: Inflammatory pathways taken from Calder (2002)

EPA and DHA supplementation on Inflammation

DHA, EPA, and arachidonic acid are all involved in maintaining cell membrane structure and function with arachidonic acid being the main components in terrestrial mammals (Sargent, Bell, McEvoy, Tocher, & Estevez, 1999). Altering the percentage of these components changes the membrane structure. N-3 PUFA supplementation increases membrane fluidity allowing more

efficient nutrient exchange (Freeman et al., 2006; Kidd, 2007). In neurons increased membrane fluidity is associated with faster neural transmission and more efficient biochemical performance resulting in improved higher brain functions including sense of well-being, reactivity, attention, cognitive performance, and mood (G. Fontani et al., 2005).

In addition to reducing free radical production and membrane fluidity, supplementation can attenuate eicosanoid production by replacement of n-6 PUFAS with less inflammatory n-3 PUFAs. Eicosanoid production regulates inflammation and originates from 20-carbon n-3 PUFAs. Inflammatory cells contain high proportions of n-6 PUFAs incorporated into the phospholipid membrane and low proportions of other 20-carbon PUFAs and as a consequence when lipid peroxidation occurs arachidonic acid is the major substrate for eicosanoid synthesis. The relative proportion of these fatty acids in the membrane can be altered through dietary modifications. Increases in dietary consumption of n-6 PUFAs or precursors such as linoleic acid have increased inflammation compared to n-3 PUFAs through increased arachidonic acid and inflammatory mediator production such as PGE2 and LBT4 (Kelley, Taylor, Nelson, & Mackey, 1998; L. D. Peterson et al., 1998; F Thies et al., 2001). Despite the increase in inflammatory mediators, production of TNF-α, IL-1β, or IL-6 by mononuclear cells did not increase or alter superoxide production (F. Thies et al., 2001). Increased dietary consumption of n-3 PUFAs such as DHA and EPA increased the composition of these fatty acids in the phospholipid membrane and decreased the percentage of n-6 PUFA in the phospholipid membrane. Additionally the same study found a decrease in eicosanoid mediator production of PGE2, thromboxane B_2, LTB_4, and 5-hydroxyeicosatetraenoic acid (Caughey, Mantzioris, Gibson, Cleland, & James, 1996; Endres et al., 1989; Lee et al., 1985). Omega fatty acids can also act as a substrate for both COX and 5-LOX, giving rise to eicosanoids such as LTB_5, LTE_5, and PGE3. These compounds have different structures than those formed from arachidonic acid and as a consequence are less inflammatory than those formed from arachidonic acid (Goldman, Pickett, & Goetzl, 1983; Lee, et al., 1985; Sperling et al., 1993; von Schacky, Kiefl, Jendraschak, & Kaminski, 1993). Additionally, EPA can generate anti-inflammatory compounds such as D and E series resolvins from COX2 (C.

N. Serhan, Arita, Hong, & Gotlinger, 2004; Charles N Serhan et al., 2000). In addition to their anti inflammatory properties, n-3 PUFA's can enhance endogenous antioxidant defense systems such as glutathione reductase and γ -glutamyl-cysteinyl ligase (Arab, Rossary, Flourié, Tourneur, & Steghens, 2006). DHA and EPA incorporate into the phospholipid bilayer of cells where they displace arachidonic acid. Arachidonic acid causes ROS generation through COX2 and xanthine oxidase inflammatory pathways (Marnett, Rowlinson, Goodwin, Kalgutkar, & Lanzo, 1999). These inflammatory pathways can be inhibited by DHA and EPA administration and the chemoattractants derived from EPA are less potent than those derived from arachidonic acid (Kim & Chung, 2007; Mayer et al., 2003). Recently, it was shown that EPA and DHA attenuated TNF-α stimulated monocyte protein gene expression by interacting with key gene inflammatory pathways such as ERK and NF-κB (Encarnacion et al., 2011; Epstein, Barnes, & Karin, 1997). EPA and DHA supplementation reduced oxidative stress, inflammation and fibrosis through the reversal of inflammatory and oxidant pathways in patients with kidney disease, heart failure, diabetes, hypertension, and dyslipidemia (An, Kim, Cho, & Vaziri, 2009; Bouzidi et al., 2010; Matsumoto, Nakayama, Ishida, Kobayashi, & Kamata, 2009; Peake, Gobe, Fassett, & Coombes, 2011; Woodman et al., 2002). Despite anti-inflammatory properties, n-3 PUFA supplementation has not improved performance of endurance athletes compared to placebo (Nieman, Henson, McAnulty, Jin, & Maxwell, 2009).

Muscle Damage Exercise Models

Damage in skeletal muscle can be elicited through eccentric exercise by downhill running. For aerobic endurance exercise downhill running is commonly used in literature as a noninvasive way of eliciting muscle damage. Variations of maximal oxygen up take and gradient have been used as a way to achieve damage during the downhill run. Literature suggests that steeper gradients and longer durations have been highly correlated with increased skeletal muscle damage (Tiidus, 2008). Studies have varied speed and gradient with the most reliable method to elicit symptoms of EIMD/ DOMS have been to have subjects run at a -16% gradient for at least 20 minutes (Stephan Sorichter, et al., 1997; Tiidus, 2008).

The mechanism of action is debated however it is thought that during the eccentric phase of movement of downhill running, the muscle absorbs energy by stretching and absorbs mechanical energy and as a consequence results in increased force production (LaStayo, Ewy, Pierotti, Johns, & Lindstedt, 2003; Lindstedt, LaStayo, & Reich, 2001). Muscle damage occurs during running while the muscles are lengthening after the foot touches the ground and center of mass is decelerating (Armstrong, Ogilvie, & Schwane, 1983). During downhill running, the joint angle is greater and the muscle has to work eccentrically at a greater length requiring more work at its longer length resulting in greater knee extensor muscle group strain compared to level running (Newham, Jones, Ghosh, & Aurora, 1988). Additionally, downhill running contributes to an increase in intramuscular pressure in a non-compliant muscle compartment, compared with concentric exercise that affects nerve nocioreceptors creating a sensation of pain which is thought to cause the sensation of DOMS (Eston, Mickleborough, & Baltzopoulos, 1995).

Maximal force loss and Muscle damage

Skeletal muscle injury results from trauma and can affect the performance of contractions themselves, especially eccentric contractions. Muscle injury results in impaired muscle function that includes decreased joint range of motion, increased swelling, and prolonged strength loss. Strength losses following eccentric exercise in excess of 40–50% baseline values with complete recovery occurring up to 30 days from the initial insult. Because of the implications of long lasting injuries on training and performance, athletes have sought to minimize injuries by utilizing various techniques such as supplementing with n-3 PUFAs.

The cause of muscular force loss post eccentric exercise has been debated with one theory suggesting force loss was a result of a physical disruption or alteration of force-generating and transmitting structures in muscle such as the actin or myosin filaments. Evidence to support this theory has traditionally come from histological lesions associated with this type of injury such as A band disruptions and z-line streaming which result in decreased force production (Tiidus, 2008). While there is some evidence to support the theory, it has been shown the myofilament disruption

typically accounts for 34% of strength loss in single fiber preparation models indicating that there is a more significant factor that affects force loss after eccentric exercise (Reznick, et al., 1998).

Another theory has focused on the inability to activate the actin and myosin microfilaments through the excitation contraction coupling. The excitation contraction-coupling pathway begins with acetylcholine release by the alpha-motor neuron at the neuromuscular junction. An action potential is generated which passes along the plasmalemma until it goes deep into the fiber via the t-tubule. T-tubular membrane depolarization induces calcium release from the sarcoplasmic reticulum into the cytosol, where it binds to troponin to initiate the actin myosin cross bridges. Warren and coworkers found that disruption of calcium homeostasis affects force production after muscle damage in mouse soleus muscles. After injury, muscles were injected with caffeine that promoted calcium release from the sarcoplasmic reticulum thereby increasing intracellular calcium levels. Any observed force loss after caffeine injection would be interpreted as intrinsic dysfunction of the sarcoplasmic reticulum or excitation contraction coupling rather than a physical disruption of the actin and myosin microfilaments. The study found that after post muscle damage the caffeine-elicited force produced by the injured muscles was not statistically different from the non damage control muscle suggesting that force loss was due to failure of the excitation-contraction coupling (Warren, et al., 1993).

Linker proteins such as desmin have been proposed as a cause of the loss of force in skeletal muscle post eccentric exercise. Desmin connects the sub-sarcolemmal cytoskeleton with the z disk and laterally connects the z disks together which maintains cellular structural integrity during contraction (Z. Li et al., 1997; Paulin & Li, 2004). There is little evidence to suggest that force loss immediately after exercise is caused by desmin loss since the rate of protein degradation of myofilament protein content is not altered until 6 hours post muscle damaging exercise (Ingalls, Warren, Williams, Ward, & Armstrong, 1998; Lowe, Warren, Ingalls, Boorstein, & Armstrong, 1995). While not a cause of immediate force loss, there is a progressive loss of contractile

protein content beginning 1 day and continuing until 5 days after muscle damage suggesting a later role in force loss (Lowe, et al., 1995).

Rajabi et al. (2013) showed that 30-day n-3 PUFA supplementation (2000 mg/day) attenuated force loss post eccentric muscle damage in 20 healthy subjects compared to control. Leg presses were performed at 75% of one-repetition maximum with 4 sets of 20 repetitions with a 180 second rest period between sets. Voluntary contractile force was measured immediately, 24, 48 and 72 hours after exercise. The results showed that there were significant differences between the control and treatment groups 48 and 72 hours post muscle-damaging exercise with the treatment group experiencing attenuation in force loss compared to control (Rajabi, Lotfi, Abdolmaleki, & Rashid-Amiri, 2013).

These results are contradicted by Pumpa et. al. (2012) who found that force attenuation after muscle damaging exercise was unaffected by PCSO-524™ supplementation compared to control. Twenty well-trained males were randomly assigned to consume PCSO-524™ (200 mg/day) or placebo for 8 weeks prior to a downhill treadmill run. Maximal force was measured by isometric torque at 60º/second for 8 maximal force contractions on the right and left legs, countermovement jump and squat jump. The results found no significant decrease for time or group effects in force production following exercise induced muscle damage for both the countermovement jump and squat jump suggesting that PCSO-524™ was ineffective at attenuating force loss compared to placebo. The study also showed that there was a non-significant decrease in force production after the downhill run in both placebo and treatment groups in the left leg and a decrease in force production in the treatment group.

DOMS

Delayed onset muscle soreness (DOMS) is transient perception of muscular pain and is often combined with tenderness and stiffness that results from muscle trauma especially from intense or unaccustomed eccentric exercise such as downhill running, resistive cycling, and ballistic stretching (L. L. Smith, 1991). The causes of DOMS have been debated and research has

centered on nociceptor compression by muscle spasms; excessive lactic acid accumulation; disrupted z-lines; connective tissue strain; inflammatory cell infiltration and resulting nociceptor activation from edema; and excessive calcium accumulation leading to inhibition of ATP generation, and activation of protease and phospholipase enzymes (Armstrong, 1984; de Vries, 1966; KT Francis & Hoobler, 1987; Gulick & Kimura, 1996; Hough, 1902).

While none of these theories is thought to be entirely responsible for DOMS, current literature suggests an integrated theory beginning with disruption of structural proteins in muscle fibers, particularly at the weakened z-lines during eccentric exercise damages the sarcolemma inhibiting cellular respiration and increasing lypolytic and proteolytic enzyme activation. These enzymes disrupt the plasma membrane allowing intracellular contents to leak into the interstitial space that attracts monocytes that differentiate into macrophages within 12 hours of muscle damage. The macrophages produce prostaglandin (PGE_2), which sensitizes nerve endings to mechanical, chemical or thermal stimulation. Additionally the products from active phagocytosis and cellular necrosis such as histamine, potassium and kinins in addition to elevated pressure from tissue edema further activate nociceptors within the muscle (Smith Jr & Jackson, 1990). One of the limitations in using DOMS in muscle damage assessment is the high rate of non responders with one study reporting that one-third of subjects undergoing maximal eccentric contractions experienced no "meaningful" muscle soreness (Sayers & Clarkson, 2001).

DOMS can affect athletic performance and may result in altered muscle function and joint mechanics which may lead to a reduction in performance and less than optimal training intensity. Significant reductions in joint range of motion have been reported after repetitive eccentric resistance exercise (Kennon Francis & Hoobler, 1988). Significant reductions in strength and power parameters during DOMS with peak torque deficits occurring 24–48 hours after DOMS-inducing exercise and are more profound and persistent during eccentric testing and may require up to 8–10 days to return to baseline levels. Evans et al. (1990) found isokinetic eccentric peak torque of the elbow flexors decreased immediately after (44%), 24 hours (39%) and 48 hours

(32%) following repetitive eccentric contractions using an isokinetic dynamometer set at 60°/second. By 14 days, eccentric peak torque had returned to normal (D. Evans, Smith, Chenier, Israel, & McCammon, 1990).

Treatment of DOMS typically involves treatment with NSAIDs which inhibit arachidonic acid metabolism via the cyclooxygenase pathway and prevent endoperoxide and prostaglandin production reducing the inflammatory response attenuating muscle edema and intramuscular pressure which are thought to be contributing factors to DOMS (S. Hasson, Wible, Reich, Barnes, & Williams, 1992; S. M. Hasson et al., 1993).

Omega-3 fatty acids also inhibit prostaglandin production by acting as less inflammatory substrates for the COX enzymes thereby reducing membrane destruction and immune response. Recently, Tartibian et al. (2009) examined the effect of 30 days of n-3 PUFA supplementation on the effects of DOMS after eccentric exercise. 27 men were randomly assigned to one of the experimental n-3 PUFA (324 mg EPA and 216 mg DHA), placebo, and control groups. Knee range of motion was determined using goniometry, self-reported levels of lower extremity pain, and thigh circumference. Measurements were taken at baseline, immediately after, 24 and 48 hours after 40 minutes of bench stepping. The results found that while no differences were observed for pain and ROM at baseline, immediately after, and 24 hours after exercise, differences were evident in perception of pain and ROM 48 hours after exercise with the n-3 PUFA group experiencing attenuated pain and loss of ROM. Differences in thigh circumference, were evident at 24 and 48 hours after muscle damaging exercise, and there was no difference before and immediately after exercise with the control group experiencing a greater increase in thigh circumference. The authors concluded that n-3 PUFA supplementation at this dose can effectively reduce the symptoms of DOMS and inflammation (B. Tartibian, B. H. Maleki, & A. Abbasi, 2009).

Chapter 3: Methods

Subjects

Forty untrained males aged 18-26 participated in this study, and were recruited from the local Bloomington, Indiana population, with 34 subjects completing the study. Reasons for subject withdrawal included personal issue (1), failure to show (1) inability to obtain blood (3) and removed for outliers for DOMS data (2). Since the menstrual cycle has been shown to affect muscle damage, females were not included in this study (Sipaviciene, Daniuseviciute, Kliziene, Kamandulis, & Skurvydas, 2013). Subjects exercised less than 3 times a week for 30 minutes a session and had a BMI under 30 kg/m^2, blood pressure of less than 140 mmHg/90 mmHg, fasting blood glucose of less than 100 mg/dL; and total cholesterol of 200 mg/dL. Additionally, subjects did not have a history of asthma or exercise-induced asthma, COPD, interstitial lung diseases, or cystic fibrosis, dyspnea, performed eccentric endurance exercise or participated in a strength training program 60 days prior to enrolling in the study, consumption of n-3 PUFAs or anti-inflammatory medication, history of hip or knee pain, seafood allergies, a familial or personal history of cardiovascular disease, ankle edema, tachycardia, known heart murmurs, shortness of breath or diabetes. Additionally subjects were not enrolled if they came from a family that had a history of heart disease, tobacco use, or dyslipidemia. Subjects were not allowed to consume fish oil or fish oil products during the study or exercise for more than 3 times a week for 30 minutes a session. Diet was monitored to ensure no significant dietary changes occurred during the study. Subjects also documented mood and exercise activity during the study.

Study Design

The study lasted over 7 weeks and was divided into pre and post supplementation phases (Figure 4). The pre supplementation phase consisted of baseline measures of muscle damage and blood biomarkers. 143 subjects were screened and 40 qualified and were randomly assigned to either a treatment or control group with a randomization ratio of 1:1. 34 subjects completed the study with 2 subjects being excluded and 6 subjects dropped for failing to comply with the requirements of the study.

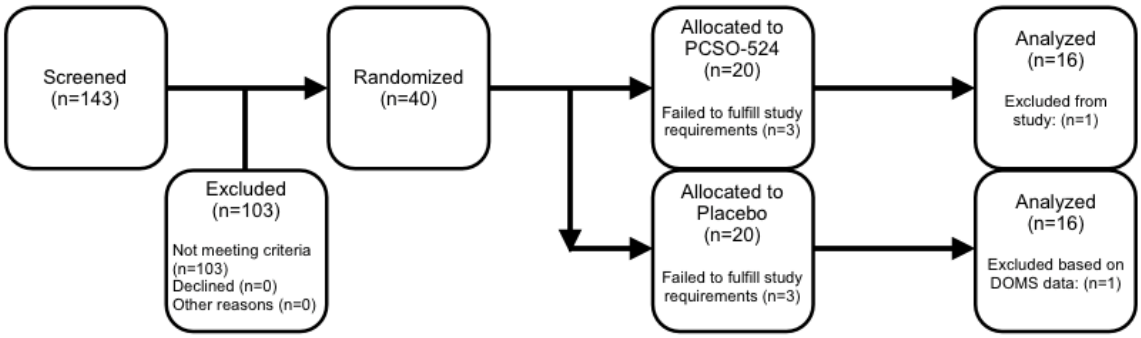


Figure 3: Flowchart of subject participation through the study

Subjects entered the study on their normal diet and after completing the baseline measures were randomly assigned to supplement for 29 days with either 8 capsules of Omega XL® (1 capsule contains 50 mg of n- 3 PUFA and 100 mg olive oil) or placebo capsules (1 capsule contains 150 mg olive oil). PCSO-524™ contains stabilized lipids from the New Zealand green-lipped mussel, Perna canaliculus, in combination with olive oil, vitamin E, sterol esters, sterols, polar lipids, triglycerides and free fatty acids (including 13% EPA, 21% DHA) (Murphy, Mooney, Mann, Nichols, & Sinclair, 2002). The placebo group of 16 subjects consumed 8 capsules/day containing olive oil for four weeks. A between-subject design was used to avoid the influence of the repeated bout effect associated with a within-subject design (McHugh, Connolly, Eston, & Gleim, 1999).

Testing was performed in a laboratory setting during the baseline and post supplementation periods with supplementation occurring away from the laboratory. Tests were conducted in the human performance and exercise biochemistry laboratories at Indiana University, Bloomington. An independent investigator not involved in data collection and having no contact with the subjects used a computerized random number generator (Random, http://www.random.org/) to create the randomization sequence with a 1:1 placebo/control allocation.

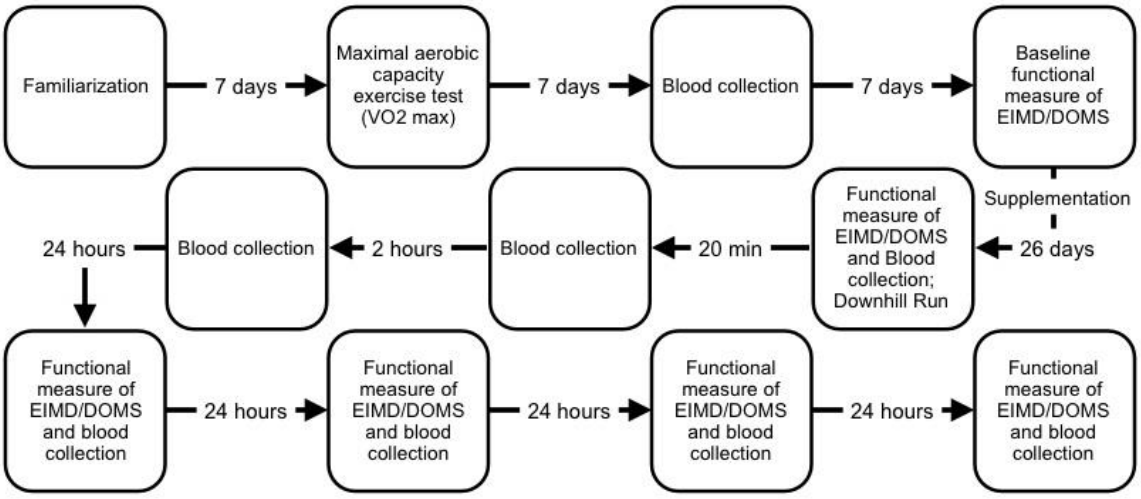


Figure 4: Schematic diagram of study protocol showing the pre and post supplementation sequential phases of the study.

Subjects performed a cycle familiarization time trial during the first lab visit. On the second visit (week 2), subjects performed a treadmill VO_2 max test. For the VO_2 max test, subjects selected a comfortable pace to run at and rested for 5 minutes while being monitored for expired O2 and CO2. The test commenced at 0% grade and a speed of 1.6 kph less than the selected speed for 2 minutes after which time, the speed was increased to the predetermined speed chosen by the subject for 2 minutes. After 2 minutes the slope of the treadmill was increased to 4% for 2 minutes while maintaining a constant speed. Slope was increased 2% every 2 minutes until exhaustion.

On the third visit (week 3), subjects were evaluated for baseline measures of muscle force, range of motion, muscle swelling, muscle pain and algometry. Additionally a blood draw established baseline measures of intracellular muscular proteins and inflammatory markers. All blood draws were taken from the antecubital vein of the arm and collected into red top Vacutainer® tubes. The tubes were placed on ice for at least 30 minutes before centrifugation at 20°C at 3000 RPM for 15 minutes. Serum was allocated to storage tubes and stored immediately at -80°C in multiple aliquots.

During the 26 day supplementation period, half of the subjects supplemented with the placebo, which was an olive oil capsule, and the treatment group supplemented with the Omega XL® capsules. Both groups consumed 8 capsules a day (4 in the morning, 4 in the evening). Dosage was carefully chosen based on studies that showed an effect with 400 mg n-3 PUFA/day (Tecklenburg-Lund et al., 2010). On the fifth visit, (47th day of the study), functional measures of exercise-induced muscle damage were assessed and blood drawn prior to the downhill muscle damaging exercise.

After the muscle damaging exercise, subjects rested for 2 hours. After two hours blood was drawn. Muscle force, range of motion, muscle swelling, knee extensor pain, algometry and blood was collected 24, 48, 72, and 96 hours after muscle induced damage.

Measurements

Maximal Voluntary Contraction

Muscle force protocol required subjects to contract their quadriceps at a knee angle of 80°. Subjects were secured into a chair and their leg secured to a force transducer (Dillion, Fairmont, MN) with a non-compliant strap. Data was measured in volts and converted to force by a regression equation (Biopac AcqKnowledge, Goleta, CA). Subjects were familiarized with the equipment by performing three warm up contractions (two submaximal, 1 maximal) separated by 10 seconds of rest. After the last maximal contraction subjects were instructed to rest for 5 minutes followed by 3, 3 second maximum voluntary contractions of the quadriceps, interspersed with 10-second rest intervals between contractions with the highest peak torque from the 3 contractions recorded. Subjects were verbally encouraged during the maximal contractions.

Range of motion

Range of motion has been shown to be an accurate method of determining the extent of muscle damage (P. M. Clarkson, et al., 1992). Subjects were instructed to lay prone on a massage table with both knees fully extended. Subjects flexed their left knee with no help from the investigator and the angle measured with a goniometer (Prestige Medical, Northridge, CA) using universal

landmarks (lateral epicondyle of the femur, lateral malleolus and greater trochanter) that were marked to ensure consistency on subsequent measures. Three measurements were averaged and reported in degrees. This method has been validated previously (Watkins, Riddle, Lamb, & Personius, 1991).

Thigh Girth

Limb circumference has been proposed as a functional measure of muscle damage (Tiidus, 2008). Limb circumference was assessed from the midpoint of the greater trochanter and the lateral epicondyle of the dominant leg with an anthropometric tape (Idass, Glastonbury, UK). Subjects were standing fully relaxed in the anatomical position. Subjects were instructed to bear the majority of their weight on their dominant leg with three averaged measurements taken on their non dominant leg. Measurement sites were marked to ensure consistency and measurements were reported in millimeters.

Algometry

Pressure algometry can be used to quantify muscle tenderness. The validity and sensitivity of pressure algometry has been established in literature (Kosek, Ekholm, & Nordemar, 1993). Pressure was measured at five specific sites on the quadriceps with a digital algometer (Force One, Wagner Instruments, Greenwich, CT.). The same investigator performed all measurements throughout the study. Specific sites for assessment were determined using established literature and landmarks (Pumpa, et al., 2011) involving two anatomical points (anterior superior iliac spine (ASIS) and superior pole of the patella (SPP)). All measurements were taken on the right side with the subject in the supine position. A longitudinal axis was created between the ASIS and the SPP from which the sites were marked with a permanent marker to ensure accuracy at each time point. The measured sites were: 15 cm distal to the ASIS, 4 cm proximal to the SPP, midpoint of the ASIS and SPP along the axis, then 2 cm lateral and 2 cm medial of this midpoint. Subjects were instructed to let the investigator know when the pressure transformed into pain at which point the amount of force was recorded in newtons.

Muscle damaging exercise

An established protocol was used to illicit muscle damage in trained athletes (Stephan Sorichter, et al., 1997) by downhill running on a treadmill (A.R. Young Company, Indianapolis, IN.) modified to run in reverse at a -16% gradient. Before the test, 5 minutes of warm up preceded 20 minutes of running, followed by a 5 minute cool down period. Once the test commenced, subjects were not permitted to stop and treadmill speed was adjusted so the subject could maintain a heart rate that corresponded to 70% VO2 max.

Blood assays

Indirect markers of muscle damage were assayed for the following biomarkers. Creatine kinase was measured by enzyme-linked immunoassay using a spectrophotometer at 450nm using a fluorometric assay kit (Abcam plc. Cambridge, England). Myoglobin (Calbiotech Inc., Spring Valley, CA), troponin I, (Abnova, Taipei, Taiwan), Skeletal muscle troponin I, (USCN Life Science, Hubei PRC.), Fatty Acid Binding Protein 3 (GenWay Biotech Inc., San Diego, CA), Interleukin-6 (Life Technologies Corporation, Grand Island, NY.), 8-OHdG (Cell Biolabs, San Diego, CA.) and TNF-alpha (Life Technologies Corporation, Grand Island, NY.). An automated plate washer (Biotek ELx405, Winooski, VT) was used to wash the 96-well microplates during the pre-assay preparations. All assays were analyzed using a Powerwave XSTM Spectrophotometer. The antibodies used in TNF-alpha, FABP3, Myoglobin, cTN1, IL-6, CK-MM, 8-OHdG, STN-I ELISA assays exhibited specificity with no detectable cross reactivity to any of the other intracellular constituents present in human serum. Concentrations on all biomarkers were analyzed and reported.

Data Analysis

Data were analyzed using SPSS 21 (IBM, Armonk, New York) with statistical significance assessed at a level of $p < 0.05$. Data were analyzed with a repeated measures design. Normality was assessed with Shapiro-Wilks test and Levene's test checked homogeneity of variance between groups. Mauchly's test was conducted to determine if sphericity was violated. If sphericity was violated, the ANOVA was corrected with a Greenhouse-Geisser adjustment factor. A Fisher protected least-squares difference post-hoc test was used to detect differences

in group means ($p < 0.05$) where a significant F ratio was found. Data are presented as mean plus or minus standard deviation with reliability presented as a 95% confidence interval.

Sample size was determined using a post-hoc power analysis of existing literature using G*Power version 3.0.5 (Universität Kiel, Germany) (Faul, Erdfelder, Lang, & Buchner, 2007). Effect sizes were presented as mean differences between groups divided by standard deviation. Previous literature suggests that an experiment-wise error rate of 0.05 required at least 15 subjects within each treatment group (Fontani et al., 2005), (Guezennec, Nadaud, Satabin, Leger, & Lafargue, 1989), (Santos, et al., 2012) (Tartibian, Maleki, & Abbasi, 2009), (Tartibian, Maleki, & Abbasi, 2011). In previous studies, ingestion of n-3 PUFA (n=9-15) for 30 days compared to placebo/control (n=9-15) significantly reduced inflammatory markers and perceived pain following eccentric exercise, with effect sizes ranging from 0.64-0.75 for a study power of 0.82 and 0.84 respectively. Additionally, following 30 days n-3 PUFA supplementation (n=10), compared to placebo (n=10), a number of inflammatory markers were decreased directly after 5 days of military survival boot camp for effect sizes ranging from 0.61-0.75 for a study power of 0.84 (Santos, et al., 2012). It is calculated that a sample size of 20 per group will provide a 96% power to detect differences in the series of proof of concept studies outlined in this proposal. Study power was maintained at 80% because fewer than five subjects withdrew or dropped from either side of the study (Adams et al., 1993).

Chapter 4: Results

Subjects

32 untrained males participated in the study (PCSO-524™: n=16, placebo: n=16) out of 40 that were recruited for the study. Mean age was 21.6 ± 2.0 years; mean height was 176.3 ± 6.6 cm and mean weight 70.8 ± 9.8 kg. All subjects exercised less than 3 times a week for 30 minutes a session. Subject characteristics are summarized in Table 1 with no statistical difference between groups ($p < .05$).

	Age (years)	Height (cm)	Weight (kg)	Systolic BP (mmHg)	Diastolic BP (mmHg)	Glucose (mg/dL)	Cholesterol (mg/dL)
Placebo (n=16)	21.5 ± 2.4	174.2 ± 6.7	66.6 ± 9.7	123.6 ± 7.5	80.0 ± 6.7	85.4 ± 8.2	173.0 ± 12.0
PCSO-524™ (n=16)	21.7 ± 1.7	178.1 ± 5.8	74.8 ± 8.8	130.3 ± 11.0	82.2 ± 3.2	83.1 ± 9.2	166.4 ± 9.2
P value	0.539	0.137	0.942	0.625	0.112	0.29	0.90

Table 1: Subject parameters

Maximal Voluntary Contraction

The test of within-subjects effects indicates that there was a significant effect of time on force from baseline ($F = 8.140$ $p < .001$). The lack of interaction between time and treatment suggests that this effect is consistent between placebo and PCSO-524™ groups ($F = .851$ $p = .470$). Finally the test of between-subjects effects indicates there is no significant effect of supplementation status on force from baseline ($F = .163$ $p = .690$). Pairwise comparisons found that there was a statistically significant difference ($p < 0.05$) in the PCSO-524™ group at 96 hours after eccentric muscle damage compared to baseline (figure 5). Additionally there was no difference in the baseline measures before or after supplementation (Table 2).

	Treatment	Mean (kg)	Std. Deviation	P value between groups
Pre supplementation	Placebo	75.4	19.3	.725
	PCSO-524™	72.8	22.2	
Post supplementation	Placebo	82.1	22.4	.988
	PCSO-524™	82.2	19.7	
24 hours post downhill run	Placebo	74.9	19.4	.861
	PCSO-524™	76.2	19.8	
48 hours post downhill run	Placebo	74.6	22.5	.596
	PCSO-524™	78.7	21.5	
72 hours post downhill run	Placebo	76.2	20.8	.608
	PCSO-524™	79.9	20.2	

96 hours post downhill run	Placebo	84.0	13.4	.926
	PCSO-524™	84.6	22.4	

Table 2 MVC force after various time points

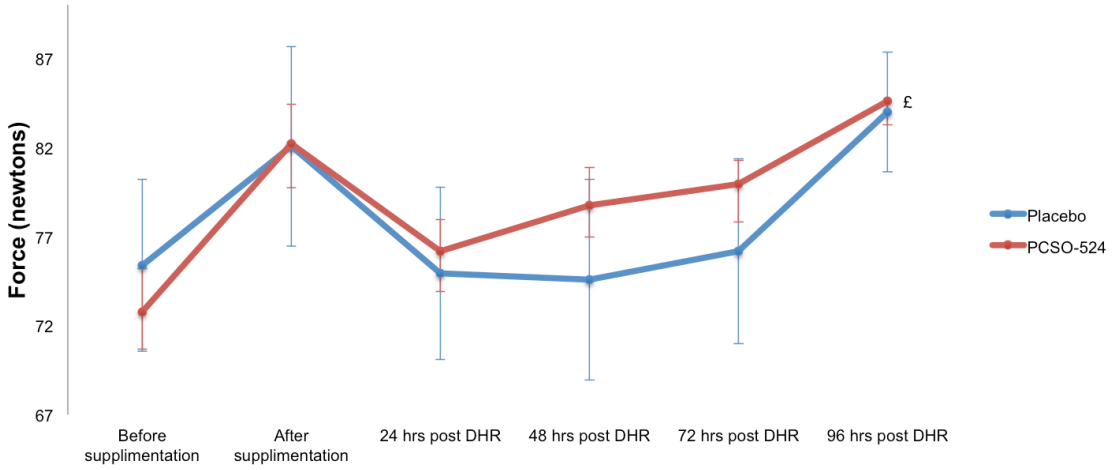


Figure 5: Maximal voluntary contraction measured in newtons. £ denotes significant difference (p < 0.05) from baseline (pre-supplementation) within groups.

Range of motion

The test of within-subjects effects indicates that there is a significant effect of time on range of motion (F = 4.105 p < .01), the lack of interaction between time and treatment suggests this effect is consistent between placebo and PCSO-524™ groups (F = 1.813 p = .114), finally the test of between-subjects effects indicates no effect of supplementation status on range of motion (F = .461 p = .502). Pairwise comparisons found that there was a statistically significant difference in range of motion between groups 96 hours after the downhill run (F = 8.236 p < .05). Additionally there was no difference in the baseline measures before or after supplementation (Table 3).

	Treatment	Mean	Std. Deviation	P value between groups
Pre supplementation	Placebo	45.75	8.37	0.505
	PCSO-524™	47.50	6.13	
Post supplementation	Placebo	46.88	9.78	0.946
	PCSO-524™	46.69	5.03	
24 hours post downhill run	Placebo	47.31	9.02	0.981
	PCSO-524™	47.38	5.16	
48 hours post downhill run	Placebo	43.44	7.11	0.611
	PCSO-524™	44.63	5.92	
72 hours post downhill run	Placebo	44.94	8.51	0.769

	PCSO-524™	45.69	5.44	
96 hours post downhill run	Placebo	41.75	5.32	0.007
	PCSO-524™	46.69	4.36	

Table 3: Range of Motion

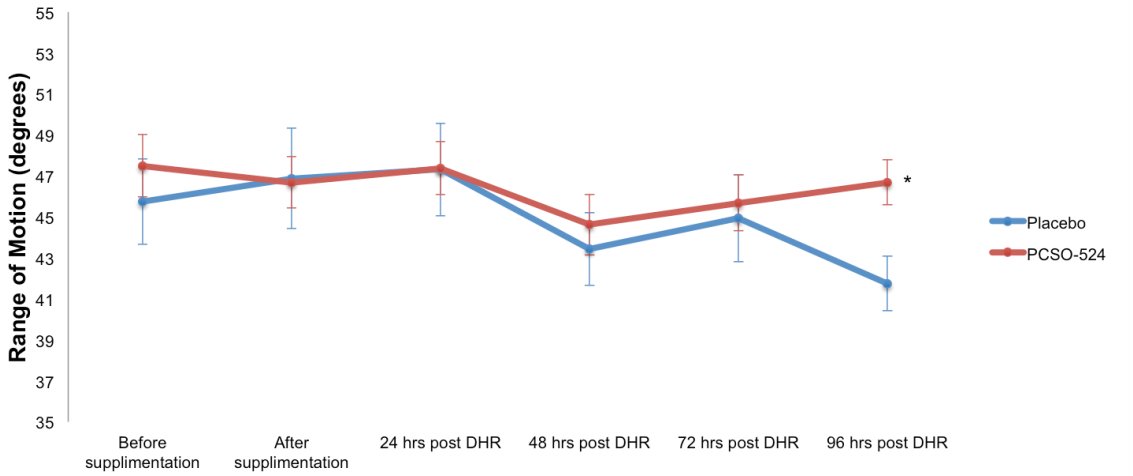


Figure 6: Range of motion vs. Time * denotes significance between placebo and PCSO-524 ($p < .05$)

Thigh girth

The test of within-subjects effects indicates that there is no effect of time on percent change in muscle swelling from baseline ($F = 1.061$ $p = .379$), the lack of interaction between time and treatment suggests that this effect is consistent between Placebo and PCSO-524™ groups ($F = .113$ $p = .978$), finally the test of between-subjects effects indicates there is no difference between supplementation status on percent change in muscle swelling from baseline ($F = .001$ $p = .997$). Pairwise comparisons found that there were no statistically significant percent changes from baseline thigh circumference between groups at any of the time points after the downhill run. Additionally there was no difference in the baseline measures before or after supplementation (Table 4).

	Treatment	Percent change from baseline	Std. Deviation	P value between groups
Baseline	Placebo	0.0078	0.02376	0.631
	PCSO-524™	0.0114	0.01808	
24 hours post muscle damage	Placebo	0.0182	0.0263	0.970
	PCSO-524™	0.0179	0.02225	
48 hours post muscle damage	Placebo	0.0146	0.02073	0.953

	PCSO-524™	0.015	0.01918	
72 hours post muscle damage	Placebo	0.0178	0.02332	0.582
	PCSO-524™	0.0136	0.01865	
96 hours post muscle damage	Placebo	0.0215	0.06243	0.971
	PCSO-524™	0.0208	0.03755	

Table 4: Percent changes from baseline in thigh circumference

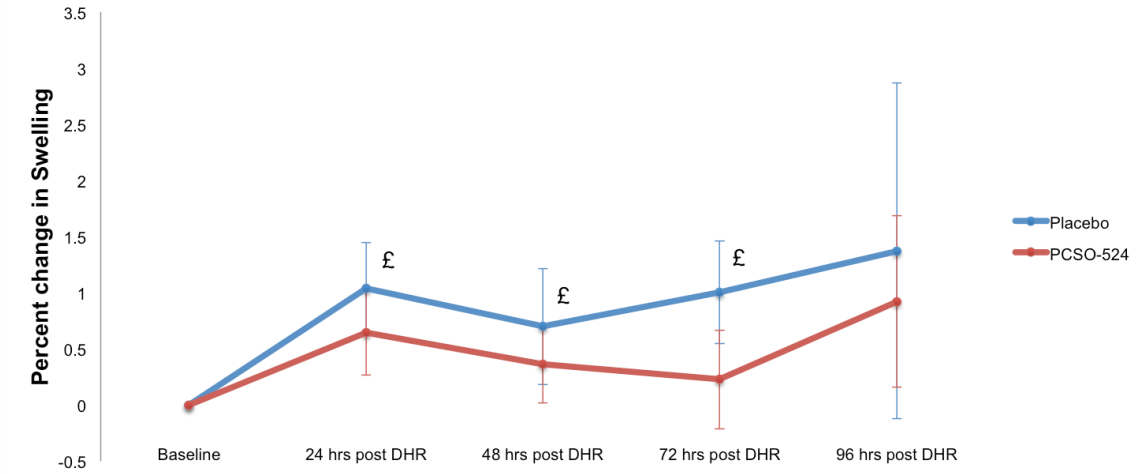


Figure 7: Percent change from baseline thigh circumference £ denotes significant difference ($p < 0.05$) from baseline (pre-supplementation) within groups.

Algometry

The test of within-subjects effects indicates that there is a significant effect of time on percent change from baseline in perception of pain by algometry ($F = 3.287$ $p < .05$), the lack of interaction between time and treatment suggests that this effect is consistent between Placebo and PCSO-524™ groups ($F = .206$ $p = .935$), finally the test of between-subjects effects indicates there is no effect of supplementation status on percent change from baseline in perception of pain by algometry ($F = .025$ $p = .877$). Pairwise comparisons found that there were no statistically significant percent changes from baseline between groups at any of the time points after the downhill run. Additionally, there was no difference in the baseline measures before or after supplementation (Table 5).

	Treatment	Mean deviation from baseline (%)	Std. Deviation	P-value between groups
Baseline	Placebo	-0.03	0.35	0.643
	PCSO-524™	-0.08	0.32	
24 hours post muscle damage	Placebo	-0.15	0.27	0.844
	PCSO-524™	-0.13	0.26	
48 hours post muscle damage	Placebo	-0.10	0.39	0.824

	PCSO-524™	-0.12	0.28	
72 hours post muscle damage	Placebo	-0.07	0.41	0.978
	PCSO-524™	-0.07	0.34	
96 hours post muscle damage	Placebo	0.01	0.50	0.896
	PCSO-524™	-0.01	0.34	

Table 5: Percent change from baseline in perceived pain by algometry

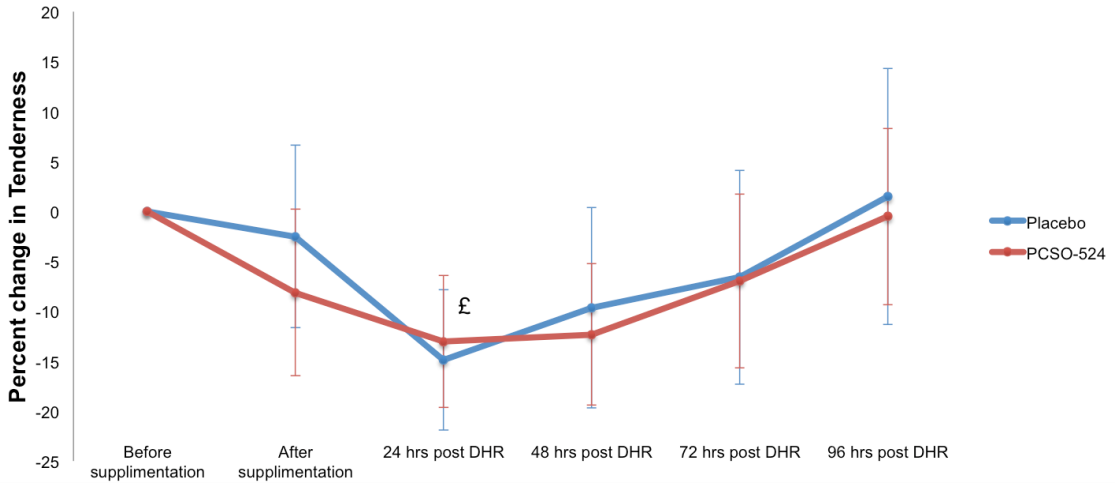


Figure 8: Percent change from baseline perceived pain by algometry. £ denotes significant difference (p < 0.05) from baseline (pre-supplementation) within groups.

Creatine Kinase - MM

The test of within-subjects effects indicates that there is a significant effect of time on creatine kinase in the blood ($F = 346.132$ $p < .001$), the interaction between time and treatment suggests that this effect is not consistent between Placebo and PCSO-524™ groups ($F = 75.554$ $p < .001$), finally the test of between-subjects effects indicates there is a significant effect of the supplementation on creatine kinase in the blood ($F = 191.321$ $p < .001$).

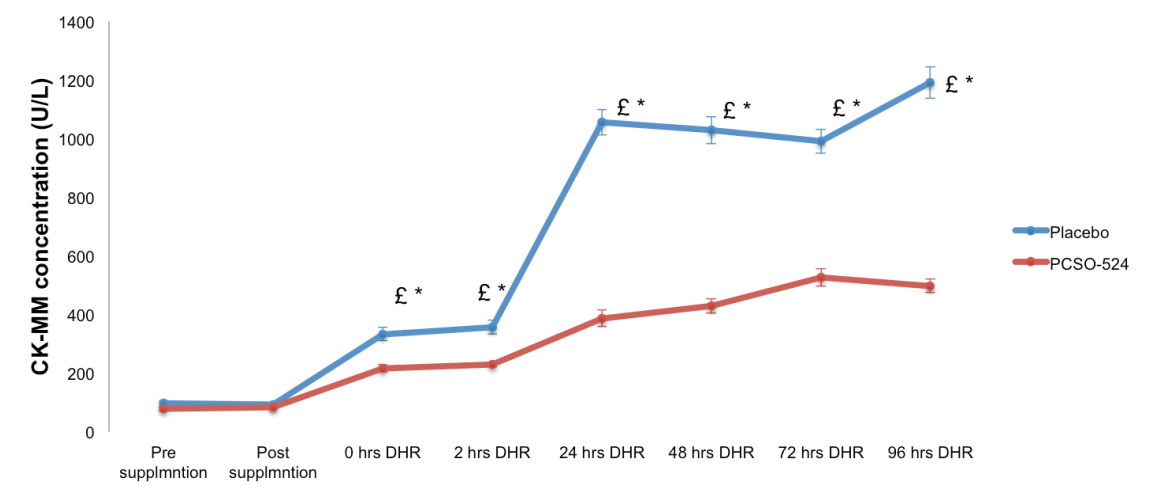


Figure 9: Creatine Kinase Appearance in Blood. * denotes significant difference between groups (p < .05); £ denotes significant difference (p < 0.05) from baseline (pre-supplementation) within groups.

Pairwise comparisons found there was a significant difference between groups immediately (t = 4.467, p < .001; 95% CI = 63.0 – 169.2), 2 (t = 5.076, p < .001; 95% CI = 76.1 – 178.6), 24 (t = 12.829, p < .001; 95% CI = 562.5 – 775.6), 48 (t = 11.477, p < .001; 95% CI = 493.2 – 706.7), 72 (t =9.118, p < .001; 95% CI = 359.8 – 567.5), and 96 hours (t = 11.935, p < .001; 95% CI = 574.4 – 811.6) post downhill run. No significant difference was observed between groups pre supplementation and post supplementation after downhill running (Table 6).

	Treatment	Mean	Std. Deviation	P value between groups
Before Supplementation	Placebo	95.4	42.3	0.205
	PCSO-524™	77.4	35.9	
After Supplementation	Placebo	92.2	33.4	0.365
	PCSO-524™	82.9	23.0	
0 hr post muscle damage	Placebo	332.5	88.8	0.000
	PCSO-524™	216.4	54.1	
2 hr post muscle damage	Placebo	355.9	93.5	0.000
	PCSO-524™	228.6	36.5	
24 hr post muscle damage	Placebo	1055.2	175.2	0.000
	PCSO-524™	386.1	113.3	
48 hr post muscle damage	Placebo	1028.0	185.4	0.000
	PCSO-524™	428.0	96.7	
72 hr post muscle damage	Placebo	989.7	163.3	0.000
	PCSO-524™	526.0	121.2	
96 hr post muscle damage	Placebo	1189.8	213.1	0.000
	PCSO-524™	496.8	92.4	

Table 6: Creatine Kinase-MM Appearance in Blood

Myoglobin

The test of within-subjects effects indicates that there is a significant effect of time on myoglobin in the blood ($F = 373.835$ $p < .001$), the interaction between time and treatment suggests this effect is not consistent between Placebo and PCSO-524™ groups ($F = 15.179$ $p < .001$), finally the test of between-subjects effects indicates there is a significant effect of the supplementation on myoglobin in the blood ($F = 34.229$ $p < .01$). Pairwise comparisons found there was a significant difference between groups at 24 ($t = 4.334$, $p < .001$; 95% CI = 22.9 – 63.7), 48 ($t = 6.402$, $p < .001$; 95% CI = 67.7 – 131.1), 72 ($t = 5.340$, $p < .001$; 95% CI = 119.0 – 266.3), and 96 hours ($t = 3.272$, $p < .01$; 95% CI = 49.0 – 212.3) post downhill run. No significant difference was observed between groups pre supplementation, post supplementation, immediately and 2 hours after downhill running (Table 7).

	Treatment	Mean	Std. Deviation	P value between groups
Before Supplementation	Placebo	28.76	11.11	0.416
	PCSO-524™	32.06	11.50	
After Supplementation	Placebo	30.84	12.02	0.349
	PCSO-524™	34.88	11.95	
0 hr post muscle damage	Placebo	51.20	11.37	0.064
	PCSO-524™	42.45	14.25	
2 hr post muscle damage	Placebo	52.16	12.82	0.079
	PCSO-524™	43.14	15.18	
24 hr post muscle damage	Placebo	127.81	21.62	<.001
	PCSO-524™	84.53	33.60	
48 hr post muscle damage	Placebo	346.24	42.16	<.001
	PCSO-524™	246.80	45.64	
72 hr post muscle damage	Placebo	580.28	103.57	<.001
	PCSO-524™	387.64	100.49	
96 hr post muscle damage	Placebo	449.62	122.51	0.003
	PCSO-524™	318.97	102.45	

Table 7: Myoglobin Appearance in Blood

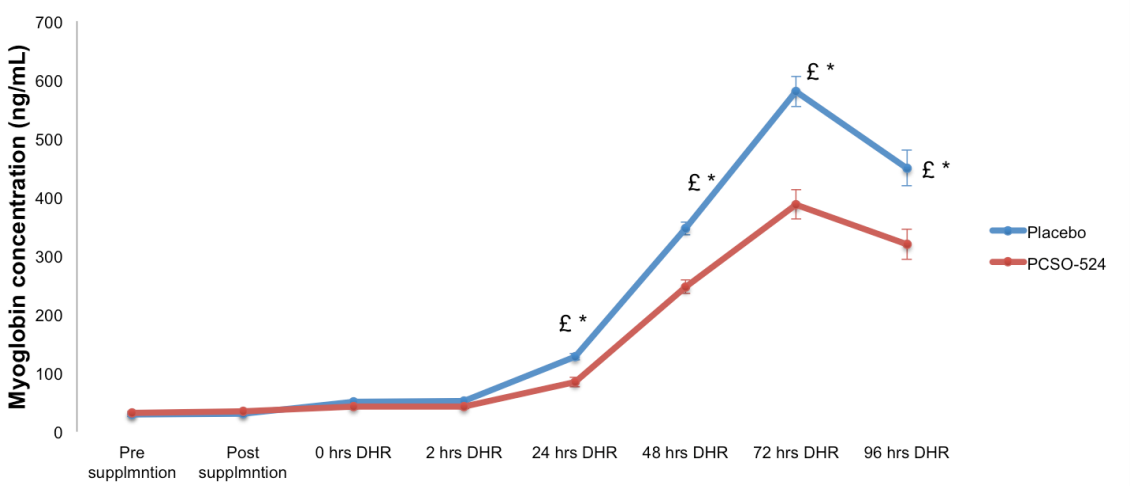


Figure 10: Myoglobin Appearance in Blood. * denotes significant difference between groups (p < .05); £ denotes significant difference (p < 0.05) from baseline (pre-supplementation) within groups.

Troponin – I (Skeletal)

The test of within-subjects effects indicates that there is a significant effect of time on skeletal muscle troponin I in the blood ($F = 102.681$ $p < .001$), the interaction between time and treatment suggests that this effect is not consistent between Placebo and PCSO-524™ groups ($F = 7.397$ $p < .001$), finally the test of between-subjects effects indicates there is a significant effect of supplementation on skeletal Muscle Troponin I in the blood ($F = 10.265$ $p < .01$). Pairwise comparisons found that there was a significant difference between groups at 2 ($t = 2.562$, $p < .05$; 95% CI = 0.9 – 8.0), 24 ($t = 3.561$, $p < .01$; 95% CI = 4.4 – 15.4), 48 ($t = 4.095$, $p < .001$; 95% CI = 4.7 – 14.0), 72 ($T = 3.222$, $p < .01$; 95% CI = 2.5 – 11.2), and 96 hours ($T = 2.177$, $p < .05$; 95% CI = 0.3 – 10.4) post downhill run. No significant difference was observed between groups pre and post supplementation and immediately after downhill running (Table 8).

	Treatment	Mean	Std. Deviation	P value between groups
Before Supplementation	Placebo	8.36	3.06	0.544
	PCSO-524™	7.67	3.33	
After Supplementation	Placebo	7.77	3.47	0.618
	PCSO-524™	7.12	3.82	
0 hr post muscle damage	Placebo	13.24	4.51	0.109
	PCSO-524™	10.68	4.25	
2 hr post muscle damage	Placebo	17.93	5.56	0.016
	PCSO-524™	13.48	4.16	
24 hr post muscle damage	Placebo	30.21	9.03	0.001
	PCSO-524™	20.33	5.98	

48 hr post muscle damage	Placebo	27.97	7.09	<.001
	PCSO-524™	18.60	5.78	
72 hr post muscle damage	Placebo	23.58	6.82	0.003
	PCSO-524™	16.74	5.04	
96 hr post muscle damage	Placebo	16.74	8.05	0.037
	PCSO-524™	11.38	5.66	

Table 8 : Slow Skeletal Troponin I Appearance in Blood

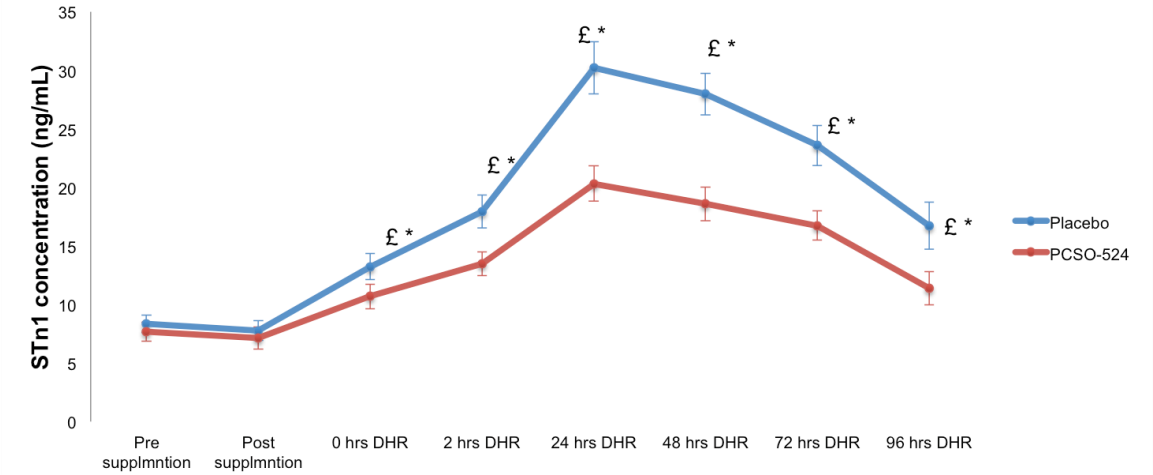


Figure 11: STN1 Appearance in Blood. * denotes significant difference between groups (p < .05); £ denotes significant difference (p < 0.05) from baseline (pre-supplementation) within groups.

Fatty acid binding protein

The test of within-subjects effects indicates that there is a significant effect of time on fatty acid binding protein in the blood (F = 6.346 p < .001), the lack of interaction between time and treatment suggests that this effect is consistent between Placebo and PCSO-524™ groups (F = 1.582 p = .142), finally the test of between-subjects effects indicates there is no effect of supplementation status on fatty acid binding protein appearance in the blood (F = .418 p = .523). Pairwise comparisons found that there was no significant difference between groups at any of the time points after a downhill. Additionally there was no difference in the baseline measures before or after supplementation (Table 9).

	Treatment	Mean	Std. Deviation	P value between groups
Before Supplementation	Placebo	1873.0	1362.6	0.498
	PCSO-524™	2266.6	1845.6	
After Supplementation	Placebo	1860.6	1669.8	0.251
	PCSO-524™	2654.9	2140.5	
0 hr post muscle damage	Placebo	2451.7	2005.6	0.162

	PCSO-524™	3483.5	2063.0	
2 hr post muscle damage	Placebo	3191.1	2146.6	0.546
	PCSO-524™	3693.9	2497.0	
24 hr post muscle damage	Placebo	2332.9	2767.8	0.287
	PCSO-524™	3534.4	3458.8	
48 hr post muscle damage	Placebo	1853.8	1987.2	0.97
	PCSO-524™	1831.2	1336.1	
72 hr post muscle damage	Placebo	1709.8	1460.4	0.582
	PCSO-524™	1465.1	980.6	
96 hr post muscle damage	Placebo	2306.2	3093.0	0.436
	PCSO-524™	1620.2	1577.6	

Table 2: Fatty acid Binding Protein Appearance in Blood

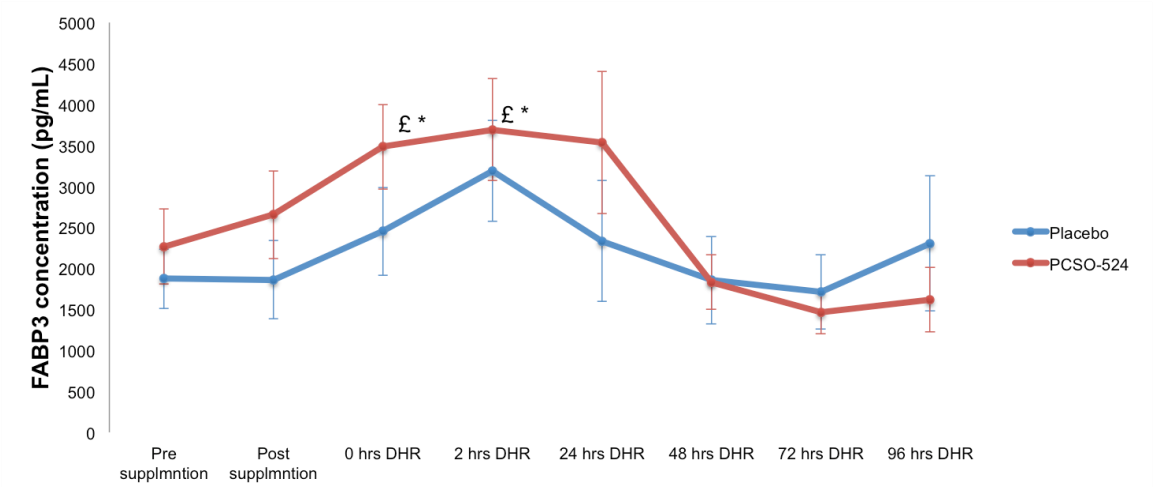


Figure 12: Fatty acid binding protein 3 appearance in blood. £ denotes significant difference ($p < 0.05$) from baseline (pre-supplementation) within groups.

Interleukin - 6

The test of within-subjects effects indicates that there is a significant effect of time on interleukin-6 concentration in the blood ($F = 128.951$ $p < .001$), the interaction between time and treatment suggests that this effect is not consistent between Placebo and PCSO-524™ groups ($F = 8.722$ $p < .001$), finally the test of between-subjects effects indicates there is a significant effect of supplementation status on interleukin-6 appearance in the blood ($F = 9.930$ $p < .01$). Pairwise comparisons found that there was a significant difference between groups at 24 ($t = 3.619$, $p < .01$; 95% CI = 3.7 – 13.3), 48 ($t = 3.993$, $p < .001$; 95% CI = 5.8 – 17.8), 72 ($t = 3.621$, $p < .01$; 95% CI = 5.0 – 17.9), and 96 hours ($t = 3.076$, $p < .01$; 95% CI = 3.1 – 15.35) post downhill run. No significant difference was observed between groups pre supplementation, post supplementation and immediately after downhill running (Table 10).

	Treatment	Mean	Std. Deviation	P value between groups
Before Supplementation	Placebo	18.05	5.04	0.381
	PCSO-524™	19.53	4.36	
After Supplementation	Placebo	19.46	4.65	0.473
	PCSO-524™	20.72	5.13	
0 hr post muscle damage	Placebo	31.28	4.67	0.058
	PCSO-524™	26.98	7.35	
2 hr post muscle damage	Placebo	31.54	6.44	0.085
	PCSO-524™	27.09	7.65	
24 hr post muscle damage	Placebo	39.22	6.39	0.001
	PCSO-524™	30.71	6.90	
48 hr post muscle damage	Placebo	52.93	8.48	<.001
	PCSO-524™	41.15	8.20	
72 hr post muscle damage	Placebo	48.48	8.59	0.001
	PCSO-524™	37.02	9.30	
96 hr post muscle damage	Placebo	42.62	8.39	0.004
	PCSO-524™	33.39	8.59	

Table 3: IL-6 Appearance in Blood

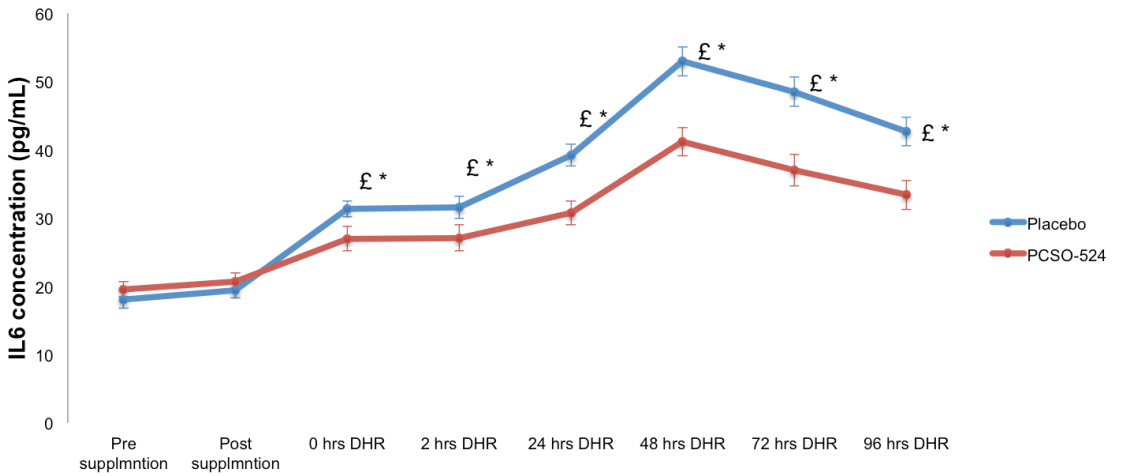


Figure 13: IL-6 Appearance in Blood. * denotes significant difference between groups (p < .05); £ denotes significant difference (p < 0.05) from baseline (pre-supplementation) within groups.

TNF - alpha

The test of within-subjects effects indicates that there is a significant effect of time on TNF alpha concentration in the blood ($F = 132.012$ $p < .001$), the interaction between time and treatment suggests that this effect is not consistent between placebo and PCSO-524™ groups ($F = 19.123$ $p < .001$), finally the test of between-subjects effects indicates there is a significant effect of supplementation status on TNF alpha appearance in the blood ($F = 21.414$ $p < .001$).

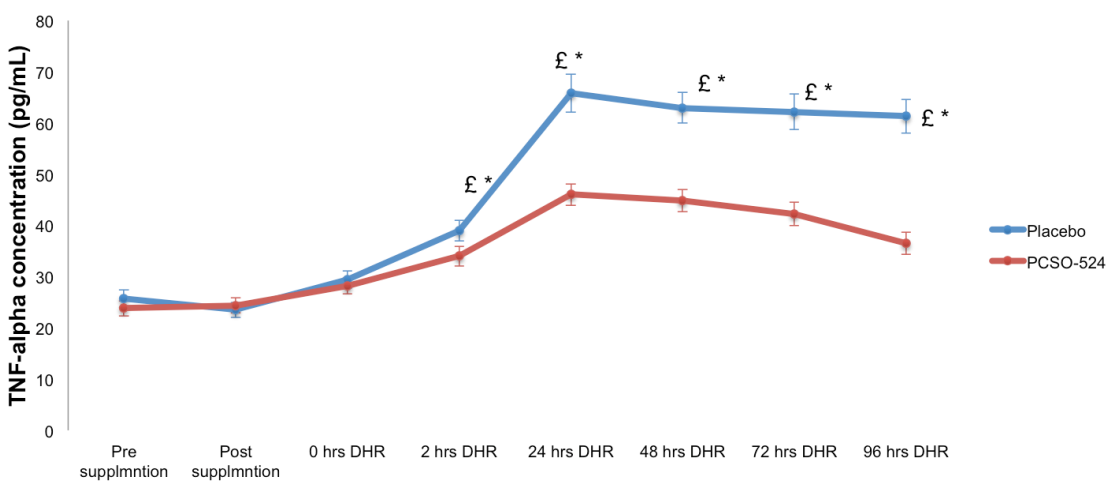


Figure 14: TNF-alpha Appearance in Blood. * denotes significant difference between groups (p < .05); £ denotes significant difference (p < 0.05) from baseline (pre-supplementation) within groups.

Pairwise comparisons found that there was a significant difference between groups at 24 (t = 4.633, p < .001; 95% CI = 11.1 – 28.5), 48 (t = 4.942, p < .001; 95% CI = 10.6 – 25.7), 72 (t = 4.747, p < .001; 95% CI = 11.3 – 28.5), and 96 hours (t = 6.349, p < .001; 95% CI = 16.8 – 32.8) post downhill run. No significant difference was observed between groups pre supplementation, post supplementation, immediately, and 2 hours after downhill running (Table 11).

	Treatment	Mean	Std. Deviation	P value between groups
Before Supplementation	Placebo	25.68	6.71	0.406
	PCSO-524™	23.80	5.88	
After Supplementation	Placebo	23.53	5.94	0.688
	PCSO-524™	24.37	5.72	
0 hr post muscle damage	Placebo	29.42	6.51	0.579
	PCSO-524™	28.17	6.10	
2 hr post muscle damage	Placebo	38.94	8.07	0.086
	PCSO-524™	33.99	7.67	
24 hr post muscle damage	Placebo	65.81	14.77	<.001
	PCSO-524™	46.01	8.60	
48 hr post muscle damage	Placebo	62.95	11.82	<.001
	PCSO-524™	44.80	8.73	
72 hr post muscle damage	Placebo	62.17	14.04	<.001
	PCSO-524™	42.27	9.17	
96 hr post muscle damage	Placebo	61.29	13.06	<.001
	PCSO-524™	36.48	8.59	

Table 11: TNF-alpha Appearance in Blood

8 - Hydroxyguanosine

The test of within-subjects effects indicates that there is not a significant effect of time on 8-OHdG concentration in the blood (F = 1.018 p .419), the lack of interaction between time and treatment suggests that this effect is consistent between placebo and PCSO-524™ groups (F = 1.005 p = .429), finally the test of between-subjects effects indicates there is no effect of supplementation status on 8-OHdG appearance in the blood (F = 3.250 p = .081). Pairwise comparisons found that there was no significant difference between groups at any of the time points after the downhill run. Additionally there was no difference in the baseline measures before or after supplementation (Table 12).

	Treatment	Mean	Std. Deviation	P value between groups
Before Supplementation	Placebo	0.17	0.08	0.402
	PCSO-524™	0.19	0.08	
After Supplementation	Placebo	0.21	0.19	0.437
	PCSO-524™	0.16	0.09	
0 hr post muscle damage	Placebo	0.17	0.07	0.905
	PCSO-524™	0.16	0.09	
2 hr post muscle damage	Placebo	0.16	0.10	0.310
	PCSO-524™	0.23	0.24	
24 hr post muscle damage	Placebo	0.21	0.21	0.413
	PCSO-524™	0.31	0.44	
48 hr post muscle damage	Placebo	0.13	0.04	0.114
	PCSO-524™	0.32	0.45	
72 hr post muscle damage	Placebo	0.13	0.04	0.111
	PCSO-524™	0.17	0.09	
96 hr post muscle damage	Placebo	0.13	0.07	0.209
	PCSO-524™	0.22	0.28	

Table 12: 8-OHdG Appearance in Blood

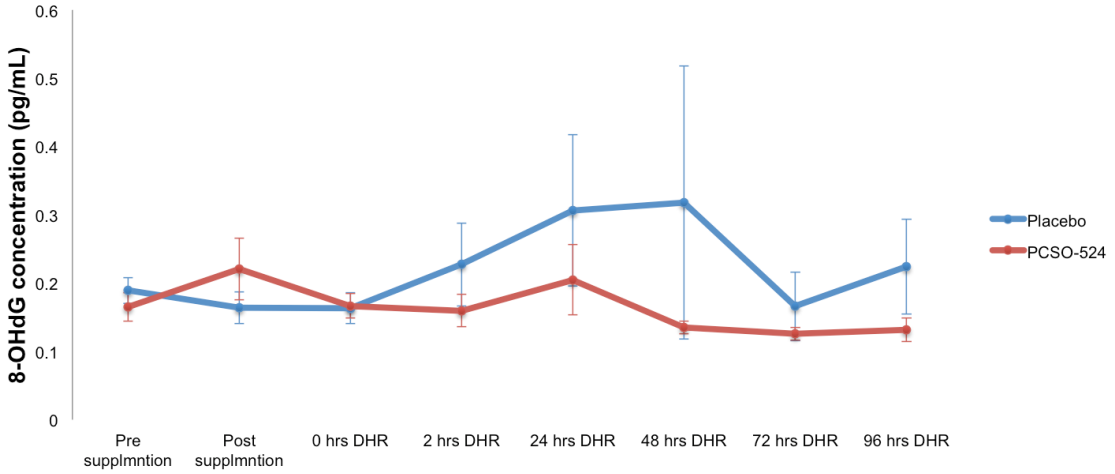


Figure 15: 8-OHdG Appearance in Blood. No significant difference (p > 0.05) was observed between or within groups at any time point.

Troponin – 1 (Cardiac)

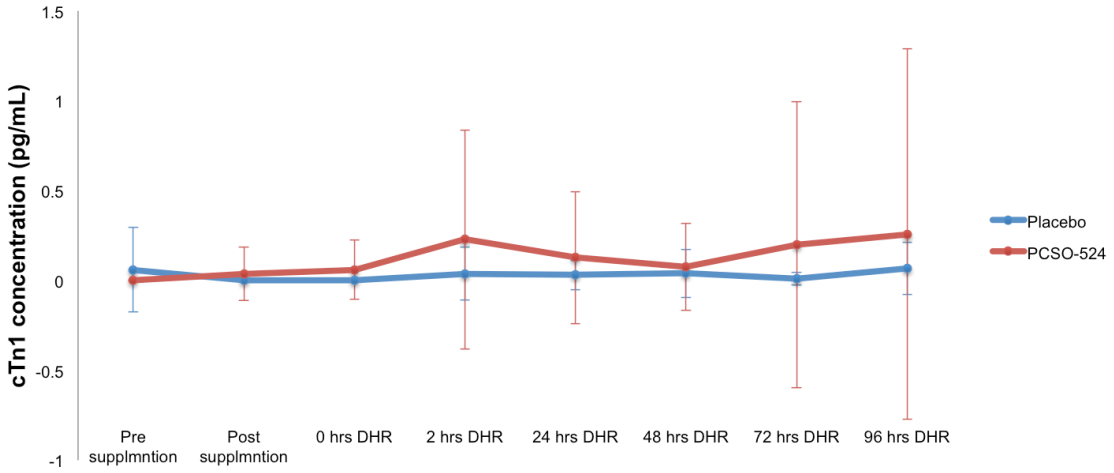
The test of within-subjects effects indicates that there is not a significant effect of time on cTn1 concentration in the blood (F = .420 p = .889), the lack of interaction between time and treatment suggests that this effect is consistent between Placebo and PCSO-524™ groups (F = .381 p = .912), finally the test of between-subjects effects indicates there is no effect of supplementation

status on cTn1 appearance in the blood (F = 2.220 p = .148). Pairwise comparisons found that there was no significant difference between groups at any of the time points after a downhill. Additionally there was no difference in the baseline measures before or after supplementation (Table 13).

	Treatment	Mean	Std. Deviation	P value between groups
Before Supplementation	Placebo	0.06	0.24	0.329
	PCSO-524™	0.00	0.00	
After Supplementation	Placebo	0.00	0.00	0.341
	PCSO-524™	0.04	0.15	
0 hr post muscle damage	Placebo	0.00	0.00	0.162
	PCSO-524™	0.06	0.16	
2 hr post muscle damage	Placebo	0.04	0.15	0.249
	PCSO-524™	0.23	0.61	
24 hr post muscle damage	Placebo	0.03	0.08	0.313
	PCSO-524™	0.13	0.37	
48 hr post muscle damage	Placebo	0.04	0.13	0.624
	PCSO-524™	0.08	0.24	
72 hr post muscle damage	Placebo	0.01	0.04	0.382
	PCSO-524™	0.20	0.80	
96 hr post muscle damage	Placebo	0.07	0.14	0.469
	PCSO-524™	0.26	1.03	

Table 13: cTN1 Appearance in Blood

Figure 16: cTn1 Appearance in Blood. No significant difference (p > 0.05) was observed between or within groups at any time point.



Decisions on the Hypotheses

1. PCSO-524™ supplementation attenuated force loss after muscle damage compared to placebo in untrained men at 96 hours post-eccentric exercise. The hypothesis that PCSO-524™ supplementation attenuates force loss after muscle damage following downhill running was

accepted.

2. PCSO-524™ supplementation attenuated loss of range of motion compared to placebo in untrained men at 96 hours post-eccentric exercise. The hypothesis that PCSO-524™ attenuates loss of range of motion following downhill running was accepted.

3. PCSO-524™ supplementation failed to lessen percent change in muscle swelling compared to placebo in untrained men following downhill running. The hypothesis that PCSO-524™ supplementation attenuates percent change in muscle swelling following downhill running was not accepted.

4. PCSO-524™ supplementation failed to lessen percent change from baseline in perception of pain by algometery compared to placebo in untrained men following downhill running. The hypothesis that PCSO-524™ supplementation attenuates percent change from baseline in perception of pain following downhill running was not accepted.

5. PCSO-524™ supplementation attenuated appearance of creatine kinase compared to placebo in untrained men following downhill running. The hypothesis that PCSO-524™ attenuates the appearance of creatine kinase following downhill running was accepted.

6. PCSO-524™ supplementation attenuated appearance of skeletal troponin I compared to placebo in untrained men following downhill running. The hypothesis that PCSO-524™ attenuates the appearance of slow skeletal troponin I following downhill running was accepted.

7. PCSO-524™ supplementation did not attenuate appearance FABP in the blood compared to placebo in untrained men following downhill running. The hypothesis that PCSO-524™ supplementation attenuates FABP in the blood following downhill running was not accepted.

8. PCSO-524™ supplementation attenuated appearance of IL-6 compared to placebo in untrained men following downhill running. The hypothesis that PCSO-524™ attenuates the appearance of IL-6 following downhill running was accepted.

9. PCSO-524™ supplementation attenuated appearance of TNF-alpha compared to placebo in untrained men following downhill running. The hypothesis that PCSO-524™ attenuates the appearance of TNF-alpha following downhill running was accepted.

10. PCSO-524™ supplementation failed to show any differences in 8-OHdG concentration compared to placebo in untrained men following downhill running. The hypothesis that PCSO-524™ supplementation attenuates 8-OHdG concentration compared to placebo following downhill running was not accepted.

11. PCSO-524™ supplementation failed to show any differences in the concentration of cTn1 compared to placebo in untrained men following downhill running. The hypothesis that PCSO-524™ supplementation attenuates appearances of cTn1 compared to placebo following downhill running was not accepted.

Chapter 5: Discussion

The purpose of this study was to evaluate the effectiveness of PCSO-524™ in attenuating markers of EIMD/DOMS such as reduced muscle force, range of motion, muscle swelling, perceived muscle pain and the biochemical markers of muscle damage including CK-MM, Myoglobin, STN1, FABP3, IL-6, TNF-alpha, 8-OHdG, and cTN1. PCSO-524™ supplementation can attenuate some of the biochemical markers of muscle damage and functional measures of muscle damage such as range of motion and maximal voluntary contraction; however supplementation with PCSO-524™ does not affect thigh girth and perceived pain through the use of algometry.

Maximal Voluntary Contraction

Strength loss after eccentric exercise is a common indicator of exercise induced muscle damage and has been used as a valid and reliable indirect marker of muscle damage after eccentric exercise in 50% of eccentric muscle damage studies in humans (Tiidus, 2008). After eccentric muscle damage there can be up to a 50% loss in strength compared to baseline with restoration occurring up to 10 days after muscle damaging exercise. Multiple hypotheses exist for the cause of force loss after eccentric exercise which include disruption of the myofilaments due to histological lesions as evidenced from muscle biopsies; attenuated neural activation patterns; and overstretched sarcomeres due to non uniform sarcomere length (P. M. Clarkson, et al., 1992).

Force loss after eccentric muscle damage, measured by maximal voluntary contractions of the quadriceps, was significantly different between groups at 96 hours for the PCSO-524™ group compared to placebo suggesting PCSO-524™ supplementation has a protective effect on muscle damage during eccentric exercise by increasing force production post muscle damage. These results are similar to Clarkson et. al. (1992) who investigated 2 bouts of high force eccentric exercise which resulted in substantial decreases in baseline force production immediately after eccentric exercise (P. M. Clarkson, et al., 1992).

Supplementation of n-3 PUFAs, the principle ingredient in PCSO-524™, has been shown to

affect muscle strength by increasing grip strength in older adults (Robinson et al., 2008) by potentially increasing protein anabolism through the Akt-mTOR-S6K1 pathway (Gingras et al., 2007a). Additionally, n-3 PUFA supplementation has been shown to maintain muscle mass during muscle atrophy due to increases in amino acid delivery and anti-inflammatory action (P. C. Calder, 2009; Fujita, Rasmussen, Cadenas, Grady, & Volpi, 2006). Another mechanism that n-3 PUFAs may attenuate force loss during conditions of atrophy is through altered signaling pathways. Incorporation of n-3 PUFAs into the muscle phospholipid bilayer has been shown to increase PIP3 potency increasing phosphorylation of p70s6k, a crucial protein in the maintenance of muscle mass after resistance exercise (Alessi et al., 1997; Baar & Esser, 1999).

The effect of PCSO-524™ supplementation on force production has been previously observed in Pumpa et. al. (2012) who investigated maximal voluntary contraction by counter movement and vertical jumping following downhill running and showed there was a non significant decrease in height and no significant difference between time points suggesting there was no affect for time or treatment in this study (Pumpa, et al., 2011). Similarly, Kreider found that n-3 PUFA supplementation failed to promote strength gains in 23 experienced, resistance-trained subjects during a randomized double-blind study with either placebo (olive oil) or 6 grams/day of n-3 PUFAs (conjugated linoleic acids) for 28 days. Despite a controlled training regimen there was no significant difference in strength compared to control (Kreider, Ferreira, Greenwood, Wilson, & Almada, 2002). The lack of significant effects in both these studies may be explained by training status and supplementation period.

The findings of Pumpa et. al. (2012) and Kreider et. al. (2002) may have been influenced by the use of trained subjects. Trained subjects have been shown to attenuate the clinical signs of muscle damage and decrease the amount of intracellular muscular proteins in the blood compared to untrained individuals (Appell, Soares, & Duarte, 1992; Ortenblad, Madsen, & Djurhuus, 1997). The current study attempts to rectify this limitation by studying the effect of PCSO-524™ in untrained individuals whose muscles are less resilient to muscle damage (Tiidus,

2008).

Range of Motion

Inflammatory muscle swelling and myofibril disruption has been postulated to cause muscle stiffness and reduced range of motion. Onset of reduced range of motion is gradual peaking around 3 days after eccentric muscle damage, and exhibits a similar time course to muscle swelling and therefore might be a result of reduced range of motion. Clinical studies suggest that range of motion is decreased after post-traumatic edema and joint range of motion can be affected by muscle size which can vary between individuals (Airaksinen, Partanen, Kolari, & Soimakallio, 1991).

Modification of the arachidonic acid pathway through the use of less inflammatory substrates such as n-3 PUFAs or the inhibition of eicosanoid synthesis by blocking COX enzymes has been shown in literature to attenuate inflammation, thereby increasing range of motion (Bjordal, Ljunggren, Klovning, & Slørdal, 2004; P. Calder & Grimble, 2002). Kjeldsen-Kragh et. al (1992) found that subject consumption of 3.8 grams EPA /day and 2.0 grams of DHA /day for 16 weeks was effective at decreasing morning stiffness despite a significant increase in morning stiffness in the placebo group (Kjeldsen-Kragh et al., 1992). Further, Tartibian et. al. (2009) found 30 days of n-3 PUFA supplementation at 324 mg EPA /day and 216 mg DHA /day attenuated loss of range of motion compared to placebo 48 hours after eccentric exercise (B. Tartibian, et al., 2009). These data are in agreement with the present study, which found that while there was initially no significant difference between the placebo and PCSO-524™ groups immediately after eccentric exercise, range of motion increased over time with significant increases in the range of motion of the PCSO-524™ group 96 hours after muscle damaging exercise.

Despite this theoretical basis of n-3 PUFA supplementation, there have been inconsistent results of eicosanoid pathway inhibition by the use of n-3 PUFA or NSAID supplementation on range of motion. Lenn and colleagues used 1.8 g/day n-3 PUFA with a 30 day supplementation period and failed to show a significant difference in relaxed arm angle after 50 maximal isokinetic eccentric

elbow flexion contractions at any time during the study (Lenn, et al., 2002). Tokmakidis reported a similar effect with the use of NSAIDS (Tokmakidis, Kokkinidis, Smilios, & Douda, 2003). Similar findings were observed by Barlas et. al. (2000) and Stone et. al. (2002) who showed no effect of eicosanoid inhibition on loss of ROM (Barlas et al., 2000; Stone, Merrick, Ingersoll, & Edwards, 2002).

The lack of significant findings in these studies differs from the present study that showed statistically significant differences in ROM 96 hours post eccentric exercise. The lack of significant effects of n-3 PUFA supplementation in the studies conducted by (Lenn et. al. (2002), Barlas et. al. (2000), and Stone et. al. (2002) may be attributed to training status. For example, Tartibian et. al. (2009) used untrained males and found 324 mg EPA and 216 mg DHA /day for 30 days attenuated loss of range of motion and found the attenuation effect increased gradually post eccentric muscle damaging exercise with the first significant difference between placebo and n-3 PUFA groups observed at the last time point measured in the study at 48 hours post eccentric muscle damaging exercise (B. Tartibian, et al., 2009).

Thigh girth

Swelling occurs within the muscle shortly after exercise and thigh circumference has been used as an indicator of exercise induced muscle damage caused by eccentric exercise due to muscle swelling and inflammation by influx of fluid into the muscle tissue (K. Nosaka & Newton, 2002). Swelling peaks 4 to 5 days post eccentric muscle damage with an initial increase in swelling occurring 0-1 hours post muscle damage in some studies.

N-3 PUFA supplementation is hypothesized to attenuate inflammation by inhibiting the eicosanoid pathway since n-3 PUFAs are less inflammatory substrates than n-6 PUFAs and as a consequence, attenuate macrophage free radical production. Diets rich in n-3 PUFAs found in the components in fish oil attenuate reactive oxygen species generation, production of inflammatory cytokines such as TNF-alpha, IL-1b and IL-6 by macrophages which lead to an attenuated response from the immune system (P. C. Calder, 1998). Increased consumption of n-

3 PUFAs such as EPA and DHA, result in the incorporation of n-3 PUFAs phospholipids in the membrane of the inflammatory cell at the expense of the more proinflamatory arachidonic acid. Because less substrate is available for synthesis of eicosanoids from arachidonic acid, fish oil supplementation decreases production of PGE2, thromboxane B2, LTB4, 5-hydroxyeicosatetraenoic acid, and LTE4 by inflammatory cells (P. Calder & Grimble, 2002). Supplementation with n-3 PUFAs after eccentric exercise reduces muscle swelling 48 hours after exercise (Lenn, et al., 2002).

However reduction in swelling by arachidonic acid pathway inhibition has not always been observed consistently in literature. For example, there was no effect on arm circumference after eccentric arm exercises after supplementation of 2,000 mg EPA and 1,000 mg DHA per day (Jouris, McDaniel, & Weiss, 2011). Similarly, Reynolds failed to demonstrate attenuation of thigh circumference following acute hamstring injuries by NSAID (300 mg/day) supplementation (Reynolds, Noakes, Schwellnus, Windt, & Bowerbank, 1995). Reduction of inflammatory compounds has been demonstrated in a number of studies by supplementation of inhibitors such as n-3 PUFAs or NSAIDs suggesting that inhibition of eicosanoid production reduces swelling (Tiidus, 2008; Yang et al., 2010).

One reason for the lack of observed difference in the present study might be that swelling was being affected by gravity. Clarkson et. al. (1996) observed that the location of swelling in the muscle appears to change over time by using MRI and found that fluid began to accumulate in the lower portions of the extremities hypothesized to be due to the effects of gravity (Kazunori Nosaka & Clarkson, 1996). The lack of significant time effects for both groups might be explained by examining other parts of the muscle for swelling rather than the midpoint. In the present study the increase in TNF - α concentration is strongly linked to inflammation and it can be assumed muscle swelling might not be noticed since the effects of edema would be most noticeable in the distal end of the leg rather than the midpoint.

Some studies have found a poor correlation between the biochemical inflammatory markers and muscle swelling indicating that the two events might occur by two independent processes and suggests that the mechanism for muscle damage is not the underlying mechanism of exercise-induced biomarker increase which would corroborate with the findings in the present study (Kazunori Nosaka & Clarkson, 1996; Sprenger et al., 1992).

Algometry

Delayed-onset muscle soreness is a hallmark manifestation of exercise-induced muscle damage a claim first made by Hough (1902). Typically, DOMS develops between 24 and 48 hours after eccentric muscle damage and peaks within 72 hours postexercise (P. M. Clarkson, et al., 1992). The dull pain that signals DOMS is hypothesized to be caused by activation of afferent neurons by swelling and the subsequent pressure activates the mechanoreceptors. Another possible cause is the increased histamines, bradykinins, and prostaglandins produced by the inflammatory cells and breakdown of myocellular components that activate nociceptors that are sensitive to chemical signals (P. M. Clarkson, et al., 1992). Algometry has been proposed as a reliable and effective way of quantifying pressure pain thresholds and muscle damage (Potter, McCarthy, & Oldham, 2006). Further, algometry is a reliable measure and enhanced when being performed by one examiner and can be used to evaluate the development and decline of experimentally induced muscle tenderness over consecutive days (Nussbaum & Downes, 1998).

Omega 3 fatty acids attenuate prostaglandin production and reduce inflammatory mediators by replacing arachidonic acid in the cell membrane. After muscle damage, concentrations of calcium activated PLA_2 increase, resulting in greater prostaglandin and leukotriene production. Additionally the antioxidants in PCSO-524™ such as furan fatty acids and vitamin E are powerful free radical scavengers, which minimize damage caused by NADPH oxidase in macrophages. DOMS has been attenuated by antioxidant supplementation such as Vitamin C and E (Bryer & Goldfarb, 2006). PCSO-524™ supplementation has been beneficial in reducing perceived muscle soreness in athletes after a 30 kilometer run (Baum, et al., 2013). Additionally, n-3 PUFA supplementation reduced the arachidonic/eicosapentaenoic acid ratio in the cell membrane and

the number of positive tender points in patients affected by musculoskeletal pain (Giuliano Fontani et al., 2010).

The benefits of n-3 PUFA supplementation are well documented in literature for the prevention of muscle pain. Studies have found 1000 mg/day supplementation of EPA/DHA was enough to cause a increase in the tender point measured with algometry (Guimarães, Guimarães, & Raposo, 2011). Additionally the n-3 PUFAs found in PCSO-524™ have been associated with decreased neuropathic pain with a study finding that between 2400-7200 mg/day of EPA and DHA was sufficient to attenuate the perception of pain by tender point algometry (Ko, Nowacki, Arseneau, Eitel, & Hum, 2010).

These findings were corroborated with Pumpa et. al. (2012) who showed supplementation of the n-3 PUFA rich compound PCSO-524™ was ineffective at showing statistically significant differences in tender point algometry in trained athletes at the midpoint of the quadriceps following a downhill run. This failure to find differences might have been the result of using trained athletes, since training confers a protective effect and as a consequence trained athletes are more resistant to muscle damage due to the repeated bout effect (Tiidus, 2008).

The current study attempts to rectify the limitations in the Pumpa study by using untrained athletes whose muscles are more susceptible to the symptoms of EIMD/DOMS following downhill running. The present study found that there was a significant effect for time, suggesting that muscle damaging exercise in the present study was effective at inducing muscle damage ($F = 3.287$ $p < .05$) and the effect was effective for both the placebo and treatment groups ($F = .206$ $p = .935$). However PCSO-524™ supplementation at the dosage and supplementation period was not effective in creating a significant difference between the control and placebo ($F = .025$ $p = .877$). These results assume that all participants were affected in the same way, however some participants claimed that their hamstrings, a muscle not measured, was more tender than their quadriceps. Flexibility can determine which muscles are recruited and running gait can be

affected causing other muscle groups to compensate for weaker or stiffer muscles which can lead to damage (Hartig & Henderson, 1999).

Creatine Kinase - MM

Blood proteins such as creatine kinase have been proposed as indirect indicators of muscle damage and are routinely studied after strenuous exercise. It is believed that after eccentric exercise the membrane of the muscle cell is damaged causing the release of creatine kinase, an enzyme that catalyzes phosphocreatine from creatine. Creatine kinase is found and cells consuming ATP rapidly such as skeletal, and smooth muscle, photoreceptor cells of the retina and neurons in the brain.

Due to multiple sources, increases in creatine kinase can be triggered by any number of events and increases have corresponded poorly with measures of EIMD/DOMS. For example, an animal model, Kuipers et. al. (1985) reported that creatine kinase activity showed a poor correlation with a histological assessment of muscle damage (H. Kuipers, Keizer, Verstappen, & Costill, 1985). Another study found that creatine kinase activity was much greater than actual tissue damage evaluated from tissue biopsy calculated by creatine kinase appearance and disappearance rates (Van der Meulen, et al., 1991). An additional confounding problem is the large variability between subjects following the same exercise. For example, Newham et. al. found that subjects who performed an eccentric exercise session had CPK activities ranging from 500 U/L to 34,500 U/L creatine kinase with variability unrelated to general fitness level and physical characteristics of the subjects (Newham, Jones, & Edwards, 1983).
A possible reason for these discrepancies is that creatine kinase is cleared by the mononuclear phagocyte system and any imbalance in production or clearance can lead to misestimating the effect of muscle damage after eccentric exercise.

A novel first for this study was the use of an isoform of creatine kinase only found in skeletal muscle after downhill running. The lack of significance of other studies in difference in creatine kinase after eccentric exercise may be to the use of the widely popular creatine kinase MB which

is found in multiple locations including muscles and the brain and can be released for multiple reasons other than muscle damage. The isoform in this study utilized the isoform CK-MM that is the major isoform of creatine kinase expressed in skeletal muscle cells with other forms such as CK-MB playing a minor role (F. S. Apple, Rogers, & Ivy, 1986). Other studies have confirmed the efficacy of CK-MM as a reliable indicator of muscle damage in both athletes and diseased individuals (F. Apple, et al., 1988; Page, Jackson, Coakley, & Edwards, 1989).

The present study has shown that PCSO-524™ can significantly attenuate CK-MM levels compared to placebo following downhill running ($F = 191.321$ $p < .001$). Significant differences were observed immediately, 24, 48, 72, and 96 hours after the downhill run ($p < .05$) with maximal values observed at 72 hours and 96 hours for the PCSO-524™ and placebo groups respectively which is in agreement with previously published literature which suggests creatine kinase increases 24 hours after muscle damaging exercise and peaks within 3-5 days (Tiidus, 2008).

The results in the present study differ from the Pumpa et. al. (2012) study which failed to show a significant difference in creatine kinase concentrations for trained athletes supplemented with PCSO-524™ compared to placebo after a downhill run. One explanation for the failure to show significance may be due to the creatine kinase isoform in the study was specific to both muscle and brain and not just skeletal muscle as in the present study. Another reason for the differences observed between studies may be due to a greater supplementation of PCSO-524™ per day, (200 mg PCSO-524™/day) in the Pumpa study in comparison to the present study (400 mg PCSO-524™/day).

Myoglobin

Myoglobin is a heme protein of striated muscle and is rapidly released after muscle damage. Myoglobin is involved in oxygen binding, storage and diffusion in cardiac and skeletal muscle fibers. It accounts for up to 5 to 10% of all cytoplasmic proteins with the highest concentration located close to the contractile apparatus and the intracellular membranous or fibular structures.

Since myoglobin is only found in skeletal muscle cells, it's appearance in the blood is hypothesized to be due to ruptured cell membranes following eccentric muscle action and the amount of protein released into the blood is correlated with the magnitude of muscle damage (Van Nieuwenhoven et al., 1995). In skeletal muscle, myoglobin is found mainly in slow-twitch fibers with current myoglobin assays unable to discriminate between myoglobin released from the heart or from skeletal muscle, and therefore myoglobin is not muscle specific (Delanghe, Chapelle, & Vanderschueren, 1990). Use of myoglobin has been problematic since production has not always been correlated with actual muscle damage. Some studies have correlated myoglobin with muscle damage through verification with muscle biopsy (Dawson et al., 2002) and through disruption of the muscle membrane (Driessen-Kletter, Amelink, Bär, & Van Gijn, 1990).

Supplementation of n-3 PUFAs has been used to attenuate the effects of muscle damage on myoglobin appearance in the blood (Tartibian, Maleki, & Abbasi, 2011). The effect observed was similar to the present study with the appearance of myoglobin in the blood increasing until 48 hours after eccentric muscle damaging exercise with levels in the study of myoglobin for control (393.4 +/- 133.2) and treatment 200.1 +/- 120.4) not statistically different than control (346.2 +/- 42.2) and treatment (246.8 +/- 45.6) in the present study at 48 hours post muscle damaging exercise. Additionally, the study findings are in agreement with other studies that have found elevated myoglobin levels 24 to 96 hours post eccentric muscle damage (Tiidus, 2008). The findings of the present study agree with previous studies that show that n-3 PUFA supplementation has been effective in attenuating muscle damage post eccentric muscle exercise (Lenn, et al., 2002).

The findings in the present study that PCSO-524™ significantly decreased myoglobin release following exercise induced muscle damage is in disagreement with Pumpa (2012) who found no difference between treatment and placebo groups of trained athletes during downhill running with supplementation of 200 mg PCSO-524™/day at any time point in the study (Pumpa, et al., 2011). The maximal values for myoglobin concentration were much earlier than the present study (6

hours versus 72 hours) after muscle damaging exercise might be attributed to excessive myoglobin clearance by the kidney. Ritter et al (1979) found that myoglobin clearance is altered after exercise training with significant increases in clearance and decreases in total myoglobin plasma concentration in 16 male subjects following strenuous physical evaluation tests. The present study attempts to rectify this deficiency by using untrained individuals which are more susceptible to EIMD/DOMS (Ritter, Stone, & Willerson, 1979).

Another explanation for the discrepancy between the present study and the study conducted by Pumpa et al (2012) may be explained by the muscle damaging protocol used in the Pumpa study was not severe enough to illicit muscle damage in trained athletes. Since trained subjects experience the repeated bout effect, which protects against subsequent muscle damage, the magnitude of damage is important in eliciting EIMD/DOMS. The current study utilizes a protocol that has been shown to illicit muscle damage in trained individuals by using a greater slope (-16% vs. -10%) and longer duration of exercise (20 minutes continuous) in contrast to the study conducted by Pumpa and colleagues. The protocol was successful in inducing muscle damage and myoglobin release as evidenced by a significant treatment effect for time ($F = 373.835$ $p < .001$).

Troponin – I (Skeletal)

Skeletal troponin I (sTnI) has been proposed as an alternative to commonly used muscle damage markers such as creatine kinase and myoglobin (Sorichter et al., 1997). Current biomarkers such as creatine kinase are highly variable both within subjects and between subjects making them a poor estimator of exercise induced muscle damage. Both creatine kinase and myoglobin are actively cleared from the blood by the reticuloendothelial system and myoglobin through the kidney, and alterations in the rates of clearance and production can lead to differences in the blood that do not accurately reflect the extent of muscle damage (Tiidus, 2008). Skeletal muscle troponin I is located on the force-producing units of the sarcomere, which is responsible for binding with calcium causing tropomyosin to shift revealing the myosin binding sites which are responsible for force production. Since this protein is unique to skeletal muscle cells, any

appearance in the blood is believed to be indicative of muscle damage (S Sorichter, Puschendorf, & Mair, 1999). The cardiac isoform of STN1 has been used as a reliable diagnostic marker for patients suffering from heart attack. Due to the unique specificity to skeletal muscle sTnI has been suggested as a possible gold standard of measuring the extent of muscle damage (Stephan Sorichter, et al., 1997).

The current study is novel in that it is the first to measure the appearance of STN1 in the blood after muscle damaging exercise. The results of the study found that the downhill run at -16% for 20 minutes was sufficient to induce elevation of sTnI serum levels with the placebo group experiencing statistically elevated levels of sTN1 compared to the PCSO-524™ group. Differences in sTnI levels were observed at 2, 24, 48, 72 and 96 hours after eccentric muscle damaging exercise. Levels of sTnI were greatest 24 hours after eccentric muscle damage and (PCSO-524™: 20.3 ± 6.0 ng/ml, placebo: 30.2 ± 9.0 ng/ml) differ slightly from a study conducted by Sorichter et. al. (1997) who found that maximal values occurred 6 hours after muscle damaging exercise. This difference might be explained since the study used trained athletes rather than the current study that used untrained subjects who are subject to the repeated bout effect. While the maximal values of stN1 are similar it is entirely possible that the subjects in the current study experienced much higher concentrations than measured at 24 hours since blood was not taken 6 hours after exercise. The data from the present study indicates the downhill running eccentric exercise protocol was successful in inducing muscle damage as evidence by appearance in the blood.

There are differences among reported sTnI levels in the literature that maximal values in trained individuals appear 6 hours post exercise and can be influenced by gender and mode of exercise. For example after a downhill run Sorichter (2001) noted that median values for females were much lower than for males (8.2 mg/ L; M: 22.0 mg/L) (S Sorichter et al., 2001). The resting baseline values of stn1 are similar to those in literature 8.7 ± 3.0 sTnI which are similar to Soricther et. al. (1999). Additionally mode of exercise showed differing amounts of STN1 release

with knee extensor exercises having a 4 fold increase compared to downhill running and it is not understood as to the reason for the increase between the two modes of exercise (D. S. Willoughby, VanEnk, & Taylor, 2003).

Fatty acid Binding Protein

An adequate supply of long-chain fatty acids for metabolism by skeletal muscle is important as fatty acids are important building blocks for membrane phospholipids which are the main substrate for energy production, especially in slow-twitch oxidative muscle fibers and serve as signaling molecules for inflammatory processes such as arachidonic acid (Van Nieuwenhoven et al., 1995). During times of cellular stress, concentrations of these molecules are elevated to maintain homeostasis. FABP3 can serve as the cytosolic equivalent of albumin in plasma by carrying fatty acids thereby increasing the overall rate of utilization inside the cell and transfer of fatty acids from the intracellular membrane (Weisiger, 2002). While there are many different isoforms of fatty acid binding protein, FABP3 has been proposed as an isoform for reliably monitoring skeletal muscle damage due to a high concentration in skeletal muscle tissue. FABP3 expression in skeletal muscle is altered in response to physiological stimuli such as endurance training and in certain pathophysiological conditions, such as metabolic situations, which increase fatty acid oxidation and result in elevated FABP3 concentrations (Pritt, et al., 2008; Storch & Thumser, 2000). Damaged muscle cell membranes leak FABP3 into the interstitial space and capillaries. FABP3 has been shown to be a reliable and early diagnostic indirect marker of skeletal muscle damage (Pritt, et al., 2008). Additionally, Fabp3 had greater diagnostic value than when combined with other markers of muscle damage such as creatine kinase-MM (Pritt, et al., 2008).

The present study is novel in that it assesses the effectiveness of PCSO-524™ supplementation on attenuation of muscle damage which was evaluated by FABP3 after exercise induced muscle damage in humans. The present study found that there was a significant effect for time and appearance of FAB3 in the blood ($F = 6.346$ $p < .001$) suggesting that muscle damage had occurred and the effect was consistent in both groups ($F = 1.582$ $p = .142$). While no statistical

significant differences were observed between control and placebo, maximal values occurred two hours after eccentric muscle damage. Pairwise comparisons revealed that there was significant increases immediately after downhill running in both groups and these results are consistent with literature suggesting that increases in plasma concentrations occur 1-24 hours post muscle damage (Schmitt et al., 2003). However there was no difference between groups when comparing PCSO-524™ to placebo groups. This may suggest that the constituents in PCSO-524™ were unable to control lipid peroxidation compared to control. The lack of observed difference between control and PCSO-524™ groups might have been due to increased rates of fatty acid metabolism during exercise that can affect availability and appearance in blood. Other studies have paired fatty acid binding protein with other biomarkers such aspartate aminotransferase to improve diagnostic value (Pritt, et al., 2008).

Interleukin - 6

Interleukin-6 is produced in response to working skeletal muscle and intensity and exercise duration can affect IL-6 release. Muscle-derived IL-6 is responsible for glucose homeostasis by exerting an effect on adipose tissue and the liver during exercise and mediating exercise induced lipolysis. Muscle-derived IL-6 also inhibits pro-inflammatory cytokines such as tumor TNF-alpha that is produced by adipose tissue and inflammatory cells and plays a role in pathogenicity in diabetes and coronary artery disease.

IL-6 can have many sources, including production by immune cells and the skeletal muscle itself. IL-6 stimulates the immune system and is produced by T cells and macrophages during infection and after trauma leading to inflammation (van der Poll et al., 1997). Additionally it has been shown that IL-6 is produced by the muscle with evidence supporting levels of other biomarkers such as creatine kinase and myoglobin were poorly correlated to IL-6 appearance in the blood (B. K. Pedersen, Steensberg, & Schjerling, 2001). It has been proposed that the large and immediate increase in IL-6 concentration in the blood in response to exhaustive exercise is independent of muscle damage, while muscle damage itself is followed by repair mechanisms which include invasion of macrophages which trigger a smaller magnitude of IL-6 release which is followed by a

second and much larger wave of IL-6 release related to muscle contractions (B. K. Pedersen, et al., 2001). Studies have found that antioxidant treatment with vitamin C and vitamin E, failed to attenuate muscle damage and suggests IL-6 is not related to muscle damage and may be a hormone action used in homeostatic maintenance of injured myofibrils (Petersen et al., 2001). Other studies have found that exercise-induced destruction of skeletal muscle fibers may trigger local production of IL-6 after prolonged running (Ostrowski, Rohde, Zacho, Asp, & Pedersen, 1998).

Supplementation with n-3 PUFAs protects muscle cell membrane integrity and attenuates inflammation by inhibiting the arachidonic acid pathway in macrophages (Machado et al., 2012). In the present study supplementation of PCSO-524™ attenuated IL-6 compared to placebo. This suggests that the muscle damaging exercise was successful in disturbing IL-6 levels from baseline. Current literature suggests that peak values for IL-6 occur between 8 and 72 hours post exercise, concurrent with this study which found significant increases in IL-6 concentrations from baseline detected between 24 and 48 hours for both placebo and treatment groups (Bruunsgaard et al., 1997; Phillips, Childs, Dreon, Phinney, & Leeuwenburgh, 2003). Other studies have found significant effects between 4 and 6 hours but no difference at 24 hours and beyond. However the protocols of muscle damage were not as robust as the one employed in this study with more severe bouts of eccentric muscle damage leading to larger and delayed IL-6 release (Febbraio & Pedersen, 2002; Steensberg et al., 2002; D. Willoughby, McFarlin, & Bois, 2003). PCSO-524™ reduced the appearance of IL-6 compared to placebo, with the majority of IL-6 production occurring in muscle rather than monocytes (Febbraio & Pedersen, 2002). This protective effect may be due to PCSO-524™ forming several potent anti-inflammatory lipid mediators such as resolvins and protectins which together suppress the activity of nuclear transcription factors, such as NF-κB, and reduce pro-inflammatory enzymes and cytokine production (Kang & Weylandt, 2008).

These findings were contradicted by Pumpa et. al. (2012) who found no difference between

placebo and treatment groups and appearance of IL-6 in plasma. It appears that training status affects the appearance of IL-6 with trained athletes showing a much lower training response than untrained subjects (Fischer, 2006). Additionally, the source of the IL-6 increase must be questioned as the maximal increase in the present study is 20 times greater than the Pumpa study (52.9 pg/ml +/- 8.5 pg/ml; placebo 41.2 pg/ml +/- 8.2 pg/ml PCSO-524™). Additionally the maximal value in the present study occurred much later (48 hours) than the Pumpa study (immediately after exercise). This suggests that IL-6 production in the present study was induced by the damaged muscle as the findings correlate with other studies regarding magnitude and timing of IL-6 production after eccentric exercise (Ostrowski, et al., 1998).

TNF - alpha

Measurement of TNF-α following post eccentric muscle damage has been used as an indirect measurement of muscle damage. Measurement of TNF-α provides a quantitative measure of early phases of atherosclerosis and inflammation (Tiidus, 2008).

Supplementation of n-3 PUFAs has been shown to decrease expression of TNF-alpha and reduce the proinflammatory effects associated with muscle damage (Lenn, et al., 2002) however the mechanism for attenuation remains unclear. Additionally, n-3 PUFA and PUFA analogs decrease TNF-α secretion by RAW 264.7 macrophages. Activated macrophages induce transcription of COX2 genes causing prostaglandin synthesis and inflammation (Wadleigh, Reddy, Kopp, Ghosh, & Herschman, 2000). It is postulated that increases in the n-3 PUFA content of the macrophage membrane decrease the excitability of the macrophages attenuating the immune response by modulating eicosanoid signaling (Norris & Dennis, 2012). Alternatively, the antioxidants in PCSO-524™ such as vitamin E and furan fatty acids minimize the formation of reactive oxygen species attenuating lipid peroxides and as a consequence tissue damage (Reznick, et al., 1998).

TNF-α was significantly increased following eccentric muscle damage in the present study with the PCSO-524™ group experiencing a greater attenuation in TNF-α production compared to

placebo. The placebo group experienced a maximal increase in TNF-α 24 hours after eccentric muscle damaging exercise (65.80 +/- 14.8) compared to PCSO-524™ (46.0 +/- 8.6). While there were no differences at baseline every point after eccentric muscle damage was greater for the control compared to PCSO-524™ which suggests that PCSO-524™ supplementation has a protective effect by attenuating the immune response as evidenced by a decrease in TNF-α production.

The levels of TNF-α observed in the present study are significantly higher than levels of TNF-α observed in other exercise studies. Signorello et al (2003) observed baseline values in rested subjects before exercise of 9.32 ng/dl +/- 2.35 ng/dl and after 5 minutes of treadmill running was 11.5 ng/dl +/- 2.45 ng/dl (Santo Signorelli et al., 2003). Similarly Pumpa et al (2012) obtained values between 7 and 9 pg/ml which are significantly lower than the present study. This discrepancy may be due to the use of trained individuals, which attenuates TNF-α release with lower levels observed in higher trained athletes (Kvernmo, Olsen, & Osterud, 1992). Additionally, the time course data in the present study suggests that muscle damage occurred since there was greater than a 60% increase in TNF-α concentration within the first 24 hours, which can be correlated to other markers of muscle damage (Mackinnon, 1999). This effect was not observed in the Pumpa study with the concentration of TNF-α peaking immediately post exercise suggesting that the eccentric exercise was not severe enough. To overcome these obstacles, the present study used a much steeper incline that has been shown to induce muscle damage. Second, the use of untrained subjects in the present study helped maximize the treatment effect of downhill running since trained runners muscles are more difficult to damage due to the repeated bout effect (McHugh, 2003).

8 - Hydroxyguanosine

Both DNA damage and inflammation have been observed after exhaustive exercise suggesting that exercise-induced DNA damage might either be a consequence of inflammatory processes or involved in inflammation and immunological alterations after strenuous prolonged exercise. It

appears that reactive oxygen species appear to be effectors which link inflammation with DNA damage.

DNA damage has been shown to be present after endurance events lasting over 4 hours such as ultramarathons and marathons (Umegaki, Daohua, Sugisawa, Kimura, & Higuchi, 2000) (Gomez-Cabrera et al., 2006) (Gomez-Cabrera, et al., 2006; Mastaloudis, et al., 2004). Studies investigating DNA damage after short duration submaximal and maximal tests in the laboratory have shown no DNA damage (Neubauer, Reichhold, Nersesyan, Konig, & Wagner, 2008). For example, DNA damage was not observed after intense treadmill running in either moderately or well trained athletes (Peters, Van Eden, Tyler, Ramautar, & Chuturgoon, 2006; K Umegaki, Higuchi, Inoue, & Esashi, 1998). Further Sato et al. (2003) showed that moderate exercise does not result in DNA damage measured by 8-OHdG (Sato, Nanri, Ohta, Kasai, & Ikeda, 2003). Researchers using a similar method of inducing muscle damage in the present study using eccentric exercise by downhill running at -16% for 45 minutes found that while subjects experienced similar increases in serum biomarkers indicative of muscle damage 8-OHdG serum levels were unaffected by exercise (Sacheck, Milbury, Cannon, Roubenoff, & Blumberg, 2003). The lack of damage associated with exercise depends on the balanced between oxidant producing mechanisms such as increases in aerobic metabolism and the rate of free radical removal by antioxidant systems (Jacob & Burri, 1996). In exercise lasting up to 40 minutes consumption of antioxidant levels in a typical athlete's diet provides sufficient protection against the effects of free radical production without additional supplementation of antioxidants or n-3 PUFAs. Therefore, we can assume in the present study subjects were properly protected from DNA damage by their current diets (Watson et al., 2005).

The lack of change in 8-OHdG post muscle damaging exercise in the current study is consistent with literature finding no significant changes in DNA damage after short bouts of exercise. There was not a significant effect of time on 8-OHdG concentration in the blood ($F = 1.018$ p .419), the lack of interaction between time and treatment suggests that this effect is consistent between

placebo and PCSO-524™ groups (F = 1.005 p = .429). Finally the test of between-subjects effects indicates there is no significant effect of supplementation status on 8-OHdG appearance in the blood (F = 3.250 p = .081) which suggests the supplement had no effect on DNA damage after exercise.

Troponin – 1 (Cardiac)

Heart troponins such as cTn1 are used as specific markers to detect damage to myocardial tissue. Since creatine kinase, fatty acid binding protein and myoglobin are also found in cardiac muscle they have been used as indicators of muscle damage following myocardial infarction. In order to rule out cardiac tissue as a source of these proteins in the current study, there must be no significant increase following muscle-damaging exercise otherwise any increase in these proteins cannot be attributed to skeletal muscle alone.

In the present study, there was no significant effect of time on cTn1 concentration in the blood (F = .420 p = .889). Additionally the lack of interaction between time and treatment suggests that this effect is consistent between placebo and PCSO-524™ groups (F = .381 p = .912), finally the test of between-subjects effects indicates there is no effect of supplementation status on cTn1 appearance in the blood (F = 2.220 p = .148). Established literature suggests that cardiac muscle damage occurs when levels of cTN1 are above 10 μ m/L (Hedström et al., 2007). Since all values for all subjects are under this level for all time points, it can be assumed any increases in muscle intracellular proteins can be attributed to disruption of skeletal muscle fibers and not cardiac muscle.

Future directions

The present study demonstrated that supplementation of PCSO-524™ attenuated the appearance in blood of TNF-alpha, STN1, Myoglobin, CK-MM, and IL-6 and attenuated loss of range of motion and enhanced maximal voluntary contraction post muscle damaging exercise. Further research could look into inducing muscle damage to a greater extent by having subjects run at a steeper grade or run for a longer duration. Additionally, it might be helpful to look at the protective effect that PCSO-524™ can play in strength training rather than endurance training.

Conclusion

In conclusion, the present study has shown that a lipid extract known as PCSO-524™ attenuates skeletal muscle damage in untrained men as measured by indirect biochemical measures of muscle damage such as sTN1, CK-MM, TNF-alpha and myoglobin, range of motion and enhanced maximal force production from baseline. This study supports data from other studies (Tartiban et. al (2009) and Bloomer et. al (2009) that chronic n-3 PUFA supplementation provides a protective effect against EIMD following eccentric exercise in untrained males (R. J. Bloomer, D. E. Larson, K. H. Fisher-Wellman, A. J. Galpin, & B. K. Schilling, 2009; B. Tartibian, et al., 2009).

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